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PLATELET Ca^{2+}-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE: EVIDENCE FOR PROTEOLYTIC ACTIVATION OF THE ENZYME IN CELLS TREATED WITH PHOSPHOLIPASE C[1]

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Summary: Incubation of human platelets with C. perfringens phospholipase C caused an increase in soluble protein kinase activity assayed in the presence of EGTA, and a decrease in Ca^{2+}/phospholipid-dependent protein kinase activity. Fractionation of extracts on DEAE-cellulose columns showed that phospholipase C treatment resulted in a new peak of protein kinase active in the presence of EGTA. On Sephadex G-100 chromatography this enzyme eluted as a single peak of protein kinase activity of MW about 50,000. An extract from untreated platelets eluted as a single peak of Ca^{2+}/phospholipid-dependent protein kinase of MW about 77,000. It was concluded that phospholipase C treatment resulted in the proteolysis of this latter enzyme to the lower MW form.

Recent studies have implicated activation of a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) in the sequence of reactions occuring in thrombin-stimulated platelets (1,2). Activation in the presence of phospholipid and low Ca^{2+} concentrations is initiated by DG[2], which transiently accumulates as a consequence of increased inositol lipid breakdown (3,4). Protein kinase C has been identified in other animal cells, and has been extensively purified (2,5,6). It has been speculated that activation of the enzyme may be a common element in the action of hormones which stimulate inositol lipid breakdown (1). Protein kinase C is also activated in vitro by phorbol ester tumor promoters (7), and the enzyme probably represents one binding site for these compounds (8-11).

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[2]Abbreviations: DMSO, dimethyl sulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate; BSA, bovine serum albumin; DG, 1,2-diacylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PDBu, phorbol-12,13-dibutyrate; PMSF, phenylmethylsulfonylfluoride.

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In platelets, promoters such as TPA directly activate protein kinase C in the absence of enhanced inositol lipid breakdown, by substituting for DG (12,13).

In addition to its reversible activation by Ca^{2+}, phospholipids and DG, protein kinase C is irreversibly activated by Ca^{2+}-dependent proteases (14). Proteolysis converts the kinase (MW about 77,000) to a fragment of MW about 51,000 which is catalytically active in the absence of Ca^{2+} and phospholipid. A protease has been purified from brain which catalyses this reaction at low Ca^{2+} concentrations and is strongly stimulated by phospholipid and DG (14). This latter observation suggests that the protease specifically attacks membrane-associated kinase, and that irreversible activation of protein kinase C may have physiological significance. In the present report we provide evidence that incubation of intact human platelets with phospholipase C stimulates the proteolytic cleavage of protein kinase C and the accumulation of Ca^{2+}/phospholipid-independent kinase activity.

MATERIALS AND METHODS

Materials: Diolein, histone (type III-S), PS, PMSF and C. perfringens phospholipase C (type XII) were obtained from the Sigma Chemical Co., St. Louis, USA. [γ-^{32}P]ATP (sp. act. 0.4-1x10^6 cpm/pmole) was prepared as described (15).

Methods: Human blood platelet concentrates were obtained fresh from a blood bank and washed platelets prepared as described (16). Platelets were washed and suspended at a final concentration of 1-2x10^9 cells/ml in platelet buffer containing glucose (5.5mM), Tris (15mM), NaCl (0.14M) and BSA (0.35%) at pH 7.4. When required, phospholipase C (2 µg/ml) was added to the platelet suspensions and incubation carried out at 37°. After incubation, platelets were collected by centrifugation (2000 g; 5 min; 4°), washed once with cold buffer and disrupted by sonication in a buffer containing sucrose (0.25M), Hepes (20mM), EDTA (2mM), EGTA (5mM), β-mercaptoethanol (10mM), leupeptin (0.01%) and PMSF (2mM) at pH 7.5. The homogenate was centrifuged (100,000 g; 30 min; 4°) and the supernatant assayed directly for protein kinase activity or chromatographed as described below.

Ion-exchange chromatography was carried out at 4° on DE-52 (Whatman) columns (8 x 2 cm) pre-equilibrated with buffer containing Hepes (10mM), EDTA (2mM), EGTA (5mM) and β-mercaptoethanol (10mM) at pH 7.5. The column was eluted with a linear gradient (100 ml total vol.) of 0-0.4M NaCl in the same buffer containing 0.001% leupeptin; 1.7 ml fractions were collected. Gel filtration was carried out on a Sephadex G-100 column (47 x 2 cm) equilibrated with the above buffer and eluted in buffer containing 0.001% leupeptin. Fractions (1.7 ml) were collected and assayed for protein kinase activity at 30° for 10 min. Assays contained enzyme extract (30 µl of column eluant or 10 µl of supernatant fractions), [γ-^{32}P]ATP (5 µM), $MgCl_2$ (5mM), type III-S histone (20 µg) and EGTA (1mM) or Ca^{2+} (5mM)

