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Phosphorylation of a Tropomyosin-Like (30 KD) Protein During Platelet Activation

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In this study, we have used the tumor promoter 12-o-tetradecanoylphorbol-13-acetate (TPA), as well as its biologically inactive analogue 4α-phorbol 12,13-didecanoate (4α-PDD), to investigate platelet protein phosphorylation and its possible correlation with platelet activation. Our data show that TPA, but not 4α-PDD, induces a preferential phosphorylation of a 30,000 dalton (30 KD) protein. This phosphoprotein is found to be physically associated with an actomyosin-containing platelet cytoskeleton complex. Further analysis using both standard two-dimensional gel electrophoresis and one-dimensional urea-SDS gel electrophoresis reveals that this 30 KD protein has several tropomyosin-like properties. Most importantly, the degree of TPA-induced phosphorylation of the 30 KD protein is directly proportional to the extent of platelet granule release and the shape change of the platelet, as well as to the degree of aggregation. We speculate that this phosphorylated tropomyosinlike protein may play a pivotal role in the regulation of actomyosin-mediated platelet contractility, which has been previously implicated in a variety of platelet functions.

Key words: phosphorylation, tropomyosin, phorbol esters, platelet activation

Phorbol esters are considered to be one of the most potent classes of tumor promoters [1,2]. Although the mechanisms that direct tumor promotion are not known, there is good evidence that phorbol esters can directly activate the enzyme protein kinase C, which is known to require both phospholipid and calcium for its activity [3–6]. It is also known that protein kinase C is activated by diacylglycerol, which is generated during hormonal stimulation of phosphatidylinositol turnover [6]. In vitro, phorbol esters appear to act as analogues of certain phospholipids (eg, phosphatidylinositol and diacylglycerol) in the activation of protein kinase C [3]. In vivo studies have shown that 12-o-tetradecanoylphorbol-13-acetate (TPA), one of the most frequently used phorbol esters, causes platelet aggregation and secretion [7–12] in addition to phosphorylation of the insulin receptor [13]. It is possible that these TPA-induced effects are all mediated by the activation of protein kinase C.

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In this study, we have used TPA and its biologically inactive analogue, 4α-phorbol 12,13-didecanoate (4α-PDD), to investigate platelet protein phosphorylation and its possible correlation with platelet activation. Our data indicate that a TPA-activated event (presumably mediated through protein kinase C) may be involved in the selective phosphorylation of a tropomyosinlike (30 KD) protein during platelet activation. We believe that this phosphorylation even could be very important in the overall regulation of platelet function.

MATERIALS AND METHODS

All phospholipids, phorbol esters, and derivatives were purchased from Sigma Chemical Co. (St Louis, MO).

Preparations of Platelets

Blood platelets were obtained by venipuncture of the anticubital vein of healthy human adults who had taken no medications for the previous two weeks. Approximately 60 ml of blood was collected into a solution of sodium citrate/citric acid/dextrose [14] and the platelets were isolated by differential centrifugation [15]. Subsequently, platelets were washed twice and resuspended in a HEPES balanced salt buffer containing 145 mM NaCl, 5 mM KCl, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 5.5 mM glucose, 3 mg/ml bovine serum albumin, 50 μg/ml apyrase, and 15 mM HEPES (pH:7.0).

^{3}H-Serotonin Release Assay

Platelets were incubated with ^{3}H-serotonin (3×10^{-7} M, 8.75 μCi/ml, New England Nuclear Co., Boston, MA) for 30 min at 25°C. Labeled platelets were washed and resuspended in HEPES balanced salt solution plus 10^{-6} M imipramine (known to prevent serotonin reuptake) (Sigma Chemical Co., St. Louis, MO). Subsequently, platelets were incubated for 10 min at room temperature in the presence of one of the following compounds: phosphoinositol (10–100 μg/ml); diolein (10–100 μg/ml); dilinolein (10–100 μg/ml); thrombin (0.1–1.0 unit/ml), TPA (10^{-5}–10^{-9} M); 4α-PDD (10^{-5}–10^{-9} M).

All phospholipids, phorbol esters, and derivatives were dissolved in dimethyl sulfoxide (DMSO) which had a final concentration of 1% in all samples. In the control sample, platelets were incubated in either 1% DMSO plus HEPES or in HEPES balanced salt solution alone. After incubation, aliquots were removed and fixed with 2% formaldehyde (to prevent further secretory activity), and the platelets were pelleted by centrifugation at 11,000g for 15 sec. The supernatants were assayed for released ^{3}H-serotonin by standard liquid scintillation counting.

