Effects of Phorbol Ester Tumor Promoters in Platelet Aggregation and Platelet Production of Cyclooxygenase Products

R. A. Mufson, P. Kulkarni, K. E. Eakins, and I. B. Weinstein

Institute of Cancer Research and Division of Environmental Sciences [R. A. M., I. B. W.] and Departments of Pharmacology [P. K., K. E. E.] and Ophthalmology [P. K.], Columbia University College of Physicians and Surgeons, New York, New York 10032

ABSTRACT

The tumor promoters, 12-O-tetradecanoylphorbol-13-acetate (TPA), phorbol-12,13-didecanoate, and mezerein, in the range of 10−8 m caused aggregation of washed rabbit platelets. The lag time between the addition of tumor promoters and the onset of aggregation was inversely proportional to the concentration of the compounds. 4α-Phorbol-12,13-didecanoate, which is inactive as a tumor promoter, did not induce platelet aggregation, and 4-0-methyl-12-O-tetradecanoylphorbol-13-acetate, which is a weak tumor promoter, had low activity in this assay. Arachidonic acid also caused platelet aggregation. Arachidonic acid-induced aggregation was rapid, reversible, and accompanied by the production of thromboxane A2-like activity, as determined by bioassay on rabbit aorta and celiac artery strips. This aggregation was blocked by the cyclooxygenase inhibitor indomethacin. TPA-induced aggregation, however, was slow and irreversible. It was not accompanied by production of thromboxane A2-like activity and was not blocked by indomethacin. TPA-induced aggregation was accompanied by enhanced production of a vasoactive material which contracted only rabbit celiac artery and, in contrast to thromboxane A2, was stable for 10 min at 37°C. This material is not adenosine 5′-diphosphate or 5-hydroxytryptamine.

The results of this study indicate that the structural requirements of diterpenes for tumor promotion are similar to those for platelet-aggregating activity. The generation of prostaglandin synthetase (cyclooxygenase) products do not appear to be required for phorbol ester-induced aggregation. Phorbol ester-induced aggregation is accompanied by the production of an unidentified vasoactive material which may be related to the mechanism of aggregation.

INTRODUCTION

Submicromolar concentrations of the potent mouse skin tumor promoter TPA3 aggregate isolated human platelets (4, 20). This aggregation is blocked by Ca2+-chelating agents, N-ethylmaleimide, and prostaglandin E1 (20), indicating that it is required for phorbol ester-induced aggregation. Phorbol ester-induced aggregation is accompanied by enhanced production of a vasoactive material which contracted only rabbit celiac artery and, in contrast to thromboxane A2, was stable for 10 min at 37°C. This material is not adenosine 5′-diphosphate or 5-hydroxytryptamine.

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MATERIALS AND METHODS

Platelet Preparation. Blood from rabbits was collected in 7.5% (v/v) buffered 77 m EdTA solution, and the platelets were isolated according to the method of Hamberg et al. (7). The mixture was centrifuged at 100 rpm for 15 min. The supernatant was collected and recentrifuged at 2300 rpm for another 15 min. The platelet pellet was collected and resuspended in 134 mM NaCl-15 mM Tris, pH 7.4-1 mM EdTA buffer and centrifuged at 2300 rpm. The platelets were collected and resuspended in Krebs-Henseleit buffer without CaCl2, such that the final platelet concentration was about 3.4 × 108/cu mm. Washed platelets were used instead of platelet-rich plasma since Rao and White (15) have shown that plasma esterases degrade TPA, and we have found washed platelets to be more sensitive and to yield more consistent responses to TPA.

Bioassay for Prostaglandin Endoperoxides and Thromboxane A2. These compounds were assayed using a superfused tissue cascade consisting of rabbit celiac artery and aorta, as described by Bunting et al. (3). Prostaglandin endoperoxides G2 and H2 contract the aorta and relax the celiac artery, whereas thromboxane A2 contracts both tissues. The endoperoxides and thromboxane A2 have half-lives of 5 min and 30 sec, respectively; platelet suspensions were therefore sampled early (15 sec) after addition of arachidonic acid. Sampling after TPA addition was as described under "Results." The absolute magnitudes of contractions cannot be compared between bioassay preparations from different experiments, since tissues from the released arachidonic acid (5, 9). These cyclooxygenase products are capable of mediating platelet aggregation.

