THE TUMOR-PROMOTER PHORBOL ESTER (12-0-TETRADECANOYL-PHORBOL-13-ACETATE), A POTENT AGGREGATING AGENT FOR BLOOD PLATELETS

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ABSTRACT

The phorbol ester 12-0-tetradecanoyl-phorbol-13-acetate, a potent tumor-promoting agent, caused irreversible platelet aggregation when more than 0.02 μM was stirred with human citrated or heparinized platelet-rich plasma (PRP). With washed platelets, 1 nM was effective. The alcohol phorbol, which has little tumor-promoting activity, failed to cause platelet aggregation. With all but low concentrations of phorbol ester, aggregation was succeeded by a rapid phase. The latter was prevented or reduced by enzymes which destroy ADP and by aspirin, was associated with a change in platelet shape, and was presumably due to released ADP. At higher concentrations, only a rapid phase was seen, and these inhibitors were not effective. Low concentrations did not aggregate platelets in PRP containing sufficient EDTA or EGTA to chelate ionized calcium or in PRP from thrombasthenic patients; higher concentrations caused slight aggregation. Both the primary, non-ADP-dependent aggregation and the rapid ADP-dependent aggregation were markedly inhibited by substances which increase cyclic AMP, metabolic inhibitors, and the sulfhydryl inhibitor *N*-ethylmaleimide. Phorbol ester reduced platelet cyclic AMP only when it had been previously elevated by prostaglandin E_1. 1 μM did not release β-glucuronidase, lactic dehydrogenase, or inflammatory material from platelets in 4–5 min despite marked aggregation, but liberated all three in 30 min. The possibility is discussed that low phorbol ester concentrations cause primary aggregation by a direct action on platelet actomyosin.

INTRODUCTION

Croton oil and its active ingredients, phorbol esters, are powerful irritants and tumor-promoting agents (1, 2). The most active is the 12-0-tetradecanoate and 13-acetate ester of the 20-carbon alcohol phorbol (Fig. 1). We investigated the effect of this phorbol ester on human blood platelets because it is structurally similar to prostaglandins, and found it to be an extremely potent aggregating agent.

Platelet aggregates form the major constituents of hemostatic plugs and arterial thrombi, and are also important in the genesis of venous

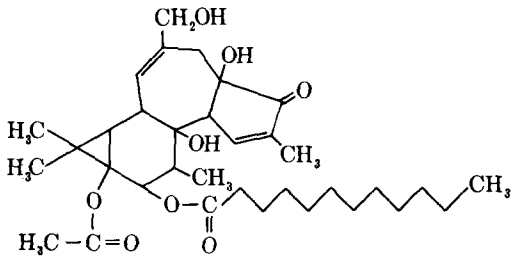

FIGURE 1 The structure of phorbol ester.

thrombi. In vivo, thrombin and contact of platelets with collagen and basement membrane probably serve as the physiologic stimuli for aggregation. In vitro, aggregation is induced when these agents or low concentrations of ADP or epinephrine are added to stirred platelet-rich plasma (PRP), a reagent prepared by slowly centrifuging blood anticoagulated with sodium citrate or heparin (3, 4, 5). ADP is considered a primary stimulus to aggregation, and ADP released from platelets is at least partly responsible for the aggregation induced by thrombin, collagen, and epinephrine. These agents induce a release reaction akin to secretion in which ADP, ATP, and serotonin (or [^{14}C]serotonin if the platelets have been tagged with this substance) are rapidly liberated from granules (6, 7). The mechanism of ADP-induced aggregation is poorly understood although use of inhibitors has provided clues (8, 9, 10). Platelet actomyosin and an ecto-ATPase have been implicated (11, 12, 13). The present investigation was undertaken to characterize aggregation induced by phorbol ester and to compare it with that induced by ADP.

MATERIALS AND METHODS

Preparation of Platelets

Venous blood from normal donors who had taken no aspirin for a week was usually collected into 0.1 vol of 3.2% (0.109 M) sodium citrate. PRP which had contacted only plastic surfaces was prepared by room temperature centrifugation at 300 *g* for 8–10 min, and incubated with [^{14}C]serotonin as previously described (14, 15). PRP was occasionally prepared from blood containing 4 U heparin/ml or 3 mM EGTA. Citrated PRP was also obtained from two patients with thrombasthenia (studied through the kindness of Dr. Harvey Weiss, Roosevelt Hospital, New York City) or from patients with typical severe von Willebrand's disease.

Some experiments were carried out with washed platelets. After $\frac{1}{25}$th vol of 135 mM neutralized sodium EDTA was added to labeled citrated PRP, the mixture was chilled and the platelets were washed as described by Cronberg and Caen (16).

Measurement of Aggregation and Release

Aggregation was studied in glass test tubes or cuvettes by incubating 8 vol (usually 0.32 ml) of PRP with 1 vol of inhibitor or its diluent and then adding 1 vol of aggregating agent or a control solution. The figures in the text are the final concentrations of inhibitors and aggregating agents. The samples were either stirred in an aggregometer (Chrono-log Corp., Broomall, Pa., or Payton Associates, Inc., Buffalo, N. Y.) which measures light transmission of PRP, or shaken in a reciprocating water bath at 37°C. At intervals, they were chilled and centrifuged, and the plasma retained for measurement of [^{14}C]serotonin or enzymes. Radioactivity was measured as previously described (14, 15), lactic dehydrogenase was assayed with a kit from Boehringer Mannheim Corp., New York, and β-glucuronidase was measured by a slight modification of the method of Talalay et al. (17) in which about 270 nmol of phenolphthalein glucuronide (Sigma Chemical Co., St. Louis, Mo.) was hydrolyzed per ml frozen and thawed PRP in 18 h. To measure release of ADP and ATP, EDTA (final concentration of 5.4 mM) was added to the aggregometer cuvette at the desired time. The PRP was chilled and centrifuged for 10 min at 12,000 *g* in the cold. After precipitating the plasma proteins with an equal volume of 95% ethanol, ADP and ATP were assayed by the firefly luminescence method (18). Cyclic AMP was measured by radioimmunoassay (19) (reagents from Collaborative Research, Waltham, Mass.) on extracts of PRP prepared by adding an equal volume of 12% trichloroacetic acid.

