Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 37-44

PROSTACYCLIN INHIBITION OF PHOSPHATIDIC ACID SYNTHESIS IN HUMAN PLATELETS IS NOT MEDIATED BY PROTEIN KINASE C

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Received February 22, 1984

SUMMARY. The activation of protein kinase C in human platelets by phorbol-12,13- dibutyrate (PDBu)* results in the phosphorylation of a 40,000 dalton protein. This phosphorylation is time- and concentration-dependent. Maximal phosphorylation is rapid and is not affected by indomethacin or prostacyclin. PDBu does not promote activation of the phosphodiesteratic cleavage (phospholipase C) of the inositol phospholipids and the subsequent formation of 1,2-diacylglycerol or its phosphorylated product, phosphatidic acid. If platelets exposed to PDBu are subsequently stimulated with thrombin, this stimulus does not initiate further 40,000 dalton protein phosphorylation but will promote the formation of phosphatidic acid and also the phosphorylation of a 20,000 dalton protein (myosin light chain). However, prostacyclin will prevent the subsequent stimulation of phosphatidic acid synthesis by thrombin in a concentration-dependent manner. The fact that prostacyclin can affect the response to thrombin, even in the presence of phorbol ester, supports the idea that the enzymes related to the formation of phosphatidic acid or inhibition of its synthesis are not related to the phosphorylated 40K protein.

Tumor-promoting phorbol esters bind to the platelet plasma membrane and directly activate protein kinase C without inducing phosphatidylinositol turnover (1). In platelets, a protein with an approximate molecular weight of 40,000 dalton (40K protein) seems to be a substrate for the protein kinase C activity (2,3). It is recognized that 40K protein phosphorylation is dependent on phosphatidylserine and Ca^{++} and markedly enhanced by unsaturated 1,2-diacylglycerol (1-3). Phorbol esters can substitute for 1,2-diacylglycerol in this activation (1-3).

Stimulation of platelets with activators such as thrombin (1-4), platelet-activating factor (5) and arachidonic acid (6) promotes the phosphorylation of the 40K protein in close parallel to the formation of phosphatidic acid. Phosphatidic acid is formed by phosphorylation of the

Abbreviations: PDBu, phorbol-12,13-dibutyrate; 40,000 dalton protein, 40K protein; 20,000 dalton protein, 20K protein.

0006-291X/84 $1.50

1,2-diacylglycerol derived by degradation of inositol phospholipids due to the activity of phospholipase C (7). Moreover, 40K protein-phosphorylation and phosphatidic acid appearance are associated with the shape change of human platelets in the total absence of the release of serotonin and platelet aggregation (4-6).

The evidence presented here indicates that concentrations of phorbol esters that produce phosphorylation of the 40K protein do not affect the thrombin-induced synthesis of phosphatidic acid or the prostacyclin dependent inhibition of phosphatidate synthesis.

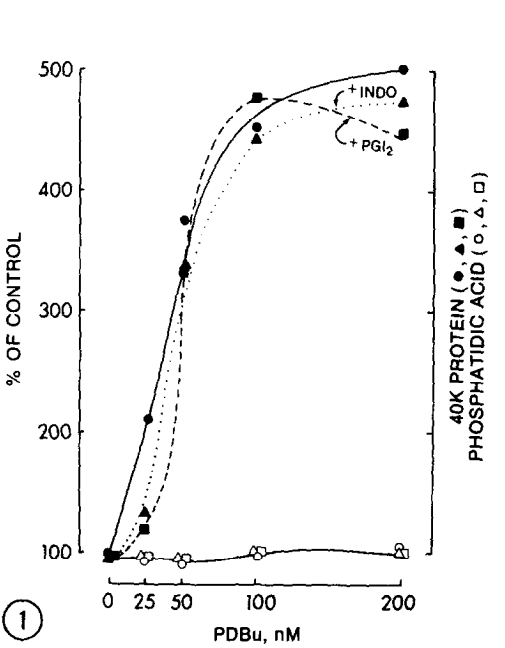
MATERIALS AND METHODS

Phorbol-12,13-dibutyrate was from P-L Biochemicals, Inc., Milwaukee, WI. Other materials were obtained as previously reported (5-8).