Table 1. Effect of phospholipase C treatment on protein kinase activity in soluble extracts from human platelets

Incubation time (min)	Protein kinase activity (cpm x 10^{-3}) + EGTA	+ Ca^{2+}/phospholipid
0	3136	21939 (7)
10	4991	18262 (3.7)
30	14133	19460 (1.4)
45	10127	9532 (0.9)

Platelets were incubated with phospholipase C (2 μg/ml) as described in the Materials and Methods. At appropriate times, platelets were washed, sonicates prepared and assayed for protein kinase activity in the presence of EGTA or Ca^{2+}/phospholipid. Each value is the mean of triplicate determinations. Values in parentheses are the ratios of kinase activity in the presence of Ca^{2+}/phospholipid to activity in the presence of EGTA.

plus PS (20 μg/ml) and diolein (4 μg/ml) in a final volume of 120 μl. Reactions were terminated and acid precipitable material collected as described (17).

Phospholipids in washed platelets were extracted and separated by 2-dimensional chromatography (18); phosphate associated with individual phospholipids was determined as described (19).

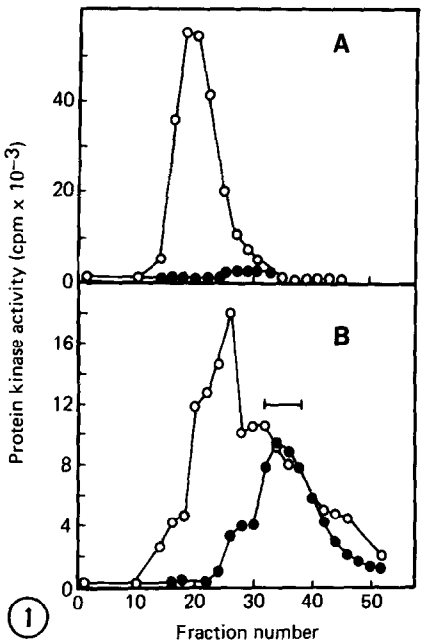
Protein was determined as described (20) using BSA as a standard.

RESULTS AND DISCUSSION

Incubation of human platelets with phospholipase C caused a decrease in the soluble protein kinase activity stimulated by Ca^{2+} and phospholipid (Table 1). For up to 30 min incubation, this was associated with an increase in activity assayed in the presence of EGTA. After 45 min, total kinase activity decreased, as did the activity of lactic dehydrogenase in soluble preparations (data not shown). It is probable that prolonged exposure to phospholipase caused the platelets to become 'leaky' to macromolecules.

One explanation of the above result is that phospholipase C treatment activated a protease which catalysed limited proteolysis of the Ca^{2+}-phospholipid dependent protein kinase, converting the enzyme into a form active in the absence of Ca^{2+} and phospholipid (14). Experiments designed to test this possibility are summarized in Figs. 1 and 2.

Fig. 1 shows the DEAE cellulose elution profiles of protein kinases in soluble preparations from untreated and phospholipase C-treated



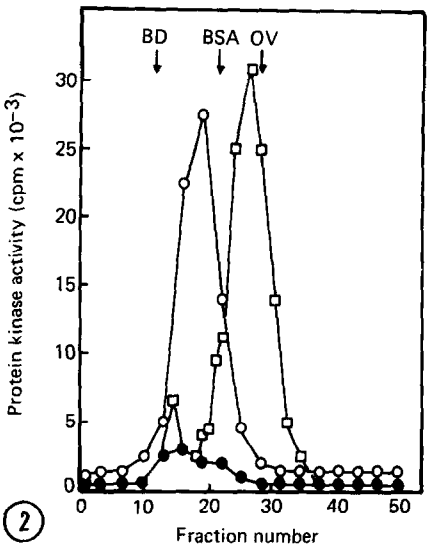


Figure 1. Effect of phospholipase C on platelet protein kinases. Panel A: Soluble extract from untreated platelets (25 mg protein) was chromatographed on DEAE cellulose as described in Materials and Methods. Aliquots were assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}/phospholipid (O).
Panel B: Platelets (10^9/ml) were incubated with phospholipase C (2 μg/ml) for 10 min. The soluble extract (43 mg protein) was chromatographed on DEAE cellulose as described in Materials and Methods. Aliquots were assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}/phospholipid (O).