Inflammatory activity of platelet preparations was estimated in rabbits. 1 ml of 5% Evans blue dye was injected intravenously, followed by the intradermal injection of 0.1 ml of the preparations in the depilated abdomen (20). 1 h later, the size of the blue spot was estimated visually.

Morphology

PRP was added to formalin fixative and the platelets were examined by phase-contrast microscopy (21).

ADP Destruction

ADP was usually removed from citrated PRP by adding either creatine phosphate (CP) (Grade A, disodium salt, Calbiochem, La Jolla, Calif.) plus creatinephosphokinase (CPK) (Sigma Chemical Co., 105 U/mg protein) at final concentrations per milliliter of 2.5 μmol and 50 U, respectively (22),

or apyrase (Grade 1, Sigma Chemical Co., 2.4 U ATPase and 2.9 U ADPase/mg protein) at a final concentration of 2 mg/ml.

Reagents

The phorbol ester and the alcohol phorbol were obtained from Consolidated Midland Corp., Brewster, N. Y. Several experiments were carried out with phorbol ester prepared by Dr. A. Sivak, Department of Environmental Medicine, New York University, with identical results. A 16 mM solution in DMSO retained full activity for at least a year at 4°C. Before use, it was diluted to 1.0 mM with DMSO and further diluted with isotonic saline. ADP (sodium salt, Sigma Chemical Co.) was kept frozen in isotonic saline at 1 mM. A maximum of 1 μl of 95% ethanol, or 2 μl of DMSO was added to 0.4 ml PRP samples since higher concentrations inhibited aggregation and release. Prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ (Upjohn Co., Kalamazoo, Mich. and Ono Pharmaceuticals, Osaka, Japan) were dissolved in 95% ethanol at 0.5 mg/ml and kept at −20°C. Theophylline (Schwarz-Mann, Div. Becton, Dickinson and Co., Orangeburg, N. Y.) and the sodium salt of N^6, 2′-O-dibutyryl adenosine-3′5′-cyclic monophosphate (dibutyryl cyclic AMP) (Calbiochem, La Jolla, Calif.) were dissolved in saline. Ouabain (injectable) was prepared by Eli Lilly and Co., Indianapolis, Ind. L-Tosylphenylalanine chloromethylketone (L-TPCK) and 1-L-chloro-2-tosylamido-7-amino-2-heptanone (L-TLCK) were obtained from Cyclo Chemical Corp., Los Angeles, Calif. L-TLCK was freshly dissolved in isotonic saline; L-TPCK was dissolved in DMSO and kept at 4°C. Aspirin (Amend Drug Co., New York.) was stored frozen at 1 mM in isotonic saline. Phentolamine (Regitine) was manufactured by Ciba Chemical Corp., Summit, N. J.

RESULTS

Aggregation Induced by Phorbol Ester

Phorbol ester aggregated platelets in citrated or heparinized PRP at concentrations as low as 0.02 μM. The alcohol phorbol did not aggregate platelets when tested in a concentration as high as 80 μM. Phorbol ester did not aggregate erythrocytes; leukocytes were not tested.

The effect of various concentrations on the pattern of aggregation depended on the aggregometer used, possibly because stirring rates were not accurately designated. At low concentrations, aggregation proceeded linearly with a slope which varied with the concentration. At about 10 min, aggregation usually ceased. However, disaggregation did not occur and the tracing remained horizontal for at least 1 h. At intermediate concentrations, the initial slow aggregation was followed by a rapid phase, giving a sigmoidal type of tracing. At high concentrations, the rapid phase occurred with little or no initial slow fall in light transmission (Fig. 2 A); the slope varied directly and the delay inversely with the temperature (Fig. 2 B). In studies with our Chronolog aggregometer, linear curves characteristic of low concentrations were observed with concentrations as high as 0.1 μM (Fig. 2 A), whereas with our Payton instrument a sigmoidal curve was almost always seen with 0.05 μM (Fig. 4) and sometimes even with 0.03 μM (Fig. 5).

When divalent cations were chelated by adding 5.4 mM EDTA to PRP, 0.05 μM phorbol ester had no visible effect and 0.5 μM caused only very slight aggregation. EGTA caused similar inhibition. Disaggregation occurred when EDTA was added soon after phorbol ester; with more than 0.1 μM phorbol ester, aggregation was resumed at a reduced rate (Fig. 3). When EDTA was added later, aggregation was arrested. Platelets in PRP from thrombasthenic patients responded like those in EDTA PRP; only slight aggregation occurred with relatively high concentrations of phorbol ester. The compound caused a normal response in PRP from patients with von Willebrand's disease. Aggregation with a low concentration of ADP was enhanced when it was added soon after the start of aggregation caused by a low concentration of phorbol ester.

Release Induced by Phorbol Ester

To determine whether released ADP was responsible for phorbol ester-induced aggregation, tests were carried out in the presence of enzymes which destroy this nucleotide. Apyrase or CP plus CPK had no effect on the slow progressive aggregation induced by low concentrations of phorbol ester, prevented or diminished the rapid second phase of aggregation produced by intermediate concentrations, and had little effect on aggregation caused by higher concentrations (Fig. 4). Doubling the concentration of CP and quintupling that of CPK and apyrase did not cause additional inhibition. This high concentration of apyrase prevented any aggregation with 50 μM ADP.