Phosphorylation of Cellular Proteins

In order to label proteins with $^{32}PO_4$, it was necessary to wash the platelets in PO_4-free HEPES balanced salt buffer. $^{32}PO_4$ (ICN, Irvine, CA, carrier free) was then added at 0.25 mCi/ml and, after 1 hr incubation with $^{32}PO_4$ at room temperature (isotopic equilibrium inside the cell is attained under this condition), the platelets were exposed to TPA or 4α-PDD (doses ranging from 10^{-5} to 10^{-9} M) for 10 min, also at room temperature. Platelets were then washed three times with HEPES balanced salt buffer to remove the free $^{32}PO_4$ and the phorbol esters.

Platelet Cytoskeleton Preparation

Detergent extraction of the ^{32}P-labeled platelets was carried out by adding 1% Triton X-100 or 1% Nonidet P-40 (NP-40) to 50 mM Tris-HCl (buffered at pH 7.4) 5 mM EGTA, 1 mM PMSF, and 10 mM iodoacetamide. Protein concentration of all samples was held constant at 0.5 mg/ml. Samples were incubated at 0°C for 20 min with frequent vortexing, and then centrifuged for 10 min at $10{,}000g_{av}$. After centrifugation, the pellets (ie, Triton X-100 or NP-40 insoluble fraction), designated the "platelet cytoskeleton" [14], were collected for further biochemical analysis as described below.

SDS-PAGE Analysis and Autoradiography

Whole platelets or the "platelet cytoskeleton" fractions isolated from platelets treated with either TPA (10^{-6} M) or 4α-PDD (10^{-6} M) contained a total of approximately 5×10^5 cpm of ^{32}Pi per 10^6 platelets. Approximately 150 μg of protein was used for analysis by either one-dimensional [16] or two-dimension polyacrylamide gel electrophoresis (PAGE) [17] as described below.

One-dimensional sodium dodecylsulfate (SDS) and SDS-urea PAGE. Electrophoresis was carried out on slab gels using either a 10% polyacrylamide gel (with or without 6 M urea) or an exponential polyacrylamide gel gradient [(7.0–17.50%) with or without 6 M urea] and the discontinuous buffer system developed by Laemmli [16]. All samples were dissolved in a buffer (pH 8.9) containing 2% SDS, 0.1 M dithiothreitol, 0.002% bromophenol blue, 20 mM Tris-HCl, and heated at 100°C for 2 min. Electrophoresis was run at a constant current of 2 mA for 18 hr at room temperature, and the polypeptide banding pattern was revealed by staining with Coomassie blue.

Two-dimensional PAGE. First-dimension isoelectric focusing gels contained 2% LKB ampholytes (pH 3.5–10). Second-dimension gels were 10% acrylamide. Gels were fixed, stained, and dried as described previously [17].

Autoradiography of both one-dimensional and two-dimensional gels was carried out with Kodak XAR-5 film at −70°C between Kodak X-Omatic intensifying screens. The relative amounts of radioactivity in phosphoproteins were determined by integration of densitometric scans of gel autoradiograms.

Scanning Microscopy

Untreated control platelets and TPA or 4α-PDD-treated (concentrations ranging from 10^{-5} to 10^{-9} M) platelets were fixed in 2% glutaraldehyde for 1 hr at room temperature, followed by dehydration through a graded ethanol series. Samples were then critical-point dried, gold coated, and examined in a JEOL-135 scanning electron microscope as described previously [18].

RESULTS

The Effect of Phospholipids and Phorbol Esters on Platelet Serotonin Release Reactions

Since both phospholipids and phorbol esters are known to serve as activators for protein kinase C, we decided to survey the relative effectiveness of several of those types of compounds on granule secretion in human platelets. Platelet secretion was monitored by the amount of serotonin released following the addition of the

various compounds [7–9]. The amount of serotonin release following thrombin activation was taken as a positive control (Table I). Addition of certain phospholipids, such as phosphatidylinositol, diolein, and dilinolein to the platelets, was found to cause very little serotonin to be released into the medium (Table I). However, addition of TPA to the platelets resulted in a very significant stimulation of serotonin release (Table I). In contrast, the biologically inactive phorbol ester analogue, 4α-PDD, did not cause any measurable effect on granule secretion (Table I). These results suggest that TPA (but not 4α-PDD or any of the phospholipids tested) could be activating protein kinase C, which then leads to granule secretion in platelets [7–9].

Phosphorylation of Cytoskeleton-Associated Proteins

We then determined whether there are any proteins that are *specifically* phosphorylated during TPA-induced platelet secretion. Using standard one-dimensional SDS-PAGE, we compared the patterns of phosphorylated proteins from TPA and 4α-PDD-treated platelets. Figure 1A and B show that a number of different platelet proteins are phosphorylated under these two treatments. Although the 42 KD and 23 KD phosphoprotein bands appear to be phosphorylated about equally under both TPA and 4α-PDD treatments (Fig. 1A,B), phosphorylation of the 40 KD, 30 KD, and 20 KD proteins appears to be significantly enhanced during TPA treatment (Fig. 1A,B). Of these three proteins, the 20 KD (possibly myosin light chain) and the 40 KD (function unknown) phosphoproteins have been previously reported to be phosphorylated by protein kinase C during platelet activation [10–12]. To our knowledge, nothing is known about the other phosphoproteins observed on the gel.

