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MODULATION OF Ca^{2+}-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN PLATELETS TREATED WITH A TUMOR-PROMOTING PHORBOL ESTER[1]

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Summary: Incubation of human platelets with 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a rapid decrease in soluble Ca^{2+}, phospholipid-dependent protein kinase activity (protein kinase C) and an increase in protein kinase C associated with the particulate fraction. TPA also induced an increased activity of a Ca^{2+}, phospholipid-independent protein kinase activity in both the soluble and the particulate fractions of platelets. This latter kinase eluted from DEAE cellulose columns at a higher salt concentration than protein kinase C, and was shown by Sephadex G-100 chromatography to have a MW of approx. 50,000 compared with an MW of 80,000 for protein kinase C. The data suggest that TPA treatment of platelets causes irreversible activation of protein kinase C by proteolysis of the enzyme to a form active in the absence of Ca^{2+} and phospholipid.

We have recently reported that brief exposure of human platelets to Clostridium perfringens phospholipase C causes a decrease in the activity of cytosolic Ca^{2+}, phospholipid-dependent protein kinase (protein kinase C) and increase in kinase activity assayed in the presence of EGTA (1). These changes were accompanied by the accumulation of a Ca^{2+}, phospholipid-independent kinase of MW about 50,000, consistent with its derivation from the dependent enzyme (MW 80,000) by proteolytic cleavage. A Ca^{2+}-dependent neutral protease has been identified in brain which catalyses the proteolysis of protein kinase C and which is stimulated by phospholipid and DG[2], suggesting that the protease preferentially attacks membrane-associated kinase (2). Consequently it is

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[2]The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; DG, 1,2-diacylglycerol; PS, phosphatidylserine; PMSF, phenylmethylsulfonylfluoride; BSA, bovine serum albumin.

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possible that the trigger for the phospholipase C-induced proteolysis (1) is the accumulation of DG in platelet membranes.

These results prompted examination of the effects of the tumor promoter TPA on platelet kinases. TPA and other promoting phorbol esters interact directly with protein kinase C and activate the enzyme by substituting for DG (3-9). It seemed possible, therefore, that TPA would also induce the proteolytic activation of platelet protein kinase C, and possibly alter the cytoplasmic-particulate distribution of the enzyme as reported before for PYS cells (10).

Materials and Methods

Materials: Histone (type III-S), PS, PMSF and leupeptin were obtained from the Sigma Chemical Co., St. Louis, U.S.A. TPA was from P-L Biochemicals Inc., Milwaukee, Wis. [γ-^{32}P]ATP (sp. act. 0.7-3x10^3 cpm/pmole) was prepared as described (11).

Methods: Human blood platelet concentrates were obtained fresh from a blood bank and washed platelets prepared as described (1). Platelets were washed and suspended at a final concentration of 1-2 x 10^9 cells/ml in platelet buffer containing glucose (5.5 mM), Iris (15 mM), NaCl (0.14M) and BSA (0.35%) at pH 7.4. When required, TPA (10^{-7}M) or thrombin (1 unit/ml) was added to the platelet suspensions and incubation carried out at 37°C. After incubation, platelets were collected by centrifugation (2000g; 5 min; 4°C), washed once with cold buffer suspended (1-2 x 10^9 cells/ml) in a buffer (pH 7.5) containing sucrose (0.25 M), Hepes (20 mM), EDTA (2 mM), EGTA (5 mM), β-mercaptoethanol (10 mM), leupeptin (0.01%) and PMSF (2 mM), and disrupted by sonication (2-6 x 10 sec; setting 7; Soniprobe type 1130A, Dawe Instruments Ltd., London). The homogenate was centrifuged (100,000 g; 20 min; 4°C) and the supernatant assayed directly for protein kinase activity or chromatographed on DEAE-cellulose or Sephadex G-100 columns as described previously (1). The particulate fraction was resuspended in the same volume of the above buffer containing 0.2% Triton X-100, incubated at 4°C for 1 h and centrifuged (100,000 g; 30 min; 4°C). The supernatant was assayed for protein kinase activity directly or chromatographed on DEAE-cellulose or Sephadex G-100 columns (1).

Protein kinase assays contained enzyme extract (30 µl), Hepes (20 mM; pH 7.5) [γ-^{32}P]ATP (5 µM), $MgCl_2$ (5 mM), type III-S histone (20 µg) and EGTA (1 mM) or $CaCl_2$ (5 mM) plus PS (20 µg/ml) in a final volume of 120 µl. Reactions were terminated after 10 min at 30°C and acid precipitable material collected as described (12).

Protein was determined as described (13) using BSA as a standard.