The concentrations of ATP, ADP, and [^{14}C] serotonin released into the plasma were measured

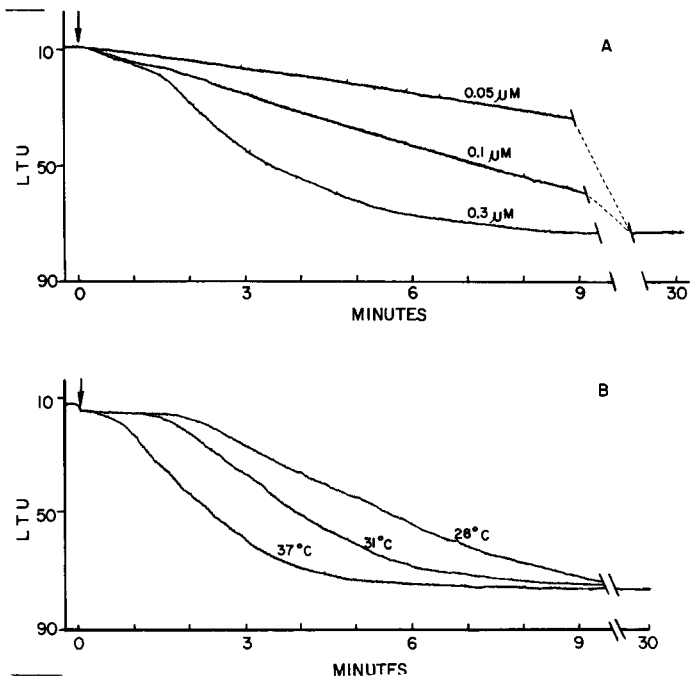

FIGURE 2 Aggregometer tracings of the response of human citrated PRP to (A) three concentrations of phorbol ester tested at 37°C and (B) 1 μM phorbol ester tested at three different temperatures. The abscissa represents light transmission units (*LTU*). PRP was set at 10 and platelet-poor plasma (PPP) at 90 (Chrono-log aggregometer).

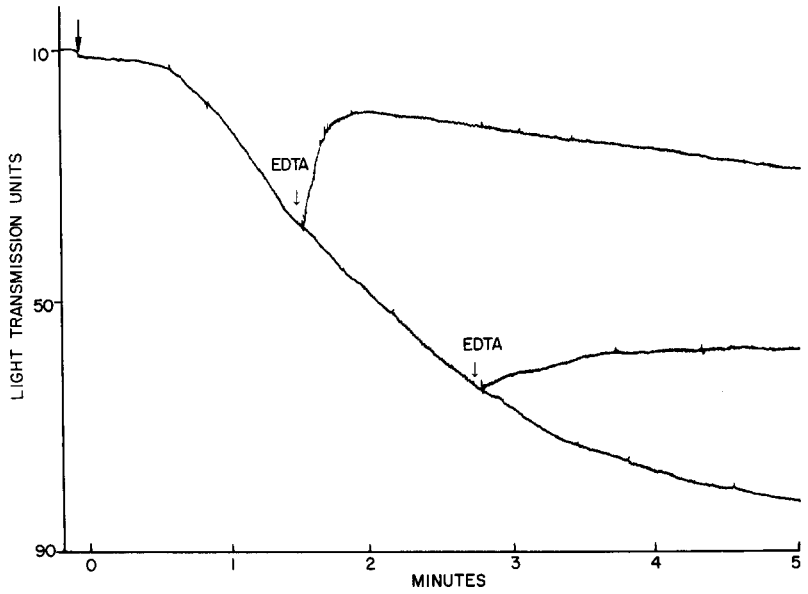

FIGURE 3 Aggregometer tracings of the effect of EDTA on the response to 5 μM phorbol ester. Three tracings are superimposed; in two of the tests, 20 μl of 0.135 M EDTA was added to 0.5 ml PRP (Chrono-log aggregometer).

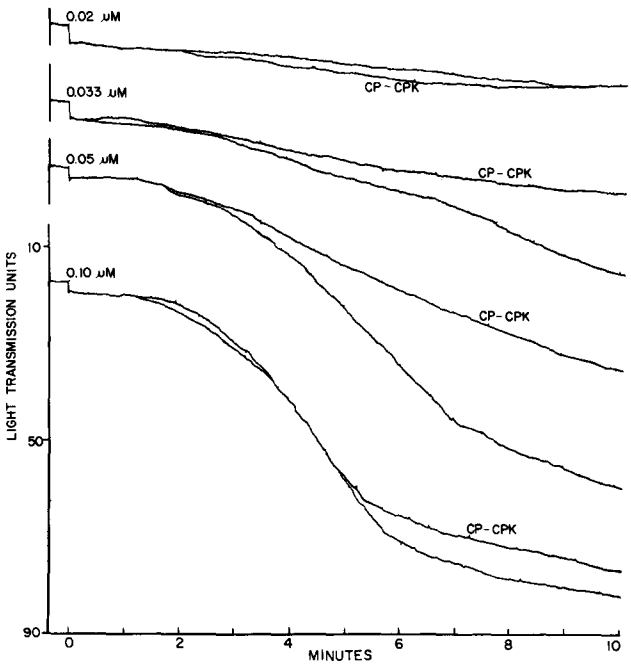

FIGURE 4 Aggregometer tracings showing aggregation induced by four concentrations of phorbol ester in the presence and absence of CP and its kinase (Payton aggregometer).

10 min after aggregation was induced with phorbol ester (Table I). Nucleotides were not found in the supernatant plasma when aggregation was induced by 0.02 μM phorbol ester but were present with 0.05 μM or more. Release of [^{14}C]serotonin was generally greater with the higher phorbol ester concentrations but marked aggregation could occur with little release of radioactivity. Enzymes which destroyed ADP usually decreased [^{14}C]serotonin release even when they inhibited aggregation only slightly. Release induced in 4 min by a high concentration of phorbol ester (1 μM) took place in the presence of EDTA or with thrombasthenic PRP, whereas ADP-induced release was abolished (Table II).