Human platelets have been isolated from platelet rich plasma in the presence of prostacyclin (9,10) and labeled with ^{32}P as has been previously reported (5-8). Platelets were finally resuspended in a modified Tyrode-Hepes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5mM Hepes, 5 mM glucose, 1 mM EGTA, pH 7.4) and platelet concentration adjusted to 7.5 x 10^8/ml. Samples (0.5 ml or 1.0 ml) were used for measurement of [^{32}P]phosphatidic acid (5-8,12) and [^{32}P]protein phosphorylation (4-6). Gels containing 11% sodium dodecyl sulfate-polyacrylamide were used for the separation of proteins (13). Release of ATP was measured in a lumiaggregometer (Chronolog) using the luciferase reaction. All experiments are representative of at least four that gave qualitatively very similar results. Results are within ±10% of the mean.

RESULTS

Phosphorylation of a 40,000 Dalton Protein by Phorbol Esters in Human Platelets. The phosphorylation of the 40K protein by PDBu (1) is concentration- and time-dependent (Figs. 1-2) and is not affected by indomethacin or prostacyclin (Fig. 1). PDBu induces maximal phosphorylation of the 40K protein in less than 1 min (Fig. 2). Phorbol esters also induce some stimulation of the phosphorylation of a 20,000 dalton (20K) protein which is related to myosin light chain (Fig. 2). It has been reported that phorbol esters do not activate phospholipase C as determined by the formation of 1,2-diacylglycerol (1). In intact platelets 1,2-diacylglycerol is rapidly phosphorylated by 1,2-diacylglycerol kinase to phosphatidic acid (5-8,12,14,15). Our results show (Figs. 1, 2 and 3) that phorbol esters do not promote formation of [^{32}P]phosphatidic acid in human platelets.



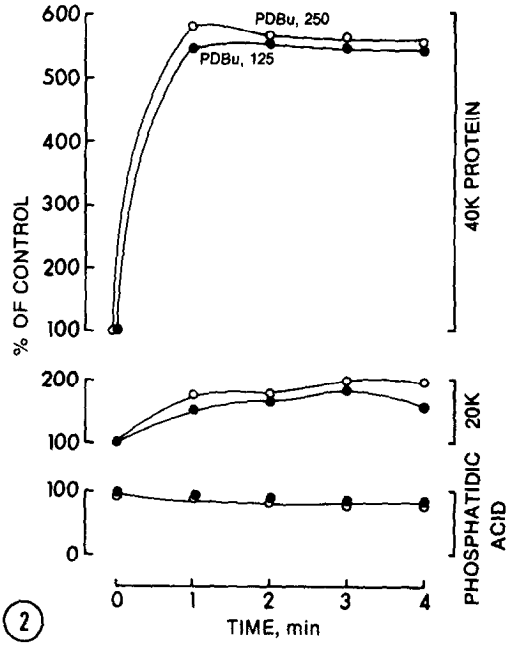


Fig. 1 Effect of various concentrations of phorbol-12,13-dibutyrate (PDBu) on phosphorylation of a 40000 dalton (40K) protein. Action of indomethacin and prostacyclin. Samples (1.0 ml) of suspensions of washed human platelets (7.5 x 10^8/ml) prelabeled with ^{32}P were preincubated without additions (○,●) or with the addition of 10 μM indomethacin (△,▲) or 4 ng/ml of prostacyclin (□,■) in the aggregometer tubes at 37°C for 3 min and subsequently stimulated with various concentrations of PDBu for 90 s. A sample (0.1 ml) was quenched for separation of proteins on 11% polyacrylamide gels and the remaining sample (0.9 ml) was extracted with chloroform/methanol for subsequent chromatographic separation of phosphatidic acid. Results are expressed as % of unstimulated controls. Closed symbols indicate [^{32}P] 40K protein while open symbols represent [^{32}P]phosphatidic acid.

Fig. 2 Effect of phorbol-12,13-dibutyrate (PDBu) on the phosphorylation of 20,000 (20K) and 40,000 (40K) dalton proteins. Samples of platelets as in Fig. 1 were stimulated with two different concentrations of PDBu, 125 nM (●) or 250 nM (○). Other details as in Fig. 1.

Consecutive Action of Phorbol Esters and Thrombin on Human Platelets.

