Nonpromoting 12-Deoxyphorbol 13-Esters Inhibit Phorbol 12-Myristate 13-Acetate Induced Tumor Promotion in CD-1 Mouse Skin

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ABSTRACT

Prostratin and 12-deoxyphorbol 13-phenylacetate (dPP) form a new class of protein kinase C activators of unique biological activity. Although they bind to and activate protein kinase C, in mouse skin they either fail to induce typical phorbol ester (PMA) effects (e.g., hyperplasia) or induce only partial response (e.g., inflammation). Furthermore, pretreatment with these agents inhibits a range of PMA induced effects (acute and chronic hyperplasia, inflammation, etc.). These observations suggested that prostratin and dPP would function as inhibitors of phorbol ester tumor promotion. Here we verify that prediction. We report that both compounds reduced both the average number of papillomas and the tumor incidence in a tumor promotion schedule in CD-1 mouse skin, in which each PMA application was preceded by 12-deoxyphorbol 13-monoester pretreatment. The highest dose of prostratin used (2.56 nmol or 1 mg/pretreatment) caused a 96% (23-fold) reduction in the average number of papillomas with a decrease of tumor incidence from 97 to 46%. The highest dose of dPP used (21.4 nmol or 10 µg/pretreatment) induced an 86% (7-fold) reduction in the average number of papillomas with a 53% reduction of tumor incidence from 100 to 47%. The inhibitory effect was dose dependent. The dose causing 50% inhibition was 11 nmol/pretreatment for prostratin and 0.8 nmol/pretreatment for dPP. Maximal inhibition of tumor promotion was accompanied by a block of epidermal hyperplasia; however, significant inhibition of tumor induction was observed at doses without any apparent effect on the PMA induced hyperplasia.

INTRODUCTION

The phorbol esters have been the object of intense research effort, initially driven by the potent tumor promoting activity of these compounds in mouse skin. These investigations revealed that the major receptor for the phorbol esters is protein kinase C, a family of serine/threonine kinases involved in cellular signal transduction (1-4). The phorbol esters function as ultrapotent analogues of sn-1,2-diacylglycerol, the lipophilic second messenger generated through hydrolysis of phosphatidylinositol 4,5-biphosphate and phosphatidylcholine (5). This signaling pathway is of central importance in control of cellular growth and differentiation and is a target of multiple oncogenes (6).

One strategy for dissecting the complexity of control of the protein kinase C pathway has been to exploit differences among phorbol esters and related natural products in the patterns of biological response which they induce (6). The existence of such differences was a critical early finding of Hecker et al. (7, 8). The most dramatic example of divergent responses is provided by the bryostatins. These compounds, while yet more potent activators of protein kinase C in vitro than the phorbol esters, induce only a subset of responses typical of the phorbol esters and block in a dominant fashion those responses which they themselves do not induce (9).

Recently, a second class of protein kinase C activators has emerged which, like the bryostatins, function at the biological level as partial agonists. The 12-deoxyphorbol 13-monoesters were initially described by Hecker (10). Whereas 12-deoxyphorbol 13-tetradecanoate was potent both for mouse ear reddening and for tumor promotion, shorter chain derivatives were found to be irritant but not promoting (10), leading Hecker to conclude that "obviously these biological activities have to be considered as virtually independent" (11). Initial analysis of short chain 12-deoxyphorbol derivatives in cellular systems, however, failed to reveal unique behavior (12, 13). We have recently reexamined this issue in more detail, motivated by the identification of prostratin (12-deoxyphorbol 13-acetate) as the constituent in the Samoan medicinal plant Homalanthus nutans responsible for its activity in a screen for anti-HIV agents (14). Prostratin had been previously identified by Hecker et al. (15) as a toxic constituent of Pimelea prostrata. It was reported to be 144-fold less potent than PMA for mouse ear reddening and, at a dose 40-fold that of PMA, not to be tumor promoting (10). In mouse skin we found that prostratin functioned as a partial agonist, inducing ornithine decarboxylase and myeloperoxidase (a marker of neutrophil granulocyte infiltration) only to 25-30% of the level induced by PMA (14). No hyperplasia was observed either in response to single or multiple treatments, and keratin K6, a marker of hyperproliferation, was not induced (16, 17).

By analogy with the bryostatins, we assumed that perhaps the partial or complete lack of response reflected suppression of the protein kinase C pathway by prostratin. Indeed, pretreatment with prostratin fully suppressed PMA induced acute and chronic hyperplasia, induction of ornithine decarboxylase, and keratin K6 expression (16, 17). Edema and myeloperoxidase were reduced approximately 70% (16, 17). The effectiveness of prostratin significantly depended on the pretreatment schedule. An optimal schedule was treatment with 256 nmol prostratin 24-48 h before PMA application and with 2.56 µmol prostratin 15 min before PMA application. Curiously, a higher dose of prostratin for the initial treatment was less effective (17).