Recently, Ohuchi and Levine (13) demonstrated with transformed canine kidney cells that TPA can also release arachidonic acid from membrane phospholipids and induce the synthesis of prostaglandins. We have also found that TPA induced release of arachidonic acid and prostaglandin synthesis with normal chick embryo and C3H10T½ mouse fibroblasts. Further, Moroney et al. (12) have shown that prostaglandin can mimic some of the membrane effects of phorbol esters on mouse fibroblasts. Since the effects of TPA on platelets may be due to effects on cell membranes which are common to the action of TPA on other cell types, we wished to investigate the role of the cyclooxygenase products in TPA-induced platelet aggregation. Further, since platelets may be a simple, relatively homogeneous model system for studying the membrane effects of phorbol esters, we wished to correlate the structure-activity relationships previously established for the tumor-promoting activity of phorbol esters with their platelet-aggregating activity.
from different rabbits have varying sensitivities to these compounds.

**Platelet Aggregation.** This measurement was made by continuous recording of light transmission (aggregometer; Chronolog Corp., Broomall, Pa.) through 500 μl of the above-described suspension of washed platelets. Platelets were maintained at 37° and continuously stirred during the measurement period.

**Addition of Phorbol Esters.** Phorbol esters were dissolved in DMSO as stock solutions of 1 mg/ml and were added to the platelet suspension in 1 μl of DMSO after proper dilution. The final concentration of DMSO (0.2%) neither caused nor interfered with aggregation.

**RESULTS**

TPA caused aggregation of washed rabbit platelets at concentrations as low as 3.2 \times 10^{-8} \text{ M}. At this concentration, aggregation began within 2 or 3 min. Increasing the dose of TPA shortened the lag period before the onset of aggregation (Chart 1A). PDD, Phorbol-12,13-didecanoate, which is a very active tumor promoter (1), caused aggregation within the same dose range, and again increasing the dose shortened the lag period before the onset of aggregation (Chart 1B). It should be noted that the phorbol ester-mediated platelet aggregation is irreversible. Mezerein, which structurally resembles the phorbol esters and is weakly active as a tumor promoter (8), also caused aggregation (Chart 1C). This finding is consistent with other studies indicating that, although mezerein is a weak tumor promoter, it is very active in producing tumor promoter-related effects on cells in vitro (18). 4α-Phorbol-12,13-didecanoate, which is inactive as a tumor promoter (1), did not cause any aggregation even when tested at 3.2 \times 10^{-7} \text{ M}, which is 10 times the minimal effective concentration needed for TPA to cause aggregation (Chart 2). 4-O-Methyl-12-O-tetradecanoylphorbol-13-acetate, which is a very weakly active phorbol ester (16, 19), caused some aggregation at 6.4 \times 10^{-7} \text{ M} but not at 6.4 \times 10^{-8} \text{ M} (Chart 2).

To examine the relationship of prostaglandin synthesis to phorbol ester-induced aggregation, we incubated washed rabbit platelets with exogenous arachidonic acid and monitored platelet aggregation. Bioassays were also performed to detect the production of prostaglandin endoperoxides G2 and H2 and thromboxane A2. Chart 3A demonstrates that 400 μM arachidonic acid caused a rapid and reversible aggregation response. This is in contrast to the slow irreversible aggregation mediated by the phorbol esters. Exposing the bioassay tissues to 50 μl of the platelet suspension 15 sec after the addition of arachidonic acid resulted in a marked contraction of both rabbit aorta and celiac artery. This indicated the production of thromboxane A2-like activity. Preincubating the platelets for 2 min with 28 μM indomethacin abolished aggregation in response to arachidonic acid, as well as production of thromboxane A2-like activity (Chart 3B).

Sampling platelet suspensions at 0.5, 1, 2.5, and 10 min after phorbol ester addition did not reveal the presence of thromboxane A2-like activity. Chart 4A demonstrates, however,
that phorbol ester-mediated platelet aggregation was accompanied by production of a material which contracted only the rabbit celiac artery. The presence of celiac artery-contracting material associated with untreated platelets is demonstrated in the control traces. In fact, much of the activity found at 30 sec was due to this background activity; however, TPA did stimulate some production of vasoactive material even 30 sec after addition (Chart 5A). The accumulation of vasoactive material was stable at 37° for 10 min, unlike thromboxane A2, which is extremely labile. Chart 4B demonstrates that neither TPA-induced aggregation nor production of the vasoactive material was blocked by preincubation of the platelets with 28 μM indomethacin. Although in some experiments a small lag was induced by preincubation with indomethacin, this effect was not reproducible, and in none of the experiments could indomethacin prevent TPA-induced aggregation; however, aggregation which was dependent on arachidonic acid-derived endoperoxides was completely suppressed by indomethacin (Chart 3).