In order to determine whether there is a relationship between these TPA-induced phosphoproteins and known cytoskeletal elements, we employed a selective extraction procedure using nonionic detergents such as Triton X-100 or NP-40 to obtain a relatively pure platelet cytoskeleton preparation [14]. Analysis of these preparations by SDS-PAGE indicates that actin, myosin, and a number of actin-binding proteins are among the major components in both of the cytoskeleton preparations (Fig. 1C,D). However, there appear to be a number of differences between the two patterns obtained from TPA- and 4α-PDD-treated platelets. One of the most obvious differences is the significant accumulation of both myosin heavy chain (200 KD) and light chain (20 KD) in the TPA-treated samples (Fig. 1C) as compared to the 4α-PDD-treated samples (Fig. 1D). Of the five phosphoproteins mentioned previously (Fig.

TABLE I. Platelet Serotonin Release*

Treatment	Serotonin released (No. fold increase over untreated control)
Untreated	1.0
Thrombin (1 unit/ml)	68.0
Phosphatidylinositol (50 μg/ml)	1.0
Diolein (50 μg/ml)	1.0
Dilinolein (50 μg/ml)	1.1
TPA (10^{-8} M)	12.0
TPA (10^{-7} M)	30.5
4 α-PDD (10^{-7} M)	1.0

*The results represent an average of five experiments with a standard deviation of ±5%.