Figure 2. Chromatography of platelet protein kinases on Sephadex G-100. Fractions 32-38 from the experiment shown in Fig. 1 (panel B) were pooled, and an aliquot (2.2 ml) was chromatographed on a Sephadex G-100 column as described in Materials and Methods. Aliquots (60 μl) were assayed for protein kinase activity in the presence of EGTA (□) using a 15 min assay time. Soluble extract from untreated platelets (2.2 ml, 29 mg protein) was separately chromatographed on the same column. Aliquots were assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}/phospholipid (O). The arrows show the elution points of blue dextran (BD), BSA and ovalbumin (OV).

platelets. The extract from untreated platelets showed a single peak of activity which was almost totally dependent on the presence of Ca^{2+} and phospholipid. Extracts prepared from platelets exposed to phospholipase C (2 μg/ml) for 10 min contained an additional peak of kinase, active in the presence of EGTA, which eluted at a higher salt concentration. Fractions with highest Ca^{2+}-phospholipid independent activity (shown with a bar in Fig. 1) were pooled, and an aliquot chromatographed on a Sephadex G-100 column (Fig. 2). A sharp peak of kinase, active in the presence of EGTA,

eluted just ahead of ovalbumin indicating a MW of about 50,000. Fig. 2 also shows the elution of kinase activity in extracts from untreated platelets. A single peak of Ca^{2+} and phospholipid-dependent protein kinase eluted just ahead of BSA, consistent with the reported MW of about 77,000 for this enzyme (14). The simplest explanation of these results is that phospholipase C treatment activates the proteolytic cleavage of protein kinase C resulting in the accumulation of a kinase of lower MW active in the absence of Ca^{2+} and phospholipid. However formal proof of this would require isolation of the other presumptive cleavage product and direct demonstration that the lower MW kinase is derived from protein kinase C (perhaps using immunological techniques). The phospholipase C used contained no detectable protease activity (21), and had no effect on kinase activity when incubated with DEAE cellulose-purified protein kinase C.

Several experiments were carried out to determine the effect of phospholipase C on the structural integrity of the platelets. In the experiments described in Figs. 1 and 2, platelets were incubated with phospholipase for 10 min. This treatment did not alter the activity of lactate dehydrogenase in soluble extracts (data not shown) indicating that lysis of the platelets did not occur. Similarly, incubation for up to 10 min caused no change in the total lipid phosphate or in the relative proportions of PC and PE (Table 2). Longer incubation times caused a decrease in total phospholipid, with a marked drop in PC content. It is noteworthy that phospholipase C treatment resulted in PA accumulation (Table 2), presumably arising from the action of DG kinase on DG, a product of the phospholipase reaction. Earlier studies have shown that incubation of platelets with C. perfringens phospholipase C causes the accumulation of DG (3).

The above results suggest that the observed changes in protein kinases are due to relatively minor perturbation of the platelet plasma membrane by phospholipase C attack.

Incubation of platelets with phospholipase C also caused some increase in protein kinase C activity extractable from the particulate fraction.

Table 2. Effect of phospholipase C on platelet phospholipids.

Incubation time (min)	Phospholipid Phosphate (nmoles/10^9 platelets) PA	PC	PE	Others	Total
0	5.4	315	112	354	786
5	10.2	339	124	390	863
10	29.1	286	120	348	783
30	39.6	163	104	287	594
60	36.9	37.8	60	258	393

Platelets (10^9/ml) were incubated with phospholipase C (2 ug/ml). After appropriate incubation times, platelets were collected by centrifugation and phospholipids extracted and separated by 2-dimensional chromatography as described in the Materials and Methods section. Each value is the mean of duplicate determinations.

After 10 min incubation the activity of Ca^{2+}/phospholipid-dependent kinase extractable with 1% NP40 (22) increased about 2-fold (data not shown). A similar increase was observed after incubation with TPA (10^{-7} M). Exposure to phospholipase C also caused a transient increase (about 2-fold) in [^{3}H]PDBu binding to the particulate fraction. Consequently, in view of the reported activation of one form of brain Ca^{2+}-dependent protease by DG and phospholipid (14), it is possible that association of soluble protein kinase C with membrane precedes its proteolytic cleavage. However, it should be stressed that it has not been established that platelets contain a protease analogous to the brain enzyme.

Irreversible activation of protein kinase C could have physiological significance if, for example, the catalytic fragment produced by proteolysis had access to different substrates than enzyme activated by association with membrane. In other experiments, we have shown that TPA causes a similar, although less marked, accumulation of Ca^{2+}/phospholipid-independent protein kinase activity in platelet extracts (manuscript in preparation). It will be of importance to determine whether this phenomenon occurs in other cell types, and, if so, its effect on protein phosphorylation patterns.

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