Platelets have been treated with concentrations of PDBu that induce maximal phosphorylation of the 40K protein and then exposed to thrombin. Figure 3 shows one of such experiments in which platelets were preincubated without or with PDBu (200 nM) for 1 min and then stimulated with different concentrations of thrombin for 30 s. PDBu produces phosphorylation of the 40K protein, a limited phosphorylation of the 20K protein and no stimulation of the formation of [^{32}P]phosphatidic acid. The subsequent action of thrombin does not increase 40K protein phosphorylation further but does

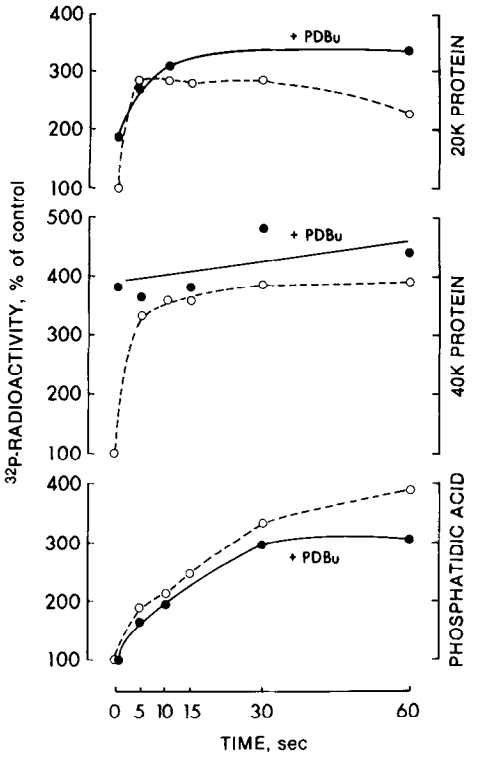


Fig. 3 Effect of preincubation with phorbol-12,13-dibutyrate (PDBu) on the stimulation of platelets with thrombin. Samples of platelets as in Fig. 1 were treated without or with PDBu (200 nM) for 1 min and then stimulated with thrombin (0.5 units/ml) for different periods of time. Other details as in Figs. 1 and 2.

induce formation of [^{32}P]phosphatidic acid and phosphorylation of the 20K protein (Fig. 3). The phosphorylation of the 40K protein that precedes the action of thrombin does not modify the enzymatic activities that trigger formation of [^{32}P]phosphatidic acid or the phosphorylation of myosin light chain (20K protein).

Effects of PDBu on Platelet Responses. Figure 4 shows the recording for aggregation and release of ATP induced by thrombin (1 unit/ml). Preincubation of those platelets for 1 min with a concentration of PDBu (200 nM) that induces maximal phosphorylation of the 40K protein (Figs. 1, 2 and 3) only shows a minute increase in light transmission and no release of ATP (Fig. 4). The subsequent addition of thrombin brings about aggregation and release of ATP, concomitantly to the formation of [^{32}P]phosphatidic acid and phosphorylation of myosin light chain (20K protein) (Fig. 3).

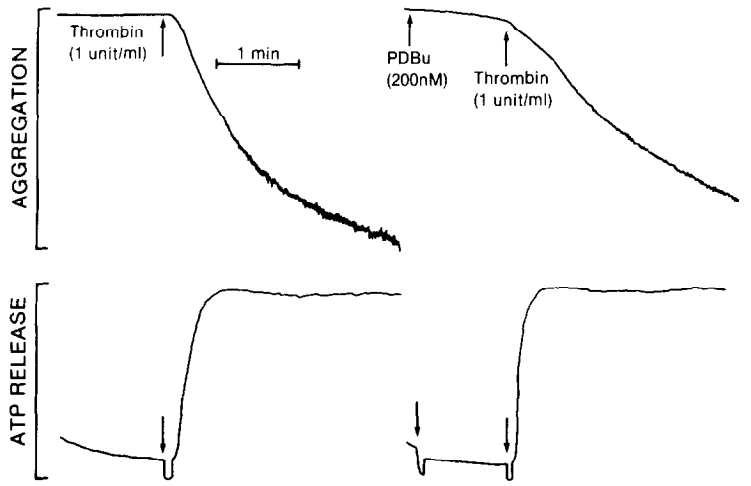


Fig. 4 Effect of thrombin and phorbol-12,13-dibutyrate (PDBu) on platelet aggregation and release of ATP. Aggregation and release of ATP measured by the luciferase reaction were simultaneously monitored using a lumiaggregometer (Chronolog). Samples of platelets (1 ml, 7.5 x 10^8 cells) were stimulated with thrombin, 1 unit/ml, or with PDBu, 200 nM, followed by thrombin, 1 unit/ml.

All experiments reported in the present study have been performed without addition of Ca^{2+} and in the presence of EGTA. It seems then that phorbol esters might be stimulating the phosphorylation of the 40K protein at a very low concentration of Ca^{++}. This concentration of Ca^{++} might be lower than the one needed to induce platelet secretion and aggregation. General mobilization of Ca^{++} in response to stimulation might be a separate and subsequent step to phosphorylation of the 40K protein and both reactions could then drive platelet aggregation.

