Neurogenic Component of Phorbol Ester-induced Mouse Skin Inflammation

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ABSTRACT

Tumor-promoting phorbol esters are potent inflammatory agents for mouse skin, and the potential mechanistic role of inflammation in tumor promotion is under active investigation. We have shown previously that resiniferatoxin, a uniquely irritant phorbol-related diterpene, acts as a capsaicin analogue to induce and then to block neurogenic inflammation. We report here that pretreatment of CD-1 mice with resiniferatoxin blocked the early (3 h) erythema and edema (6 h) in response to phorbol 12-myristate 13-acetate (PMA), whereas the edema at later times (12–24 h) was only partially blocked. Since the efficiency of resiniferatoxin pretreatment decreased as a function of time if PMA was applied 24, 48, or 96 h after resiniferatoxin administration, the late edema response to PMA may be a combination of increasing edema of nonneurogenic origin and the recovering neurogenic response due to the decreasing desensitization. For other phorbol esters, 12-deoxyphorbol mono- and diesters, and mezerein, differing kinetics of edema and differing degrees of blockade of edema following resiniferatoxin pretreatment were observed, as expected from the discrepancies between their inflammatory and tumor-promoting activities. PMA-induced skin hyperplasia, unlike edema, was not inhibited by resiniferatoxin pretreatment, suggesting that the early component of neurogenic inflammation was not essential for hyperplasia under our conditions. Distinction between inflammatory mechanisms may help to clarify the role of inflammation in tumor promotion.

INTRODUCTION

The phorbol esters are the most extensively studied class of mouse skin tumor promoters (1). Their biochemical mechanism is thought to be through activation of their receptor, protein kinase C, which mediates one arm of the phosphatidylinositol signal transduction pathway (2–5). In skin, phorbol ester treatment initiates a series of acute responses, including erythema, edema, infiltration of polymorphonuclear leukocytes, and hyperplasia (6). The relationship of these acute responses to the promoting activity of the phorbol esters has been an area of extensive investigation.

Inflammation, quantitated by ear reddening (7), has been the standard assay for detecting phorbol esters and related derivatives during purification from the Euphorbiaceae and the Thymelaeaceae and provides the only available quantitative, in vivo measure of potency for most phorbol derivatives. Structure-activity analysis suggests that inflammatory activity correlates with tumor promotion but that this correlation is only partial. Whereas all tumor-promoting phorbol esters are inflammatory, some derivatives are highly inflammatory but either nonpromoting or weakly promoting (8). The kinetics of erythema differ, moreover, among derivatives (9). These discrepancies have been postulated to reflect an ill-defined combination of pharmacokinetics and different contributions through two inflammatory pathways (10).

The strongest evidence for independent inflammatory pathways was that RTX, a 20-homovanillyl resinsiferanol derivative, induced a transient erythema lasting for only 1–2 h, in contrast to the 24-h duration for PMA-induced erythema (11, 12) and that RTX did not bind to protein kinase C or induce protein kinase C-mediated responses at biologically effective concentrations (13, 14). We have recently identified the mechanism of RTX action (15, 16). We find that RTX is an ultrapotent (103–104 fold more potent) analogue of capsaicin. Capsaicin, the pungent component in red pepper, activates and then desensitizes polymodal nociceptors (17). This neural pathway mediates neurogenic inflammation and pain perception via afferent C fibers. Denervation abolishes the inflammatory response to RTX, measured by either Evans blue extravasation or water content of skin (15), as described previously for capsaicin (19).

In this report, we use desensitization of mice to RTX to explore the possible contribution of neurogenic inflammation to the edema in response to phorbol esters and related derivatives. Consistent with RTX not being a tumor promoter, desensitization of at least the early phase of neurogenic inflammation did not inhibit the hyperplastic response of mouse skin to phorbol ester treatment.

MATERIALS AND METHODS

Female CD-1 mice between 6 and 7 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). Animals were given food and water ad libitum during the course of the experiments.