When release was assessed at intervals during aggregation, it was found to occur mainly during the latter part of the steep phase of aggregation (Fig. 5). When PRP was stirred with 1 μM phorbol ester, aggregation was complete within 5 min, but radioactivity continued to be liberated from [^{14}C]serotonin-labeled platelets until almost all of the label had been released in 2 h (Fig. 6). Platelet β-glucuronidase and lactic dehydrogenase had not been released at 4 min but by 2 h about 25% and 95%, respectively, of these enzymes were found in the supernatant plasma. When 1 μM phorbol ester was incubated with PRP without stirring, no aggregation occurred, and a much lower percentage of radioactivity or enzymes was released in 2 h. Phorbol ester added to unstirred PRP immediately before freezing had no effect on the β-glucuronidase activity of frozen and thawed PRP but decreased its lactic dehydrogenase activity by 10–16%.

Platelet Shape Change

Platelets did not change from disks to spiny spheres when aggregation was induced by very low concentrations of phorbol ester or by somewhat higher concentrations (e.g., 0.05 μM) in the presence of CP plus CPK. When ADP was not removed enzymatically, phorbol ester induced a change in platelet shape with all but the lowest concentrations. Shape change but not aggregation occurred when 1 μM phorbol ester was added to EDTA or thrombasthenic PRP.

Effect of Inhibitors on Phorbol Ester-Induced Aggregation and Release

Table III illustrates the effect of various inhibitors of aggregation. They were tested on PRP shaken in a water bath at 37°C with ADP or rela-

TABLE I

Aggregation and Release of [^{14}C]Serotonin, ADP, and ATP Induced by Phorbol Ester with and without Enzymes which Destroy ADP

Expt.	Concentration of phorbol ester																	
	0.02 μM						0.05 μM						0.1–0.5 μM					
	Aggregation*		Release of:				Aggregation*		Release of:				Aggregation*		Release of:			
			^{14}C		ADP	ATP			^{14}C		ADP	ATP			^{14}C		ADP	ATP
		(Enz‡)		(Enz‡)				(Enz‡)		(Enz‡)				(Enz‡)		(Enz‡)		
	%	%	%	%	μM	μM	%	%	%	%	μM	μM	%	%	%	%	μM	μM
1 C	42	21	0	0	0	0							62	59	28	16	1.6	0.5
2 C							74	38	19	11	1.8	1.1						
3 C	13	11	5	0	0	0	77	45	33	0	3.9	0.4	70	66	28	13	12.1	1.3
4 C	13	7	0	0	0	0	57	38	2	1	0.6	0.4	85	75	7	8	2.6	1.4
5 C							75	67	2	0	—	—						
6 C	11	8	10	4	—	—							44	24	10	4	—	—
7 C													66	66	0	0	—	—
8 C													74	66	22	14	—	—
1 A	35	23	0	0	0	0							72	68	14	35	2.4§	
5 A							75	55	2	2	—	—						
8 A													74	75	22	18	—	—
9 A	59	4	15	0	—	—	50	24	6	0	—	—						

* Change in light transmission units as percent of difference in light transmission of PRP and PPP in 10 min.

‡ Columns headed Enz. indicate results in the presence of CP plus CPK (Exps. 1 C–8 C) or apyrase (Exps. 1 A–9 A). Experiments with the same number were carried out on the same PRP.

§ ADP plus ATP.

TABLE II

Release of [^{14}C]Serotonin in EDTA-Treated and Thrombasthenic PRP after 4 Min Shaking with Phorbol Ester or ADP

Condition of PRP	Phorbol ester (1 μM)		ADP (10 μM)	
	Aggregation	^{14}C in supernatant	Aggregation	^{14}C in supernatant
		%		%
Citrated normal	4+	12	4+	25
Citrated normal with 5.4 mM EDTA	±	11	0	0
Citrated thrombasthenic	±	12	0	0

tively high concentrations of phorbol ester. Aggregation observed at 4 min was 4+ without inhibitors. The inhibitors had similar effects on aggregation induced by the two agents but were more active against ADP. Ouabain (0.1, 1.0, or 10 μM incubated for 120 min at room temperature or for 15 min at 37°C) did not inhibit phorbol ester-induced aggregation.

When the level of cyclic AMP in platelets was increased by incubating the PRP with prostaglandin E_1 (PGE_1), stirring the samples with 1 μM phorbol ester for 3 min reduced the level of cyclic AMP in a dose-dependent manner. The ester had no effect without PGE_1 (Table IV).

To assess the effect of inhibitors on the initial phase of aggregation, studies were carried out in

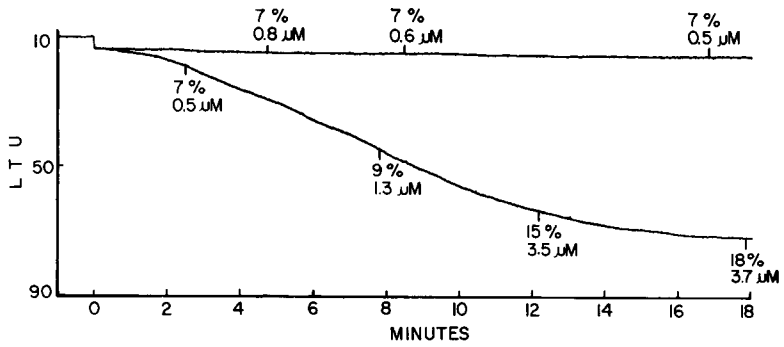

FIGURE 5 Aggregometer tracings of [^{14}C]serotonin-labeled PRP stirred with isotonic saline (top line) or 0.03 μM phorbol ester in a large cuvette (Chrono-log aggregometer). At intervals, samples were removed and the plasma analyzed for ^{14}C (given in percent of total) and ADP and ATP (combined micromolar value given). A small number of residual platelets accounts for the nucleotides in the control plasma. *LTU*, light transmission units.