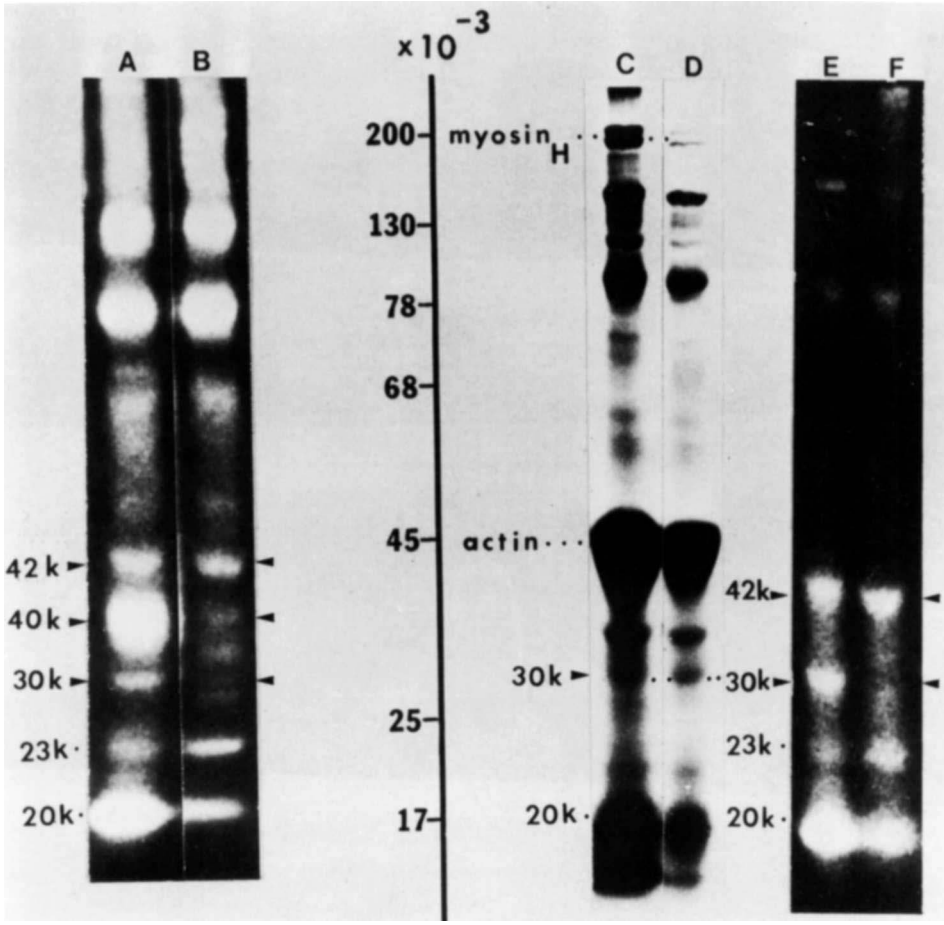
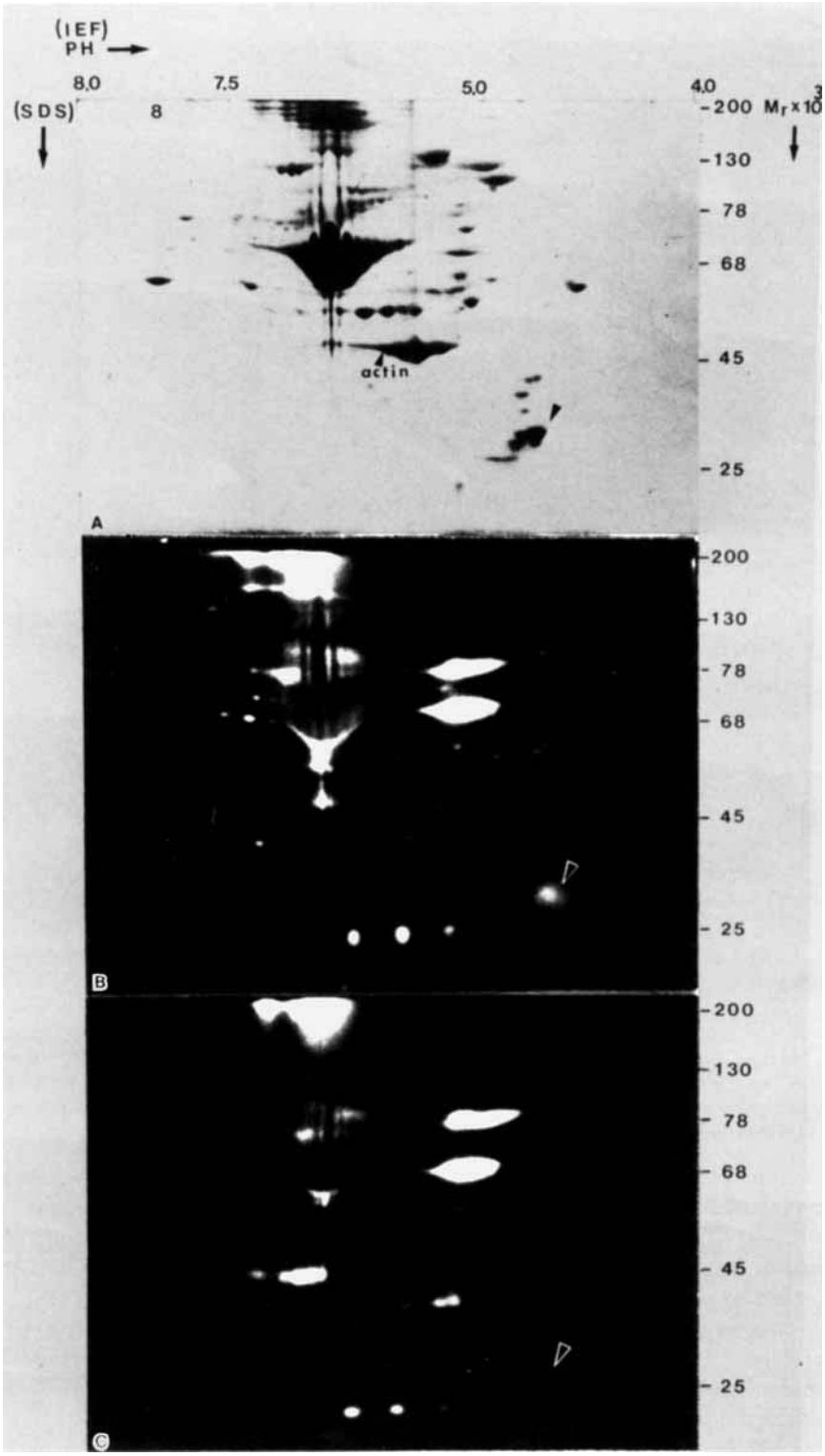


Fig. 1. One-dimensional SDS-PAGE analysis of the whole platelet proteins and platelet cytoskeleton proteins. A,B) Autoradiograms of ^{32}P-labeled whole platelet proteins treated with TPA (A) and 4α-PDD (B). C,E) Coomassie blue staining patterns (C) and autoradiogram (E) of ^{32}P-labeled platelet cytoskeleton-associated proteins isolated from platelets treated with TPA. D,F) Coomassie blue staining patterns (D) and autoradiogram (F) of ^{32}P-labeled platelet cytoskeleton-associated proteins isolated from platelets treated with 4α-PDD. Molecular weight markers are 200 KD, myosin heavy chain; 130 KD, β-galactosidase; 78 KD, lactoperoxidase; 68 KD, bovine albumin; 45 KD, actin; 25 KD, concanavalin A; 17 KD, trypsin inhibitor).