Because prostratin displayed relatively low potency, we also evaluated dPP. dPP is comparably potent to PMA for mouse ear reddening (7), although it shows markedly different kinetics (18). In mouse skin, dPP behaved in general similarly to prostratin both for induction of acute responses and for inhibition of response to PMA, but with 100-fold greater potency. Interestingly, however, the suppression of PMA induced chronic hyperplasia was incomplete (17).

Chronic hyperplasia is considered the best correlate of tumor promoting activity in mouse skin (19, 20). The suppression of hyperplasia by prostratin strongly predicted, therefore, that prostratin would be an anti-tumor promoting phorbol ester.

In this article, we evaluate that prediction and compare the responses to prostratin and dPP. We conclude that these 12-deoxyphorbol derivatives are indeed antipromoting.

MATERIALS AND METHODS

Female Charles River CD-1 mice, 5 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. Animals were shaved at 6 weeks of age, and mice in the resting phase of the hair cycle were used for tumor promotion experiments. DMBA was obtained from Eastman, Rochester, NY. PMA, prostratin, and 12-deoxyphorbol 13-phenylacetate were obtained from LC Services, Woburn, MA. Prostratin was repurified before use.

On the 7th, 12th, and 22nd week of the second set of tumor promotion experiments (5, 10, and 20 weeks after the first PMA treatment), hyperplasia...
was assessed by histological examination. The dorsal skin of two mice from each group was removed and fixed in either Bouin fixative or 70% ethanol. It was then sectioned and stained with hematoxylin-eosin by American Histolabs, Gaithersburg, MD. Under each set of conditions, two portions of the treated skin were excised/animal, and three sections were prepared from each portion of skin.

Tumor induction experiments were performed in two sets. In the first set of experiments each group contained 30 mice. In the second set of experiments, initially each group contained 40 mice, except for group 9, which contained 100 mice. On the fifth, 10th, and 20th week after the first PMA treatment, 2 mice/group were sacrificed for histological and biochemical (subject of another study in preparation) examinations. In both sets of experiments mice were initiated by topical application to the shaved backskin of 20 nmol (50 μg) DMBA, dissolved in 200 μl acetone. All other treatments were applied in 100 μl acetone. Two weeks after initiation different doses of 12-deoxyphorbol esters were applied to the initiated skin area. Each PMA treatment were summarized schematically in Fig. 1. For clarity, the overall schedules for treatment are depicted in Fig. 1.

RESULTS

According to previous results, (16, 17) multiple prostratin pretreatments of 2.56 μmol were required for complete inhibition of PMA induced chronic hyperplasia in CD-1 mouse skin. In the first set of experiments the presumed effective doses of prostratin were estimated to be in the same dose range. It turned out to be a significant underestimation of the anti-promoting potency of prostratin and therefore a second set of experiments was needed to complete the dose response curve of the inhibitory activity.

In both sets of experiments, 8.1 nmol PMA applied topically twice/week for 20 weeks induced tumors in 97–100% of the experimental animals by the 20th week (Figs. 2A and 3, A and C; groups 2 and 9). In the positive control group of the first set of experiments (group 2) the maximum of the average number of papillomas was 16.5/mouse (after 28 weeks of treatment) and in the second set of experiments (group 9) the maximum was 13.9/mouse (after 26 weeks of treatment). Prostratin (2.56 μmol/treatment; twice/week; for 20 weeks) and dPP (21.4 μmol/treatment, twice/week for 20 weeks) showed neither complete carcinogenic nor tumor promoting activity; i.e., no tumors appeared in any of the groups (groups 6, 7, 10, and 21) with the above treatment schedule whether they had been initiated with 20 nmol DMBA or not (data not shown). When PMA application was preceded by pretreatment with different doses of either prostratin or dPP, both the average number of papillomas and the tumor incidence were reduced in a dose dependent manner. The unusual treatment schedule which we used (Fig. 1A) was based on the most effective protocol inhibiting PMA induced chronic hyperplasia (16, 17); i.e., all mice were pretreated by either 256 nmol prostratin or 2.14 nmol dPP 2 days before the first PMA treatment (the beginning of the promotion) and during the promotion period every single PMA treatment was preceded by a corresponding prostratin or dPP pretreatment at a 15-min interval. The highest dose of prostratin pretreatment (2.56 μmol/treatment) reduced the average number of papillomas 96% (23-fold) relative to the positive control group (Fig. 2B, group 3). The tumor incidence was reduced from 97 to 40%. Furthermore, 21.4 nmol dPP/pretreatment reduced the average number of papillomas 86%
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Fig. 1. General treatment schedules for the tumor promotion experiments. Initiation means treatment with either 20 nmol DMBA or vehicle (100 μl acetone for control groups). Pretreatment includes all the treatments with 12-deoxyphorbol esters or vehicle preceding the promoting treatments. Promoter means all PMA or vehicle treatments administered according to the usual tumor promoting schedules (once or twice/week). A, groups 1 through 21 (Tables 1 and 2); B, group 22 (Table 2); C, groups 23 and 24 (Table 2).