Phosphorylation of the 40K Protein Does not Affect the Inhibitory Effect of Prostacyclin on Thrombin-Induced Formation of Phosphatidic Acid. Prostacyclin, or elevation of cyclic AMP, inhibits protein phosphorylation and formation of phosphatidic acid induced by thrombin, platelet-activating factor and arachidonic acid (5,6,14-16). Figure 5 shows that treatment of platelets with prostacyclin does not affect the PDBu-induction of 40K protein phosphorylation but, will prevent the subsequent stimulation of phosphatidic acid synthesis by thrombin, in a concentration-dependent manner. The fact that prostacyclin can affect the response to thrombin, even in the presence of phorbol ester, supports the idea that the enzymes

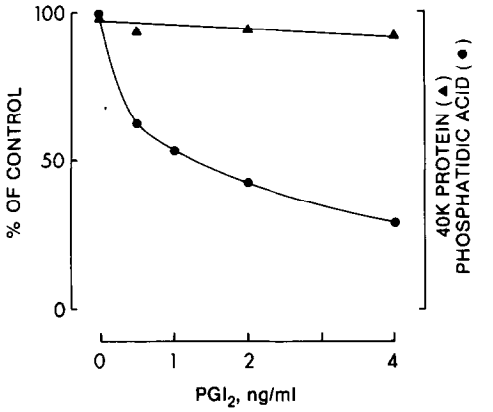


Fig. 5 Prostacyclin inhibits the formation of phosphatidic acid induced by thrombin in platelets sequentially treated with prostacyclin, phorbol-12,13-dibutyrate (PDBu) and thrombin. Samples of platelets as in Fig. 1 were incubated with different amounts of prostacyclin for 2 min, then treated with PDBu (200 nM) for 30 s and subsequently with thrombin (0.3 units/ml) for 30 s. Other details as in Figs. 1-3.

related to the formation of phosphatidic acid are not affected by phorbol esters or the phosphorylated 40K protein.

DISCUSSION

Stimulation of platelets by thrombin activates the phosphodiesteratic cleavage (phospholipase C) of the inositol phospholipids such as phosphatidylinositol, phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-bisphosphate (17,18). Phospholipase C seems to be a single enzyme (7,19,20) that degrades any of the three inositol phospholipids producing 1,2-diacylglycerol and myoinositol phosphates (myoinositol-1-monophosphate, myoinositol-1,4-bisphosphate and myoinositol-1,4,5-trisphosphate). 1,2-Diacylglycerol could then activate protein kinase C (1-3) and is also phosphorylated to phosphatidic acid which might be involved in the activation of phospholipases of the A_2 type (21). Recently, myoinositol-1,4,5-trisphosphate has been suggested to be a second messenger for mobilizing intracellular Ca^{2+} (22).

Formation of phosphatidic acid is a firm reflection of the stimulation of the phosphodiesteratic cleavage of the inositol phospholipids and there is a strict temporal and quantitative correlation between its formation and

phosphorylation of the 40K protein during the shape change of human platelets stimulated with platelet-activating factor (5), arachidonic acid (6), or thrombin (4).

Our present study describes conditions in which maximal activation of the kinase C that phosphorylates a 40K protein is dissociated from subsequent thrombin-induced formation of phosphatidic acid or platelet activation. Phosphorylation of the 40K protein does not affect the action of thrombin on the formation of phosphatidic acid, phosphorylation of myosin light chain (20K protein) and platelet activation. Moreover, we have observed that phosphorylation of the 40K protein by phorbol ester does not change the liberation of [^{14}C]arachidonic acid and the formation of [^{14}C]arachidonate metabolites derived from cyclooxygenase and lipoxygenase activities (not shown) in platelets that have been prelabeled, as previously described (16), with [^{14}C]arachidonic acid. Pretreatment of platelets with phorbol ester does neither influence the inhibitory effect of prostacyclin on thrombin-induced formation of phosphatidic acid. These observations indicate that the phosphorylation of the 40K protein does not affect the activity of enzymes such as phospholipase C, 1,2-diacylglycerol kinase (which are involved in the formation of phosphatidic acid), phospholipase A_2, cyclooxygenase and lipoxygenase (which are involved in the liberation and metabolism of arachidonic acid).

ACKNOWLEDGMENTS

Mr. Bryan Reep has provided excellent technical help.

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