1A), four (42 KD, 30 KD, 23 KD, and 20 KD) are detected in the cytoskeleton complex (detergent-insoluble fraction). Most of the platelet phosphoproteins, including the 40 KD protein, are found in the soluble fraction following nonionic detergent treatment (data not shown). The 30 KD protein is the only cytoskeleton-associated protein phosphorylated during TPA treatment but not 4α-PDD treatment (Fig. 1E,F). It is conceivable that the phosphorylation of 30 KD protein may be involved in the preferential recruitment of myosin molecules into the cytoskeleton (Fig. 1C–F). Consequently, we felt that is was important to characterize further this actomyosin-associated 30 KD phosphoprotein.


(IEF)
PH
8.0
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8
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- 68
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- 25
actin
A
-200
-130
- 78
- 68
- 45
- 25
B
-200
-130
- 78
- 68
- 45
- 25
C

Fig. 2

Initial Characterization of the 30 KD Phosphoprotein

Two-dimensional gel electrophoresis. Figure 2 shows the two-dimensional electrophoresis patterns for cytoskeleton preparations from both TPA- and 4α-PDD-treated platelets. Of particular interest is the set of proteins (indicated by the arrowhead) with an approximate molecular weight of 30,000 dalton (30 KD) and a pI = 4.6. These characteristics closely resemble those previously described for nonmuscle tropomyosin [19]. Furthermore, one or more of the proteins in this set appears to be phosphorylated by TPA treatment (Fig. 2B), but not by 4α-PDD treatment (Fig. 2C).

One-dimensional urea-SDS gel electrophoresis. Previous studies have also determined that all tropomyosins, including platelet tropomyosin (mol wt 30,000 daltons), have the following rather unique characteristic: their electrophoretic mobility is significantly reduced on urea-SDS gels (ie, appears in the 42 KD range) compared to the standard SDS gel [20]. In this study we observed the same apparent reduction in mobility of the 30 KD phosphoprotein on urea-SDS gels (Fig. 3). Specifically, on the urea-SDS gel the intensity of the 30 KD band is significantly reduced and a new composite band appears in the 42–45 KD region (Fig. 3C,D).

Correlation of 30 KD Tropomyosinlike Protein Phosphorylation and Platelet Activation

When phosphorylation of 30 KD tropomyosinlike protein is minimal in resting (untreated) platelets or those treated with a low dose of TPA (10^{-9} M) or 4α-PDD (Fig. 5), the platelets display a regular discoid shape (Fig. 4A,B) and a minimal capability for secretion (Fig. 5). Increasing the TPA concentration to 10^{-8} M causes detectable amounts of 30 KD phosphorylation (Fig. 5) along with platelet granule release (Fig. 5) and a definite shape change characterized by numerous surface projections (Fig. 4C). In the presence of 10^{-7} M TPA, phosphorylation of the 30 KD tropomyosinlike protein, and serotonin secretion increase concomitantly (Fig. 5). Small platelet aggregates are initially observed under this condition (Fig. 4D). At TPA doses of 10^{-6}–10^{-5} M, phosphorylation of 30 KD tropomyosinlike protein appears to be reaching a maximal level along with granule secretion (Fig. 5), whereas platelet aggregation (Fig. 4) appears to be signifiantly increased. In contrast, 4α-PDD up to a concentration of 10^{-5} M does not induce any significant amount of 30 KD phosphorylation, serotonin release, or any obvious shape changes in the platelets (Fig. 4B,5). These data suggest that phosphorylation of the 30 KD protein *may be* required for platelet activation.

DISCUSSION

Tropomyosin is an important component of the contractile apparatus of both muscle and nonmuscle cells. In skeletal muscle, there are two forms of the tropo-

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Fig. 2. Two-dimensional SDS-PAGE analysis of platelet cytoskeleton-associated proteins isolated from ^{32}P-labeled platelets treated with TPA (A, Coomassie blue staining; B, autoradiogram) and 4α-PDD (C, autoradiogram). The Coomassie blue staining pattern observed in 4α-PDD treated samples was the same protein as that shown in (A). The arrowhead indicates the Coomassie-stained 30 KD (A) and its phosphorylated form (B) detected in the TPA-treated platelet cytoskeleton material. With 4α-PDD-treated platelets, no ^{32}P-labeled 30 KD was detected in the cytoskeleton material, even when twice the normal amount of protein (~300 μg) was loaded on the gels (C).