Fig. 2. Inhibition of PMA induced tumor promotion by prostratin (first set of tumor promotion experiments). CD-1 mice were initiated by 20 nmol DMBA and then promoted by either PMA alone (8.1 nmol) or by a combination of different doses of prostratin for pretreatment followed by PMA treatment (8.1 nmol) as described in "Materials and Methods" (see Table 1 for detailed treatment schedules of the designated groups). Mice were treated twice/week at a 15-min interval with the following doses: 100 μl acetone/8.1 nmol PMA (group 2); 2.56 μmol prostratin/8.1 nmol PMA (group 3); 256 nmol prostratin/8.1 nmol PMA (group 4); 25.6 nmol prostratin/8.1 nmol PMA (group 5). Percent with papillomas is the percentage of surviving animals bearing one or more papillomas at every second week (A). Papillomas/mouse is the total number of papillomas counted divided by the number of mice surviving at every second week (B).

DISCUSSION

Our findings with prostratin and dPP dramatically extend the range of behavior described for phorbol ester congeners in mouse skin. Whereas variation in the effectiveness of phorbol derivatives as tumor promoters has achieved acceptance (8), prostratin and dPP provide the first persuasive example for antipromotion. In addition, our findings...

(7-fold) with a decrease in the tumor incidence from 97 to 47%. (Fig. 3, A and B, group 11).

Data were converted into dose response curves at week 26, when all groups reached a plateau in the level of both tumor incidence and average number of papillomas (Fig. 4, A and B). Since the results obtained from groups of identical (prostratin) treatments in the first and second set of experiments were very similar, we pooled the data for determining the dose response curve of the prostratin cotreated groups. The 50% inhibitory dose for prostratin was 11 nmol/treatment; the 50% inhibitory dose for dPP was 0.8 nmol/treatment.

According to previous experiments, two 12-deoxyphorbol monoster pretreatments were needed for maximal inhibition of acute hyperplasia (16, 17). In the optimal protocol the first pretreatment preceded the second one by 24–48 h, and the dose of the first treatment was 10-fold lower than that of the second pretreatment. Since we do not as yet understand the mechanistic basis for this optimal short term schedule, we also explored several variants for the tumor promotion experiments. The underlying concept was that the effect of the initial low dose of 12-deoxyphorbol might be lost as the duration of the experiment was extended. Therefore, for group 22 the schedule included a first, low dose dPP pretreatment (2.14 nmol/treatment) every week (Fig. 1B), but otherwise the treatment pattern was the same as above. In 2 groups (groups 23 and 24) a single, 2-fold increased dose (16.2 nmol/treatment) of PMA treatment/week was used for promotion. Every PMA treatment was preceded by a lower dose dPP pretreatment (2.14 nmol/treatment) at a 48-h interval and a higher dose dPP pretreatment (21.4 nmol/treatment) at a 15-min interval (Fig. 1C). The latter protocol allowed a larger interval between the treatment with PMA and the subsequent low dose treatment with dPP. Neither variation of the schedule appreciably altered the inhibitory potency of the dPP pretreatments (Fig. 5).

Samples were taken for examining hyperplasia after 7, 12, and 22 weeks of total treatment (5, 10, and 20 weeks after the beginning of the PMA treatments) during the second set of experiments. The chronic PMA treatment used for tumor promotion induced very significant hyperplasia in the positive control animals (group 9). After 7 and 12 weeks of treatment significant inhibition of hyperplasia was detected in those groups in which the reduction of the average number of papillomas was the most prominent, i.e., in groups with 256 nmol prostratin or 21.4 nmol dPP pretreatment (groups 11, 16, 22, and 23) (Fig. 6). No inhibition could be observed in those groups with less significant inhibition of the number of papillomas. After 22 weeks of treatment the evaluation of the sections became more complicated because of the frequent appearance of papillomas. However, in the above mentioned groups of maximal inhibition (groups 11, 16, 22, and 23) those parts of the sections which were far from papillomas or micropapillomas showed clear inhibition of hyperplasia.