Results and Discussion

Initial experiments established that incubation of human platelets with TPA caused rapid changes in the activity of soluble and particulate protein kinases (data not shown). In the soluble fraction, kinase activity assayed in the presence of EGTA increased (about 4-fold) and Ca^{2+}, PS-dependent activity

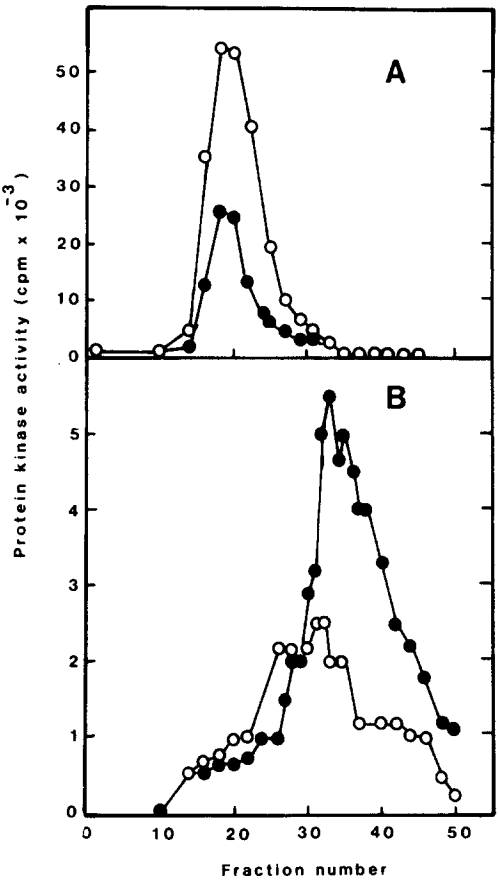

Figure 1. DEAE cellulose chromatography of soluble platelet protein kinases.

Platelets were incubated with DMSO or TPA (10^{-7}M) for 10 min, soluble extracts were prepared and aliquots (26 mg protein) were subjected to DEAE cellulose chromatography as described under Materials and Methods. Fractions were assayed for protein kinase activity in the presence of Ca^{2+}, PS (panel A) or EGTA (panel B). O—O, control platelets; ●—●, TPA-treated platelets.

decreased. Both activities increased rapidly (about 5-fold) in the Triton X-100 extract of the particulate fraction. The changes were observed after 1 min and were maintained for at least 10 min. These changes were not observed after incubation of platelets with 1 unit/ml of thrombin for between 0.5 and 5 min (data not shown).

The protein kinases present in soluble and particulate extracts from control and TPA-treated platelets were analysed by DEAE-cellulose chromatography (Figs 1 and 2). In these experiments it was repeatedly observed that the detectable Ca^{2+}, PS-dependent protein kinase activity was considerably

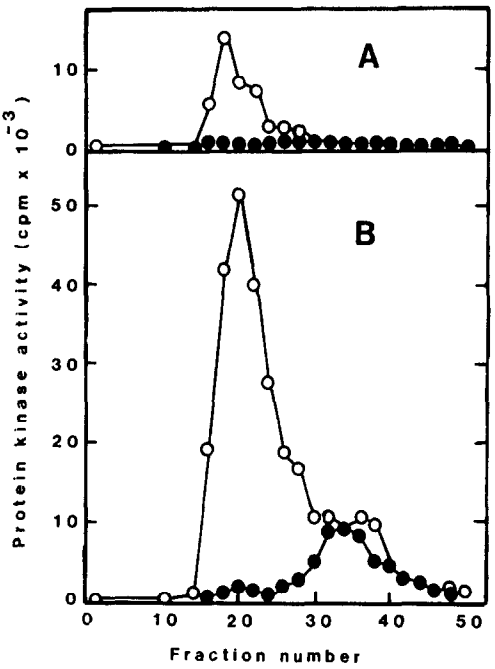

Figure 2. DEAE cellulose chromatography of particulate platelet protein kinases.

Platelets were incubated with DMSO or TPA (10^{-7}M) for 10 min, particulate fractions were prepared and extracted with Triton X-100 as described under Materials and Methods. Aliquots of the detergent extracts (11 mg protein) were chromatographed on DEAE cellulose columns. Column fractions from control (panel A) and TPA-treated (panel B) platelets were assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}, PS (O).

increased after DEAE-cellulose chromatography. This was presumably because of the presence of factors in the crude extracts which interfered with the protein kinase assay, as reported by several authors (14, 15). Incubation of platelets with TPA caused a marked decrease in soluble Ca^{2+}, PS-dependent kinase activity (Fig. 1) and an increase in activity extractable from the particulate fraction with Triton X-100 (Fig. 2). These data suggest that TPA induces an association of protein kinase C with cellular membranes as reported before for PYS cells (10).