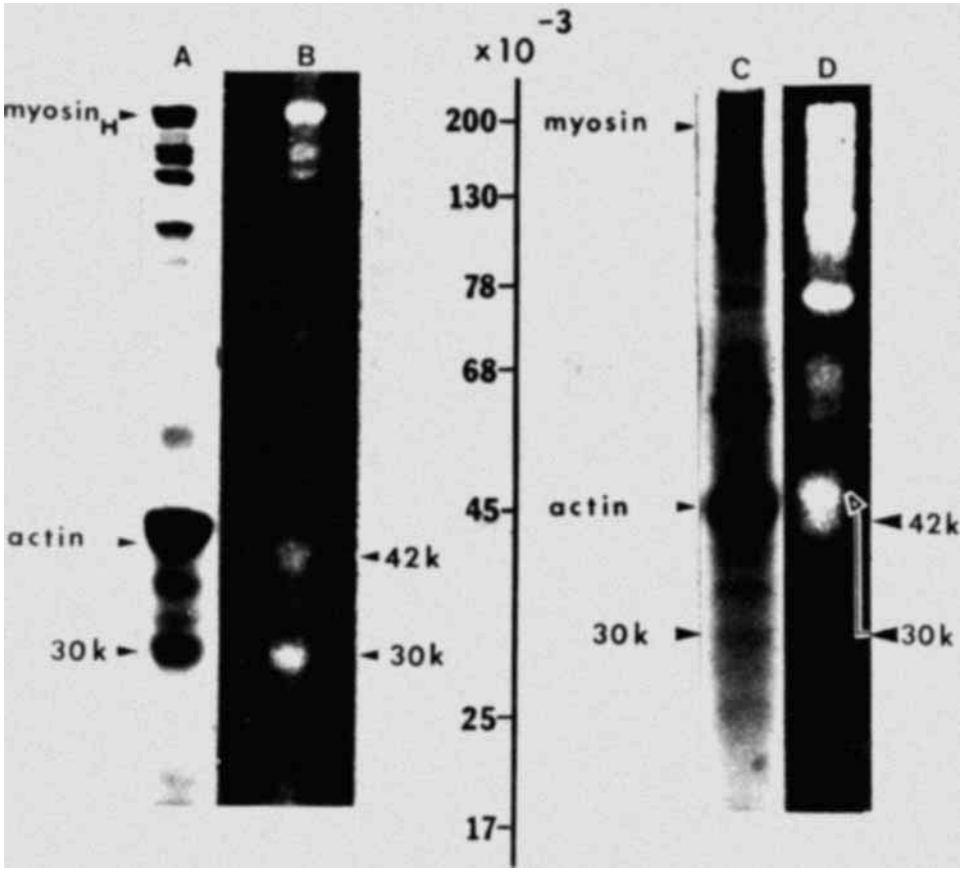

Fig. 3. One-dimensional SDS gel and SDS-urea gejl analysis of platelet cytoskeleton-associated proteins isolated from platelets treated with TPA. A,B) Coomassie blue staining pattern (A) and autoradiogram (B) of ^{32}P-labeled platelet cytoskeleton isolated from TPA-treated material using standard SDS-PAGE. C,D) Coomassie blue staining pattern (C) and autoradiogram (D) of ^{32}P-labeled platelet cytoskeleton isolated from TPA-treated material using SDS-PAGE gel plus 6 M urea.

myosin subunits, designated and with apparent mol wt close to 33,000 daltons and 36,000 daltons, respectively [21–23]. Muscle tropomyosin, together with troponin, plays a central role in regulating the sliding of myosin heads along the actin filaments [24–27]. Smooth muscle tropomyosin has been found to have either one or two subunits (mol wt ~35,000 daltons) depending on the tissue analysed [28]. Tropomyosinlike proteins have also been isolated from platelets [20,29], brain [30,31], pancreas [31], and fibroblasts [31–33]. All of the nonmuscle tropomyosins have a smaller mol wt, ~30,000 daltons. Tropomyosin in both smooth muscle and nonmuscle systems does not appear to interact with the sliding thin filament mechanism, but instead is regulated through myosin-linked processes [34,35]. It has been reported that platelet tropomyosin enhances the Mg^{2+}-ATPase activity of reconstituted skeletal actin and platelet myosin [36]. Molecular details concerning nonmuscle tropomyosin regulation of actomyosin interactions are not well understood at the present time.