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provide great confidence that the failure to observe promotion by prostratin is not simply a consequence of an inadequate dosage level. Earlier findings with bryostatin, which unambiguously antagonizes a subset of phorbol ester responses in intact cells (21), yielded only limited evidence for inhibition of promotion (22, 23). The impressive antagonism of PMA promotion in NMRI mice by short-chain phorbol diesters remains the one other example of such antagonism in the literature (24). Unfortunately, this latter report has not been independently verified and several of the compounds are either themselves promoting or are not antipromoting in other mouse strains (25-27).

We have examined such short-chain substituted phorbol diesters and reported that they do not inhibit hyperplasia, in contrast to the 12-deoxyphorbol 13-monoesters examined here (17). The mechanism for the antagonism of promotion remains to be determined. Our in vitro studies reveal that prostratin and dPP show little selectivity for high affinity binding between protein kinase C isozymes α, β, γ, δ, ε, and η. On the other hand, we have previously reported that protein kinase C shows two functional binding sites for phorbol esters with distinct structure-activity relations (28). The 12-deoxyphorbol 13-monoesters show differentially weak affinity for the secondary site involved in insertion of protein kinase C into the membrane (28). In addition, in several cell types including mouse primary epidermal cells, prostratin and dPP show selective downregulation of protein kinase C δ compared to protein kinase C α, in contrast to PMA. Emerging evidence indicates that different protein kinase C isozymes fulfill different cellular functions. In rat basophilic leukemia RBL-2H3 cells, for example, protein kinase C isozymes β and δ reconstitute the secretory response (29), whereas protein kinase C isozymes α and ε modulate phosphatidylinositol hydrolysis (30). At least in concept, differential down-regulation of isozymes with distinct biological functions provides a plausible model for the antipromoting activity of prostratin and dPP. Extensive and detailed experimentation will be required to evaluate whether this model is correct.

Because of its central role in cellular signal transduction, protein kinase C represents an attractive target for therapeutic intervention. The difficult issue has been how to achieve selectivity. Our findings

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1 M. G. Kazanianet, L. B. Areces, A. Bahador, H. Mischak, J. Goodnight, J. F. Mushinski, and P. M. Blumberg, submitted for publication.

4 Z. Szallasi, C. B. Smith, G. R. Pettit, and P. M. Blumberg, manuscript in preparation.
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Fig. 4. Dose-dependent inhibition of PMA induced tumor promotion by pretreatment with 12-deoxyphorbol 13-monoesters. CD-1 mice were initiated and promoted as described in "Materials and Methods." Each PMA treatment was preceded by a 12-deoxyphorbol 13-monoester pretreatment at the different doses. Average numbers of papillomas were calculated after the 26th week of treatment. A, dose dependent inhibition of tumor promotion by prostratin, data of the first and second set of experiments were pooled to determine the dose response curve. B, dose dependent inhibition of tumor promotion by dPP.

with prostratin and dPP, together with our earlier work with bryostatin (9), demonstrate that dissection of subpathways of protein kinase C response is possible. Agents targeted to the regulatory domain of protein kinase C have proven to be a successful strategy, complementing efforts to obtain selectivity for the catalytic domain. The concept that protein kinase C activators are necessarily tumor promoters and therefore unsuitable as therapeutic agents is no longer a valid one.

Fig. 5. Inhibition of PMA induced tumor promotion by multiple dPP treatments. CD-1 mice were initiated and promoted as described in "Materials and Methods." Detailed treatment schedules are described in Table 2. Results are presented as described in Fig. 1. The promoting treatment was 100 μl acetone/8.1 nmol PMA twice weekly for group 9, 21.4 nmol dPP/8.1 nmol PMA twice weekly for group 22, 100 μl acetone/16.2 nmol PMA once weekly for group 24, and 21.4 nmol dPP/16.2 nmol PMA once weekly for group 23. Note that group 9 is the control for group 22 and group 24 is the control for group 23.

Fig. 6. Effect of high dose prostratin or dPP pretreatment on PMA induced hyperplasia during tumor induction treatments. Mice were initiated and promoted as described in "Materials and Methods." For individual treatment schedules see Table 2. In A, typical section derived from a mouse of group 9 after 12 weeks of treatment, no 12-deoxyphorbol monoester pretreatment was applied. B, typical section derived from a mouse of group 10 after 12 weeks of treatment; mice were pretreated with 21.4 nmol dPP/treatment. C, typical section derived from a mouse of group 16 after 12 weeks of treatment; mice were pretreated with 256 nmol prostratin/treatment (x 400).
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REFERENCES


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