Chart 5A demonstrates that the production of the celiac artery vasoactive material was dependent on the TPA concentration and that a dose of TPA (8 × 10^{-7} M) which did not cause platelet aggregation also failed to produce accumulation of celiac artery-contracting material greater than control level at either 30 sec or 10 min after addition. Phorbol-12,13-didecanoate, which also produces platelet aggregation, was found to induce the same concentration-related accumulation of vasoactive material (Chart 5B). On the other hand, 4α-phorbol-12,13-didecanoate, which did not cause aggregation, did not induce this vasoactive material. It should be noted that the magnitude of the contractions cannot be compared between experiments. The bioassay tissues prepared from different rabbits had widely differing absolute sensitivities; however, the magnitudes of contraction within an experiment are directly comparable.

A release reaction has been shown to accompany TPA-induced platelet aggregation (4, 20), and it was possible that celiac artery contraction resulted from the release by platelets of 5-hydroxytryptamine or ADP. This proved not to be the case, since exogenous ADP relaxed the celiac artery and exogenous 5-hydroxytryptamine had no effect on this tissue due to the methysergide in the superfusing buffer. The phorbol esters had no effect on the contraction of either rabbit aorta or celiac artery when added directly to the bioassay tissues at the same concentrations which induced platelet aggregation.

DISCUSSION

Although Zucker et al. (20) have demonstrated that TPA caused aggregation of human platelets, these workers did not examine the aggregating activity of a series of related compounds which vary in potency as tumor promoters. The present study with rabbit platelets has demonstrated a correlation between the structural requirements for the tumor-promoting activity of various macrocyclic plant diterpenes and their platelet-aggregating activity. These and other structure-function studies in vitro (18) suggest that TPA acts via a common mechanism in diverse biological systems. Platelets may be an excellent model for specifically studying the interactions of these compounds with cell membranes, since platelets have no nuclei and they can be isolated as very homogeneous populations.

Thrombin, collagen, and ionophore A23187, which caused platelet aggregation, all have at least 2 other functions in common with the phorbol esters. They can: (a) deacylate arachidonic acid from phospholipids; and (b) enhance prostaglandin synthesis. As a first approach to determining the mechanism by which TPA induces platelet aggregation, we have examined the relationship between cyclooxygenase-generated...
thromboxane A$_2$-like activity and phorbol ester-mediated platelet aggregation. Using 3 criteria, we have found the characteristics of aggregation mediated by arachidonic acid-derived thromboxane A$_2$-like activity to be different from the characteristics of phorbol ester-mediated aggregation: (a) arachidonic acid-induced aggregation is rapid and reversible, while that induced by phorbol esters is slow and irreversible; (b) concentrations of indomethacin which completely inhibit formation of thromboxane A$_2$-like activity and arachidonic acid-induced platelet aggregation do not prevent phorbol ester-induced aggregation; (c) generation of arachidonic acid-derived thromboxane A$_2$-like activity does not accompany phorbol ester-induced aggregation.

Phorbol ester-induced aggregation, however, was accompanied by the formation of a vasoactive material which is stable for at least 10 min at 37° and is pharmacologically distinct from ADP or 5-hydroxytryptamine. This material is clearly not thromboxane A$_2$, since it did not contract the rabbit aorta strips. Although the platelets seem to produce basal level of this material, TPA stimulated an accumulation of it during the 10-min aggregation period. Whether this material is related to the mechanism by which TPA induces aggregation remains to be determined.

Although our investigation indicates that phorbol ester-induced platelet aggregation is not mediated via arachidonic acid metabolites produced by the cyclooxygenase enzyme, it is likely that a process related to deacylation of arachidonic acid-containing phospholipids may be an early event in this phenomenon. Lapetina et al. (10) have shown that platelet aggregation induced by ionophore A23187 or thrombin is accompanied by arachidonic acid release and prostaglandin synthesis; however, inhibitors of cyclooxygenase and lipoxygenase had no effect on the aggregation induced by these agents. These authors suggest that, if arachidonic acid is involved in platelet aggregation, it is at a point prior to synthesis of prostaglandin endoperoxides. Our results with phorbol esters and the new vasoactive material described in this study may provide clues to alternative pathways.

REFERENCES


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