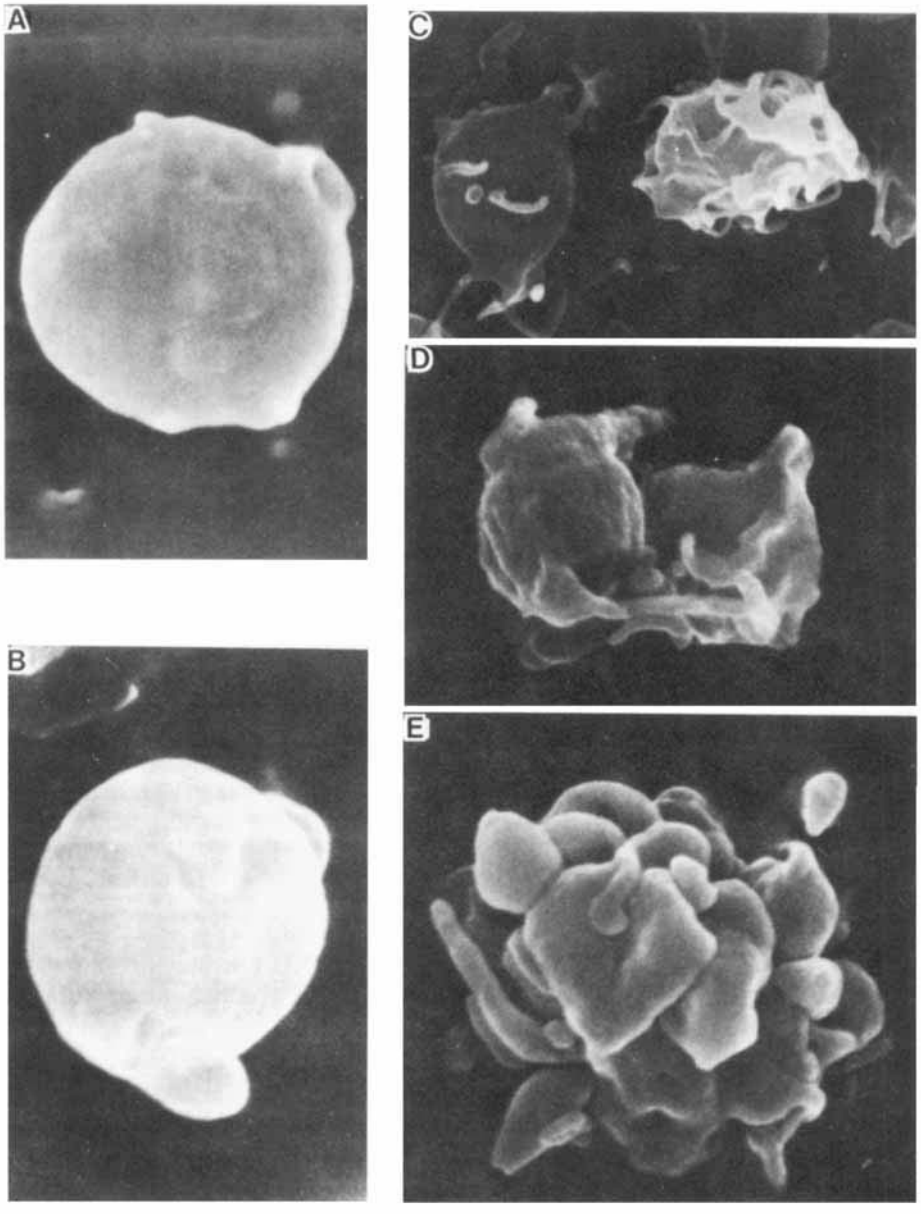

Fig. 4. Scanning electron micrographs of treated and untreated human platelets. A) Resting platelets with no treatment. B) Platelets treated with 4α-PDD (at doses ranging from 10^{-5} M to 10^{-9} M). C) Platelets treated with TPA (10^{-8} M). D) Platelets treated with TPA (10^{-7} M). E) Platelets treated with TPA (10^{-6}–10^{-5} M). All magnigifications are ×11,000.