RTX was obtained from Chemicals for Cancer Research, Inc. (Chaska, MN), and from Chemsyn Science Laboratories (Lenexa, KS); capsaicin was from Polysciences (Warrington, PA). PMA, PDBu, and mezerein were purchased from Sigma Chemical Co. (St. Louis, MO); the 12-deoxyphorbol derivatives dPP and dPPA were from LC Services (Woburn, MA).

Inflammation was characterized qualitatively by observing erythema (ear reddening) and quantitatively by measuring tissue swelling (ear plug weights). Compounds were dissolved in acetone and applied with the aid of a microliter pipet to the inner surface of the right ear in a volume of 20 l. The left ear was treated with solvent as a control. At the indicated times after application, the animals were killed by cervical dislocation and the ears were removed. Tissue plugs were obtained from the tips of each ear with a 6-mm punch and were weighed (18). Solvent alone caused no ear edema.

For determination of hyperplasia, mice in the resting phase of the hair growth cycle were clipped. Two days later the compounds in a volume of 0.2 ml acetone were applied to the back skin. The animals were killed by cervical dislocation and the ears were removed. Tissue plugs were obtained from the tips of each ear with a 6-mm punch and were weighed (18). Solvent alone caused no ear edema.

For desensitization of neurogenic inflammation, RTX or capsaicin was applied as indicated either topically in acetone or s.c. under the back skin in 10% ethanol/10% Tween 80/80% physiological saline.

RESULTS

RTX applied topically to the ears of CD-1 mice induced ear reddening, as reported previously (11, 12), together with a fast and transient tissue swelling. The edema could be conveniently quantitated by a modification of the procedure of Gschwendt et al. (18). Edema reached a peak at 30 min following RTX treatment and then subsided, approaching baseline by 3 h (Fig.
Desensitization of neurogenic inflammation by topical application of RTX was through desensitization of neurogenic inflammation by 12-24 h (Fig. 2). Thus, inhibition of edema was 94% at 4 h and approximately 40% at 24 h.

As confirmation that the action of RTX on the PMA-induced edema was through desensitization of neurogenic inflammation, we also examined the effect of pretreatment with capsaicin. Topical capsaicin treatment (1 mg/ear) abolished PMA-induced inflammation in a fashion similar to RTX pretreatment (data not shown).

The decreasing inhibition of edema as a function of time after RTX treatment could be explained either by a time-dependent decrease in the efficiency of desensitization with RTX or by an increasing contribution to the edema of a nonneurogenic inflammatory pathway for PMA. To distinguish between these possibilities, we treated with RTX 4, 24, 48, and 96 h before PMA application and assayed edema at a fixed time, 6 h after PMA application (Table 1). Inhibition was 76% at 24 h and decreased gradually thereafter. It should be noted, however, that there was significant variation, consistent with previous results of others in desensitization to capsaicin in other species (20, 21). Comparison of the 76% inhibition of the early (6 h) edema response to PMA 24 h after RTX pretreatment with the 40% inhibition of the late (24 h) edema response to PMA applied 4 h after RTX pretreatment suggests that the late edema response to PMA represents a combination of both neurogenic and nonneurogenic mechanisms. We are currently exploring other desensitization protocols to resolve this issue definitively.

In addition to examining the duration of desensitization, we also determined how short a pretreatment interval with RTX sufficed to inhibit the early edema response (6 h) to PMA. Prior or simultaneous addition of RTX was effective, whereas diminished inhibition was seen if the RTX application was delayed until 1-3 h after PMA (Table 1). These data are consistent with establishment of a desensitized state by RTX within 2-3 h (see Fig. 1) and a similar time interval being required before the induction of neurogenic inflammation by PMA (see Fig. 2).