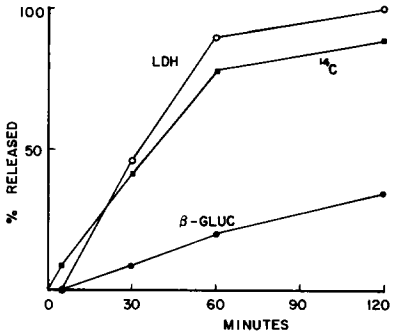

FIGURE 6 Progressive release of [^{14}C]serotonin (■——■), β-glucuronidase (β-GLUC) (●——●), and lactic dehydrogenase (LDH) (○——○) after stirring PRP in an aggregometer with 1.0 μM phorbol ester. Less than 11% of the [^{14}C]serotonin, 35% of the β-glucuronidase and 20% of the lactic dehydrogenase present in frozen and thawed PRP was found in the supernatant of control samples stirred without phorbol ester, and these values did not change during 120 min incubation.

the aggregometer either with a concentration of phorbol ester too low to cause a second phase of aggregation within the 7–10 min of observation, or with a low concentration in the presence of apyrase or CP plus CPK. Phentolamine (0.05 mg/ml) and ouabain (10 μM), incubated 10 min had no effect. Adenosine (0.1 mM incubated for 30 s), 0.14 μM PGE_1, and 0.5 mM *N*-ethylmaleimide (both incubated for 5 min) virtually abolished aggregation. Metabolic inhibitors were incubated with PRP for 30 min before adding CP plus CPK and phorbol ester. Sodium cyanide (2 mM) and 2-deoxyglucose (9 mM) had no effect alone but together prevented aggregation. Aspirin (0.1 mM, incubated for at least 5 min) did not inhibit the effect of these low phorbol ester concentrations, inhibited the steep phase of aggregation with slightly higher levels, and had no effect on aggregation with high (e.g., 0.5 μM) levels. Its effect thus resembled that of ADP-destroying enzymes. Usually, there was no additive effect when the enzymes and aspirin were both present. When aggregation was associated with appreciable release of [^{14}C]serotonin and was inhibited by aspirin, release of radioactivity was usually also reduced.

Experiments with Washed Platelets

Washed platelets were more sensitive to phorbol ester than platelets in PRP. Thus, even 0.001 μM phorbol ester released 12% [^{14}C]serotonin and caused a sigmoidal aggregation curve; these observations strongly suggested that ADP had been released. A sigmoidal curve and over 20% [^{14}C] serotonin release were observed with 0.01 μM phorbol ester in the presence of CP plus CPK, or with PRP from a donor who had recently ingested aspirin. When these inhibitors were added together, 0.01 μM released negligible radioactivity, and aggregation proceeded in a linear fashion, suggesting that released ADP did not play a part.

Frozen and thawed washed platelets at a concentration of $10^6/\mu l$ caused a moderate inflammatory response as judged from the size and color of the local accumulation of Evans blue dye in rabbit skin. No greater response was noted with twice as many platelets, and a lesser response

TABLE III

The Effect of Inhibitors on Aggregation Induced by Phorbol Ester or ADP after 4 Min Shaking

Inhibitor			Phorbol ester		ADP	
	Concentration	Incubation	Concentration	Aggregation*	Concentration	Aggregation*
	mM	*min*	*μM*		*μM*	
PGE_1	0.0003	5	2.7	3+	1.0	0
PGE_1	0.0003	5	0.3	1+	3.0	0
PGE_1	0.003	5	0.95	2+	12.5	0
PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$	0.003	5	0.95	4+	12.5	2+
Theophylline	2	5	0.3	2+	5	0
Dibutyryl cyclic AMP	5	5	0.3	2+	5	0
Theophylline + dibutyryl cAMP	2 + 5	5	0.3	0	5	0
N-Ethylmaleimide	0.5	5	0.67	0	1.0	0
Iodoacetate	0.5	30	0.67	2+	1.25	2+
Sodium cyanide	2	30	0.67	4+	1.25	4+
Iodoacetate + cyanide	0.5 + 2	30	0.67	0	1.25	0
L-TLCK	0.1	5	2.7	4+	10	4+
L-TLCK	1.0	5	2.7	0	10	1+
L-TPCK	0.15	15	5	4+	10	0
L-TPCK	0.30	15	5	2+	10	0

* Degree of aggregation without inhibitor was always 4+.

TABLE IV

Effect of Phorbol Ester and PGE_1 on Platelet Cyclic AMP

PRP with:		Cyclic AMP
PGE_1	Phorbol ester	
μM	*μM*	*pmol/10^9 platelets*
0	0	140
0	3.0	148
0.3	0	250
0.3	0.3	226
0.3	1.0	218
0.3	3.0	198

1.6 ml PRP was incubated alone or with PGE_1 for 5 min. It was then stirred with or without phorbol ester for 3 min, and an equal volume of 12% trichloracetic acid was added.