In addition, TPA treatment resulted in the accumulation of protein kinase active in the absence of Ca^{2+} and PS in both the soluble and particulate fractions. This kinase eluted from DEAE cellulose at a higher salt concentration than the Ca^{2+}, PS-dependent enzyme (see Figs. 1 and 2). The relative amounts of the dependent and independent kinases present after TPA

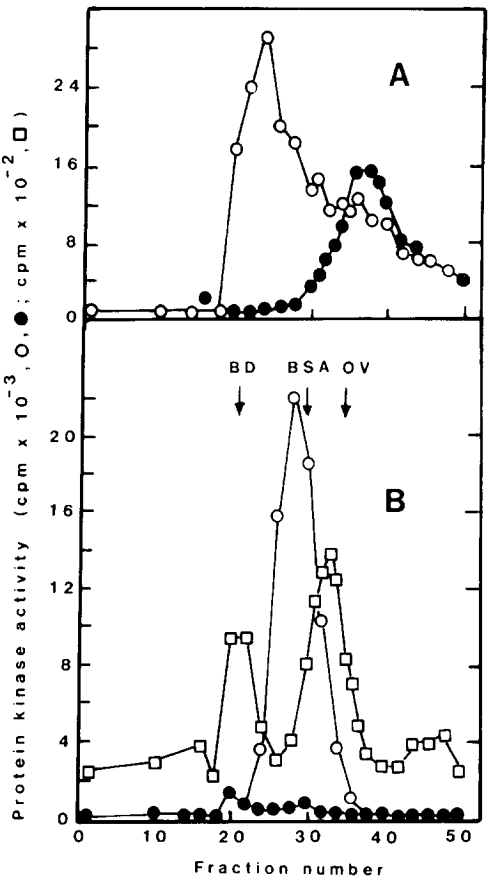

Figure 3. Sephadex G-100 chromatography of Ca^{2+}, PS-independent protein kinase.

Panel A. Platelets were incubated with TPA (10^{-7}M) for 10 min, the particulate fraction prepared and extracted with Triton X-100. An aliquot (32 mg protein) was chromatographed on a DEAE cellulose column and fractions assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}, PS (O).

Panel B. Fractions from the experiment in panel A with high Ca^{2+}, PS-independent activity (35-38) were pooled and an aliquot (2.2 ml; 1.1 mg protein) chromatographed on a Sephadex G-100 column (47 x 2 cm). Fractions were assayed for protein kinase activity in the presence of EGTA (□). Soluble extract from untreated platelets (13 mg protein) was separately chromatographed on the same column. Aliquots were assayed for protein kinase activity in the presence of EGTA (●) or Ca^{2+}, PS (O). The arrows show the elution points of blue dextran (BD), BSA and ovalbumin (OV).

treatment varied considerably between experiments (e.g. compare Figs. 2 and 3). In the experiment described in Fig. 3, fractions containing the highest Ca^{2+}, PS-independent activity were pooled and an aliquot chromatographed on a Sephadex G-100 column. A peak of protein kinase, active in the absence of Ca^{2+} and PS, eluted just ahead of ovalbumiun indicating a MW of about 50,000. A

similar result was previously reported for a soluble Ca^{2+}, PS-independent kinase which accumulated after incubation of platelets with phospholipase C (1). Fig. 3 also shows the elution of kinase activity in soluble extracts of untreated platelets. A single peak of Ca^{2+}, PS-dependent protein kinase eluted just ahead of BSA, consistent with a MW of about 80,000 for this enzyme (2).

In the present experiments, TPA treatment caused increased Ca^{2+}, PS-independent kinase activity in both the soluble and particulate fractions. Although there are other possible explanations, the most likely is that TPA induced the irreversible activation of protein kinase C by proteolytic cleavage to a protein of MW about 50,000 which is active in the absence of Ca^{2+} and phospholipid. This reaction has previously been shown *in vitro* using a Ca^{2+}-dependent neutral protease from brain (2, 16). Proteolysis occurred at relatively low Ca^{2+} concentrations (less than 10^{-5}M) and was stimulated by phospholipid and DG, suggesting that the membrane-associated form of protein kinase C was the preferred substrate (2). Consequently, although a similar protease has not been reported in platelets, the present results are consistent with a model in which the TPA-induced membrane-association of protein kinase C precedes its proteolytic cleavage.

The data do not establish with certainty that the lower MW kinase, active in the absence of Ca^{2+} and PS is derived from protein kinase C. This question is currently being explored using analysis of tryptic peptides derived from proteins phosphorylated *in vitro*.

Recognition that phorbol esters activate protein kinase C *in vitro* (3, 4) and *in vivo* (9, 17) is a major advance in understanding the biological actions of these compounds. The possibility that both irreversible (proteolysis) and reversible (membrane association) activation of the enzyme can occur therefore is of interest, particularly if the two forms of the enzyme have access to different substrates. In addition to the proteolysis product containing protein kinase activity, the non-catalytic fragment may also have functional significance. As discussed before (2) this fragment presumably carries the phospholipid-binding site of protein kinase C. Consequently it will be

important to establish unequivocally that the lower MW, Ca^{2+}, PS-independent kinase reported here is derived from protein kinase C and to determine whether a similar response to phorbol esters occurs in a range of cultured cell lines.

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