The concentration of RTX required to inhibit the early (6 h) edema response to PMA was determined for RTX administered either topically or s.c. 4 h before PMA (Fig. 3). The ED50 for topical application of RTX was $8 \times 10^{-7}$ g/kg (1.3 nmol/kg) or $3 \times 10^{-8}$ g (0.048 nmol)/ear. The ED50 for s.c. administered RTX was $8 \times 10^{-6}$ g/kg (13 nmol/kg), a factor of 10 higher. This latter ED50 is similar to that which we had obtained for desensitization of neurogenic inflammation in Sprague-Dawley rats by RTX administered s.c. (15). The dose of topically applied RTX which we used routinely for desensitization in the experiments described in this study, 10 μg (16 nmol)/ear, is 320-fold the ED50 for desensitization.

Since our data suggested that the early inflammatory response to PMA was neurogenic, whereas perhaps one-half of the late response (24 h) was nonneurogenic, we wished to determine whether the two responses showed similar or distinct kinetics from the edema in response to RTX (Fig. 2). Little edema was observed over the first 2 h. Edema then increased markedly by 4 h, remained elevated for 24 h, and then declined. The maximal level of edema (250-300% of control) was very much greater than that observed for RTX (140% of control).

The development of edema in response to treatment of control mice with PMA at 10 nmol/ear showed quite different kinetics from the edema in response to RTX (Fig. 2). Little edema was observed over the first 2 h. Edema then increased markedly by 4 h, remained elevated for 24 h, and then declined. The maximal level of edema (250-300% of control) was very much greater than that observed for RTX (140% of control).

Table 1 Inhibition of PMA-induced edema as a function of time of RTX treatment

<table>
<thead>
<tr>
<th>Time (h) of treatment</th>
<th>Edema (% of control)</th>
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<tbody>
<tr>
<td>PMA</td>
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<td>96</td>
<td>64 (31-96)</td>
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<td>48</td>
<td>47 (10-76)</td>
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<td>24</td>
<td>34 (4-65)</td>
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<td>3</td>
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<td>1</td>
<td>4 (0-14)</td>
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<tr>
<td>0</td>
<td>10 (3-18)</td>
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<tr>
<td>1</td>
<td>41 (26-71)</td>
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<td>2</td>
<td>47 (38-62)</td>
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<td>3</td>
<td>71 (52-96)</td>
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Fig. 3. Inhibition of PMA-induced edema as a function of the dose of RTX used for pretreatment. RTX was applied topically to the right ears of mice (C) or was injected s.c. under the back skin (•) at the indicated dosages. Four h later 10 nmol PMA were applied to the right ears and edema was measured 6 h after PMA application. Values were calculated from the combined ear punch weights for 5 animals at each RTX dosage. A second experiment gave similar results. A dosage of 10 μg (16 nmol)/ear corresponds to 4 x 10^{-5} g/kg (640 nmol/kg).

Fig. 4. Comparison of dose dependency for induction of early and late ear edema by 12-O-tetradecanoylphorbol-13-acetate in control and RTX-pretreated animals. RTX at a dose of 10 μg (16 nmol) (•, △) or control solvent (C, ▲) was applied to the right ears of mice, and PMA at the indicated doses was administered 4 h later. Ear edema was determined 6 h (△, △) or 24 h (C, •) after PMA application. Values were calculated from the combined ear punch weights for 5 animals at each experimental condition. A second experiment gave similar results.

The existence of at least two mechanisms of phorbol ester-induced inflammation had been postulated previously, based on the rapid kinetics and desensitization observed for RTX. Our emerging understanding of RTX action, however, suggests that RTX and closely related derivatives substituted at C-20 with the homovanillyl analogues are unique, in that the homovanillyl substitution inhibits interaction at protein kinase C (26) and is essential for conferring capsaicin-like activity (12, 26, 27). In contrast, all of the other derivatives are active on protein kinase C as an intermediate in this pathway. In support of this model, Burgess et al. (28) have reported recently that RTX can be used to probe at the whole animal level the biological regulation. Our results presented here indicate that RTX can be used to probe at the whole animal level the involvement of neurogenic inflammation.