TABLE V

Release of Inflammatory Activity from Washed Platelets by Phorbol Ester (PhE)

Material stirred with platelets	Duration of stirring	Supernatant: Inflammatory response	Supernatant: [^{14}C]serotonin (% of total)	Supernatant: β-glucuronidase (% of total)
	min			
1:1,000 DMSO	5	±	4	5.3
1:1,000 DMSO, 1 μM PhE	5	±	43	2.5
1:1,000 DMSO	30	Neg	3	3.8
1:1,000 DMSO, 1 μM PhE	30	2+	41	12.5

0.45 ml platelet suspension ($2 \times 10^6/\mu l$) was stirred at 37°C with 0.05 ml 1:100 DMSO with or without 10 μM phorbol ester for 5 or 30 min. A second set was made with [^{14}C]serotonin-labeled platelets, and all of the mixtures were centrifuged in a Serofuge (Clay Adams, Div. Becton, Dickinson and Co., Parsippany, N. J.) for 10 min. The supernatants of the unlabeled platelets were tested for their inflammatory effect, radioactivity was measured in the supernatants of the labeled set, and supernatants of both sets were combined and frozen for subsequent determination of β-glucuronidase.

or none with 500,000 platelets/μl. 1 μM phorbol ester alone had little or no inflammatory effect. When platelets at a concentration of $2 \times 10^6/\mu l$ were stirred for 30 min with 1 μM phorbol ester and the mixture was centrifuged, the supernatant had a moderate inflammatory effect. There was a negligible response to the supernatant of the control 30 min sample without phorbol ester or of a sample stirred for only 5 min with phorbol ester (Table V). As noted

earlier (Fig. 6) β-glucuronidase was released only after the longer period of stirring.

DISCUSSION

Interest in phorbol ester stems from its activity as a tumor-promoting agent. Mice whose skin has been prepared by an application of 7,12-dimethylbenz(a)anthracene begin to develop tumors in 6 wk when phorbol ester is applied three times weekly in a concentration of 0.1–1.0 μg per 10 μl of solvent (16–160 μM) (1, 2, 23). Croton oil or phorbol ester stimulates mitoses (24) and DNA (25, 26) and RNA (27) synthesis in mouse skin. In stationary cultures of 3T3 fibroblasts, phorbol ester causes RNA synthesis, removes density-dependent inhibition of cell division (28), and induces a change in cell refractility (29). The ester binds to cell membranes in a form which is removed by methanol (30) and, in very low concentrations, enhances the incorporation of choline into lecithin in cultured cells (31) and of phosphate into lecithin in mouse skin (32). It also causes an immediate fourfold increase in the activity of Na-K-dependent ATPase when added to isolated cell membranes in a concentration of 3.2 μM. The activity of 5′-nucleotidase in the membrane is increased 70%, but there is no effect on NADH diaphorase or glucose-6-phosphatase (33).

Although the tumor-promoting potential of phorbol ester was thought to be related to its lytic effect on lysosomes (34), skin lysosomes have proved relatively resistant (29) and attention is now focused on its effects on cell membranes and the mechanisms by which these effects are transmitted to the cell interior. Application of 10 μM phorbol ester to mouse skin resulted in a rapid fall in cyclic AMP. The minimum level was reached in 2 h, and the value did not return to normal for 48 h. Dibutyryl cyclic AMP plus theophylline markedly inhibited phorbol ester's potential for inducing tumors (35) and epidermal mitoses (Belman and Troll, unpublished observations). These results are significant in relation to the fall in cyclic AMP associated with mitosis (36). The possible role of proteases in the action of phorbol ester is suggested by the fact that local application of L-TPCK inhibits tumor development (37).

Our observation that low concentrations of phorbol ester cause platelet aggregation provides new evidence for the interaction of this substance with cell membranes. Phorbol ester is active at 0.02 μM with PRP, and at 1 nM with washed platelets, making it the most active platelet-aggregating agent known.

Released ADP plays an important role in the aggregation produced by intermediate concentrations of phorbol ester. ADP and ATP were found in the supernatant plasma 10 min after aggregation was induced by at least 0.05 μM phorbol ester. Furthermore, at concentrations between 0.03 and 0.1 μM, aggregation was markedly inhibited by enzymes which destroy ADP. The change in platelet shape caused by all but low concentrations of phorbol ester is presumably due to released ADP. As with ADP (38, 39), the change occurs in thrombasthenia or when aggregation is prevented by EDTA.

Released ADP does not appear to be important in aggregation induced by low concentrations of phorbol ester. No released ADP could be detected after 10 min, and slow progressive aggregation occurred even in the presence of CP plus CPK or apyrase. The failure of these enzymes to inhibit the effect of low concentrations of phorbol ester while inhibiting much of the effect of somewhat higher concentrations indicates that phorbol ester has a primary effect on platelets for which released ADP cannot be responsible. This primary effect was not associated with a change in platelet shape and was not inhibited by aspirin, ouabain, or phentolamine.

The effect of high concentrations of phorbol ester (i.e., over 0.1 μM) is also probably not mediated by released ADP since the slight inhibition of aggregation caused by apyrase or CP plus CPK is not increased by increasing the concentration of these ADP-destroying enzymes to levels which prevent the effect of 50 μM ADP—considerably more than could be released by the platelets in PRP. However, the possibility cannot be ruled out that released ADP exerts its effect as it leaves the platelet, before it is accessible to the added enzymes.

Inhibitors of ADP-induced aggregation are effective at all levels of phorbol ester. Aggregation induced by ADP and by low and intermediate concentrations of phorbol ester is abolished by chelating calcium ions with EDTA or EGTA and is absent in PRP from patients with thrombasthenia (38, 40). High concentrations (e.g., 0.5 μM phorbol ester) have a slight effect under these conditions.

The effect of other inhibitors on the primary effect of low phorbol ester concentrations was tested in the presence of CP plus CPK to eliminate any effect of released ADP. Aggregation under these conditions was markedly inhibited by adenosine, PGE_1, *N*-ethylmaleimide, and metabolic poisons. These inhibitors, as well as others, were also effective against intermediate and high concentrations of phorbol ester (Table III). As with ADP (8, 9, 41), aggregation induced by high concentrations of phorbol ester is inhibited by dibutyryl cyclic AMP and substances which raise cyclic AMP such as theophylline and PGE_1. As with ADP (41), phorbol ester lowers the cyclic AMP concentration of platelets when it has been elevated by exposure to PGE_1. Aggregation by both ADP (7, 8, 9, 10) and phorbol ester is inhibited by the sulfhydryl inhibitor *N*-ethylmaleimide, by the protease inhibitors L-TLCK and L-TPCK, and by inhibition of glycolysis, especially when respiration is also blocked.