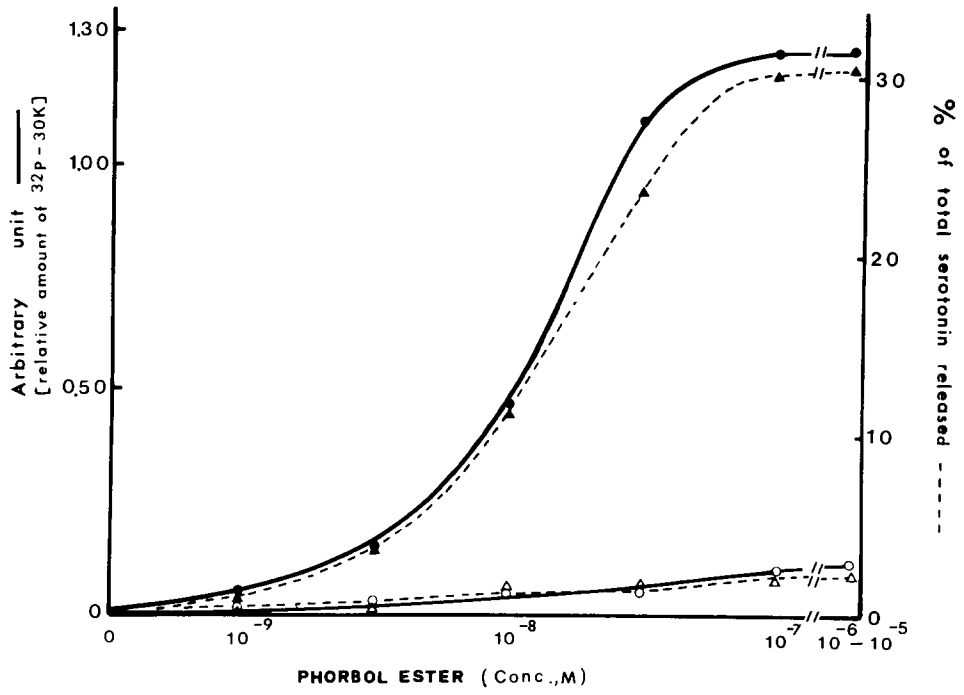

Fig. 5. Correlation of 30 KD protein phosphorylation and ^{3}H-serotonin released. The solid lines indicate the relative amount of phosphorylation of 30 KD protein (in arbitrary units) measured from densitometric tracings of the autoradiograms of ^{32}P-labeled platelet proteins treated with TPA (●—●) or 4α-PDD (○—○). The dotted lines represent the precentage of total ^{3}H-serotonin released during treatment with various concentrations of TPA (▲---▲) or 4α-PDD (△---△).

In this study, our initial characterization of the 30 KD phosphoprotein indicates that this protein displays at least five of the properties reported for tropomyosin isolated from platelets and other nonmuscle cultured cells. For example, 1) the 30 KD protein has a mol wt of 30,000 daltons on one- and two-dimensional SDS-PAGE (Fig. 1) [20,29]; 2) it displays a typical tropomyosin isoelectric point, pI of 4.6 (Fig. 2) [19]; 3) it exhibits a characteristically slower migration rate on SDS-PAGE in the presence of 6 M urea (Fig. 3) [20]; 4) it is closely associated with an actomyosin-containing cytoskeleton (Figs. 1–3) [32]; and 5) the 30 KD protein is phosphorylated (Fig. 2A,B) [19,37,38]. Considering the five aforementioned similarities with nonmuscle tropomyosin, we would like to propose that the 30 KD phosphoprotein is, in fact, platelet tropomyosin. Further work, including immunological cross-reactivity studies of the 30 KD protein with nonmuscle antitropomyosin and peptide mapping analyses of 30 KD and known platelet tropomyosin, is underway in order to make a definitive identification.

The phorbol ester TPA is mitogenic for a number of fibroblasts [1] and causes differentiation in other types of cells [2]. Biochemical analyses indicate that the phospholipid-dependent protein kinase C is the primary cellular binding site or receptor for TPA. TPA is also known to be an effective activator of protein kinase C

in vitro, and can substitute for the physiological activator, diacylglycerol, which is produced during horomone-mediated stimulation of phosphatidylinositol turnover [3].

Previous work has shown that TPA induces platelet secretion and aggregation [7–9] and that TPA-induced activation of platelets is accompanied by the time-dependent incorporation of ^{32}P into both 20 KD and 40 KD polypeptides, presumably because of the stimulation of protein kinase C [11–13]. The identity and function of the 40 KD phosphoprotein is still unknown. However, the 20 KD phosphoprotein has been identified as the regulatory light chain of myosin [39]. It has also been reported that TPA-stimulated protein kinase C phosphorylates sites on the myosin light chain different from those phosphorylated by myosin light chain kinase [39].

In this study, we have also observed the TPA-induced phosphorylation of 20 KD and 40 KD proteins (Fig. 1A,B). The 40 KD protein is a detergent-soluble protein and the 20 KD protein, which is most likely myosin light chain, remains associated with the cytoskeleton (Fig. C–F). Most importantly, we have found that TPA also induces the phosphorylation of a 30 KD tropomyosinlike protein (Figs. 1–3) (probably also mediated through protein kinase C activity). We believe the reason the 30 KD protein was not observed previously is that the gel system in those studies did not have sufficient resolution to resolve all three proteins (ie, 20 KD, 30 KD, 40KD). Our recent preliminary data indicate that 30 KD protein phosphorylation occurs following thrombin activation of platelets.

Although phosphorylation of tropomyosin has been reported in skeletal muscle [37], chick embryo muscle [19], and rat kidney cells [38], the functional importance of phosphorylated tropomyosin is not known at this time. Our current results indicate that, along with phosphorylation of the 30 KD tropomyosinlike protein, there is a concurrent accumulation of myosin molecules (both heavy and light chains) into the cytoskeleton (Fig. 1C–F). This result suggests that the phosphorylation of the 30 KD tropomyosin may be important for some myosin-linked process that is required for platelet contractility during platelet activation. Furthermore, the fact that the degree of phosphorylation of the 30 KD tropomyosinlike protein is exactly correlated with the amount of granule secretion and platelet shape change occurring during activation also strongly suggests the existence of a close relationship between 30 KD phosphorylation and platelet activation. Of course, it is possible that a complex mechanism involving the coordination of the phosphorylated 20 KD (myosin light chain), the 40 KD (a soluble protein), and the 30 KD (tropomyosinlike) proteins could take place during the overall platelet activation process. Further studies with regard to the regulation of platelet activation are under way.

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