The complexity of phorbol ester-induced inflammation is hardly unexpected, given the central role of protein kinase C in biological regulation. Our results presented here indicate that RTX can be used to probe at the whole animal level the involvement of neurogenic inflammation.

The existence of at least two mechanisms of phorbol ester-induced inflammation had been postulated previously, based on the rapid kinetics and desensitization observed for RTX. Our emerging understanding of RTX action, however, suggests that RTX and closely related derivatives substituted at C-20 with homovanillyl analogues are unique, in that the homovanillyl substitution inhibits interaction at protein kinase C (26) and is essential for conferring capsaicin-like activity (12, 26, 27). In contrast, all of the other derivatives are active on protein kinase C, either as measured directly or as determined by activation of protein kinase C-mediated responses. Their induction of neurogenic inflammation may therefore reflect the involvement of protein kinase C as an intermediate in this pathway. In support of this model, Burgess et al. (28) have reported recently that...
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Fig. 5. Induction of ear edema by phorbol esters and related diterpenes as a function of time and of RTX pretreatment. Control mice (C) or mice pretreated 4 h before with 10 μg (16 nmol)/ear of RTX (•) were treated with (A) 40 nmol/ear PDBu, (B) 10 nmol/ear mezerein, (C) 22 nmol/ear dPP, or (D) 67 nmol/ear of dPPA, and edema was then determined as a function of time. Values were determined for the ear punch weights of 5 animals for each treatment condition in each experiment. Points, average for 2 experiments; bars, range. Ears of RTX-pretreated mice showed enhanced toxic effects (necrosis) by 48 h following dPP administration or by 24 h after dPPA treatment; we did not determine ear plug weights in these mice.

Fig. 6. Dose dependency for induction of edema by phorbol esters and related derivatives. Mezerein (C), PDBu (•), dPP (A), and dPPA (△) were applied to the right ears of mice and edema was measured 6 h later. Values were calculated from the ear punch weights for 5 animals at each experimental condition in a single experiment. Points, mean; bars, SEM.

Phorbol ester treatment caused membrane depolarization and calcium influx in cultured dorsal root ganglion cells, the target cells for capsaicin action. Moreover, capsaicin pretreatment abolished the depolarization of ventral roots in response to PDBu administration (29).

The ability to induce chronic hyperplasia represents one of the better correlations with tumor-promoting activity (30, 31). The retention of the hyperplastic response upon inhibition of the early neurogenic inflammation suggests that the neurogenic pathway may not be necessary for tumor promotion, but definitive evaluation will require complete inhibition of the late neurogenic response. Approaches are currently being assessed for achieving complete long-term desensitization. Other factors dictating caution in extrapolation of the present results to tumor promotion are possible differences in response between back skin and ears of mice and the lower dose of PMA typically used in promotion protocols.

The involvement of inflammation in tumor promotion by the phorbol esters has been controversial. On the one hand, many antiinflammatory agents are inhibitors of tumor promotion (32), prostaglandins play a role in the mitogenic response of epidermal cells to the phorbol esters (33), and differences in arachidonic acid metabolism represent one of the few characterized differences between Sencar and NMRI mice (34). On the other hand, significant discrepancies exist between the inflammatory activity of phorbol esters as quantitated by erythema and their tumor-promoting activity. In light of the divergent kinetics of edema formation and of the different contributions of early neurogenic inflammation, reevaluation of the relationship between the nonneurogenic component of the inflammatory response and tumor promotion may be of interest.

A difficulty in the separate analysis of the involvement of neurogenic and nonneurogenic inflammation is that the two processes are not mutually independent. Rather, the early neurogenic component of the phorbol ester response appeared to protect against phorbol ester toxicity, as reflected in greater toxicity in the RTX-desensitized animals. This toxicity may influence the attainable promotion response either positively or negatively, and a difference in tumor yield for different derivatives might reflect different degrees of protection by the differing proportions of early neurogenic inflammation.

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