Platelets contain a high concentration of contractile protein (i.e., platelet actomyosin; thrombosthenin) which is involved in clot retraction and perhaps also in aggregation and the release reaction (11, 12, 13, 42). At least some of this protein is localized at the cell surface (40, 43, 44), and is presumably responsible for the ouabain-insensitive ecto-ATPase activity of platelets (12). Because actomyosin has been implicated in platelet aggregation, Puszkin and Zucker (45) tested the effect of phorbol ester on actomyosin isolated from platelets and skeletal muscle. It was found to stimulate ATPase activity and enhance superprecipitation at very low concentrations. Higher concentrations inhibited superprecipitation. Furthermore, phorbol ester, like ADP, caused retraction of fibrin clots produced by the enzyme Reptilase. Unlike clots produced by thrombin, those produced by Reptilase do not retract in the absence of phorbol ester or ADP. Evidence was provided that released ADP was not responsible for most of the clot-retracting effect of phorbol ester (45).

These findings suggest that the primary aggregating effect of phorbol ester is mediated by platelet actomyosin. Recent studies have shown that added ADP enhances superprecipitation (46) and that ADP derived from ATP by the ATPase action of actomyosin seems essential for superprecipitation (45), additional evidence for the role of actomyosin in ADP-induced aggregation as well. Interference with the level of ionized calcium, energy metabolism, sulfhydryl groups, and cyclic AMP levels could thus inhibit both primary ADP- and phorbol ester-induced aggregation because they affect the contractile mechanism. This unified theory is supported by the observations that ADP and phorbol ester cause platelets to retract Reptilase-induced clots and that platelets of patients with thrombasthenia fail to promote clot retraction (38) and do not aggregate with ADP and only slightly with high concentrations of phorbol ester.

It is of interest that both ADP and phorbol ester accelerate phospholipid synthesis; ADP augments the incorporation of ^{32}P into phosphatidic acid and di- and triphosphatidyl inositol within 30 s (47) and, in other cells, phorbol ester enhances incorporation of choline and phosphate into lecithin (31, 32).

Release induced by phorbol ester has features in common with the platelet release reaction caused by ADP, epinephrine, and collagen (6–10). Aspirin, a known inhibitor of the release reaction (6, 9, 10), inhibited the steep phase of aggregation induced by low concentrations of the tumor promoter. When aggregation was associated with release of [^{14}C]serotonin, aspirin usually reduced the amount of radioactivity released. As with other agents which induce the release reaction (6), the rapid early release of [^{14}C]serotonin and nucleotides caused by 1 μM phorbol ester was not accompanied by release of β-glucuronidase, an enzyme found in alpha granules, or of lactic dehydrogenase, a cytoplasmic enzyme. ADP enhanced phorbol ester-induced release, as shown by the diminished release of [^{14}C]serotonin in the presence of ADP-destroying enzymes. A similar effect has been shown for other releasing agents (22). On the other hand, the release of [^{14}C]serotonin induced by 1 μM phorbol ester differed from that caused by ADP (7), since it was not inhibited by EDTA and occurred in thrombasthenia. Furthermore, β-glucuronidase and lactic dehydrogenase were liberated when stirring was continued for longer periods. Liberation of these enzymes was apparently not simply due to lysis, since there was much less release when aggregation was prevented by calcium chelation or lack of stirring. White et al. (48) also observed that phorbol ester causes release and degranulation. The unusual characteristics of release are in harmony with their electron microscope studies showing that degranulation is associated with a

type of granule swelling which does not occur during a typical release reaction. Dissimilarities between the release reaction induced by phorbol ester and other aggregating agents led us to the earlier erroneous conclusion that it did not induce release (49).

Inflammatory activity was released with β-glucuronidase when washed platelets were incubated for 30 min with phorbol ester, and can probably be attributed to a cationic protein with inflammatory properties which has been isolated from human platelets (20). Because human plasma itself caused inflammation, experiments could not be carried out on PRP, but it seems likely that in plasma, release of inflammatory material from platelets would also accompany the release of β-glucuronidase. However, the role of platelets in the inflammatory reaction to phorbol ester remains to be established, since platelet aggregates were not noted in histologic sections of the ears of mice prepared 30 min to 24 h after application of inflammatory concentrations of phorbol ester (50). Whether or not platelets prove to be related to phorbol ester's inflammatory and tumor-promoting activity, their rapid and profound response to low concentrations of this compound provides a new means of investigating its action on cells as well as the general mechanism of cell aggregation.

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REFERENCES

1. HECKER, E. 1968. *Cancer Res.* **28**:2338.
2. VAN DUUREN, B. L. 1969. *Prog. Exp. Tumor Res.* **11**:31.
3. MARCUS, A. J. 1969. *N. Engl. J. Med.* **280**:1213, 1278, 1330.
4. MUSTARD, J. F., and M. A. PACKHAM. 1970. *Circulation.* **42**:1.
5. ALEDORT, L. M. 1971. *In* The Circulating Platelet. S. A. Johnson, editor. Academic Press Inc., New York. 259.
6. DAY, H. J., and H. HOLMSEN. 1971. *Ser. Haematol.* 4(1):3.
7. ZUCKER, M. B. 1972. *Thromb. Diath. Haemorrh.* **28**:393.
8. MICHAL, F., and B. G. FIRKIN. 1969. *Annu. Rev. Pharmacol.* **9**:95.
9. MUSTARD, J. F., and M. A. PACKHAM. 1970. *Pharmacol. Rev.* **22**:97.
10. WEISS, H. J. 1972. *In* Progress in Hemostasis and Thrombosis. T. H. Spaet, editor. Grune and Stratton, Inc., New York. 199.
11. DAVEY, M. G., and E. F. LÜSCHER. 1968. *Semin. Hematol.* **5**:5.
12. CHAMBERS, D. A., E. W. SALZMAN, and L. L. NERI. 1967. *Arch. Biochem. Biophys.* **119**:173.
13. BOOYSE, F. M., and M. E. RAFELSON, JR. 1972. *Microvasc. Res.* **4**:207.
14. JERUSHALMY, Z., and M. B. ZUCKER. 1966. *Thromb. Diath. Haemorrh.* **15**:413.
15. VALDORF-HANSEN, J. F., and M. B. ZUCKER. 1971. *Am. J. Physiol.* **220**:105.
16. CRONBERG, S., and J. P. CAEN. 1971. *Scand. J. Haematol.* **8**:151.
17. TALALAY, P., W. H. FISHMAN, and C. HUGGINS. 1946. *J. Biol. Chem.* **166**:457.
18. HOLMSEN, H., I. HOLMSEN, and A. BERNHARDSEN. 1966. *Anal. Biochem.* **17**:456.
19. STEINER, A. L., C. W. PARKER, and D. M. KIPNIS. 1972. *J. Biol. Chem.* **247**:1106.
20. NACHMAN, R. L., B. WEKSLER, and B. FERRIS. 1970. *J. Clin. Invest.* **49**:274.
21. ZUCKER, M. B., and J. BORRELLI. 1954. *Blood.* **9**:602.
22. PACKHAM, M. A., M. A. GUCCIONI, P.-L. CHANG, and J. F. MUSTARD. 1973. *Am. J. Physiol.* **225**:38.
23. BAIRD, W. M., and R. K. Boutwell. 1971. *Cancer Res.* **31**:1074.
24. BELMAN, S., and W. TROLL. 1972. *Cancer Res.* **32**:450.
25. HENNINGS, H., and R. K. BOUTWELL. 1970. *Cancer Res.* **30**:312.
26. RAICK, A. N., K. THUMM, and B. R. CHIVERS. 1972. *Cancer Res.* **32**:1562.
27. BAIRD, W. M., P. W. MELERA, and R. K. BOUTWELL. 1972. *Cancer Res.* **32**:781.
28. SIVAK, A., and B. L. VAN DUUREN. 1970. *Cancer Res.* **30**:1203.
29. SIVAK, A., and B. L. VAN DUUREN. 1969. *Cancer Res.* **29**:624.
30. SIVAK, A., and B. L. VAN DUUREN. 1971. *Chem.-Biol. Interactions.* **3**:401.
31. SÜSS, R., G. KREIBICH, and V. KINZEL. 1972. *Eur. J. Cancer.* **8**:299.
32. ROHRSCHNEIDER, L. R., D. H. O'BRIEN, and R. K. BOUTWELL. 1972. *Biochim. Biophys. Acta.* **280**:57.
33. SIVAK, A., B. T. MOSSMAN, and B. L. VAN DUUREN. 1972. *Biochem. Biophys. Res. Commun.* **46**:605.
34. WEISSMAN, G., W. TROLL, B. L. VAN DUUREN,

and G. SESSA. 1968. *Biochem. Pharmacol.* **17**:2421.
35. BELMAN, S., and W. TROLL. 1973. *Proc. Am. Assoc. Cancer Res.* **14**:21.
36. BURGER, M. M., B. M. BOMBIK, B. McL. BRECKENRIDGE, and J. R. SHEPPARD. 1972. *Nat. New Biol.* **239**:161.
37. TROLL, W., A. KLASSEN, and A. JANOFF. 1970. *Science (Wash. D.C.).* **169**:1211.
38. ZUCKER, M. B., J. H. PERT, and M. W. HILGARTNER. 1966. *Blood.* **28**:524.
39. ZUCKER, M. B., and J. B. ZACCARDI. 1964. *Fed. Proc.* **23**:299.
40. BOOYSE, F., D. KISIELESKI, R. SEELER, and M. RAFELSON, JR. 1972. *Blood.* **39**:377.
41. SALZMAN, E. W. 1972. *N. Engl. J. Med.* **286**:358.
42. HOLMSEN, H. 1974. *In* Platelets. Production, Function, Transfusion and Storage. M. G. Baldini and S. Ebbe, editors. Grune and Stratton, Inc., New York.
43. NACHMAN, R. L., and A. J. MARCUS. 1968. *Br. J. Haematol.* **15**:181.
44. ZUCKER-FRANKLIN, D., and G. GRUSKY. 1972. *J. Clin. Invest.* **51**:419.
45. PUSZKIN, E. G., and M. B. ZUCKER. 1973. *Nat. New. Biol.* **245**:277.
46. PUSZKIN, S., E. PUSZKIN, J. HANSON, and L. M. ALEDORT. 1973. *Fed. Proc.* **32**:220. (Abstr.).
47. LLOYD, J. V., E. E. NISHIZAWA, J. HALDAR, and J. F. MUSTARD. 1972. *Br. J. Haematol.* **23**:571.
48. ESTENSEN, R. D., and J. G. WHITE. *Am. J. Pathol.* In press.
49. ZUCKER, M. B., S.-J. KIM, S. BELMAN, and W. TROLL. 1972. Abstracts of the 3rd Congress International Society on Thrombosis and Haemostasis, Washington, D.C. 266.
50. JANOFF, A., A. KLASSEN, and W. TROLL. 1970. *Cancer Res.* **30**:2568.