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# PHOSPHOLIPID TURNOVER AS A POSSIBLE TRANSMEMBRANE SIGNAL FOR PROTEIN PHOSPHORYLATION DURING HUMAN PLATELET ACTIVATION BY THROMBIN*

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SUMMARY: Human platelets contain a large amount of $Ca^{2+}$-activated, phospholipid-dependent protein kinase (protein kinase C). The activation of this enzyme is initiated by unsaturated diacylglycerol which results from the thrombin-induced phosphatidylinositol hydrolysis. Protein kinase C preferentially phosphorylates *in vitro* a polypeptide having a molecular weight of about 40,000 (40K protein). This protein is labelled rapidly in platelets stimulated by thrombin as well as by exogenous phospholipase C, and diacylglycerol formation always accompanies 40K protein phosphorylation. The phosphorylation of 40K protein *in vivo* induced by thrombin is selectively inhibited by chlorpromazine and dibucaine, which are potent inhibitors for protein kinase C. Thus, phosphatidylinositol turnover provoked by thrombin seems to serve as a transmembrane signal for protein phosphorylation during platelet activation.

Since the stimulation of platelets by thrombin may be analogous to hormonal control of cellular processes, attempts have been made to clarify the regulatory mechanisms of several intracellular events eventually leading to aggregation and release reaction (for a review see Ref. 1). Among these events attention has been paid to the phosphorylation of some endogenous proteins which may be observed specifically upon stimulation by thrombin (2-5). In particular, two proteins having

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1/ The abbreviations used are: protein kinase A, cyclic AMP-dependent protein kinase; protein kinase G, cyclic GMP-dependent protein kinase; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethylether)N,N,N',N'-tetraacetic acid.

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molecular weights of about 40,000 (40K protein) and 20,000 (20K protein) have been shown to be heavily phosphorylated in platelets which are stimulated by thrombin, and the phosphorylation of 40K protein is proposed to be intimately related to release reaction (2-5). Although the phosphorylation of 20K protein has been proposed to be catalyzed by myosin light chain kinase (4), the enzyme responsible for the phosphorylation of 40K protein has not yet been defined. Rittenhouse-Simmons (6) and Bell and Majerus (7) have shown recently that diacylglycerol is a product of phosphatidylinositol turnover which is provoked by thrombin. Preceding reports from this laboratory have shown that the $Ca^{2+}$-activated, phospholipid-dependent protein kinase (protein kinase C) found in many mammalian tissues is activated by unsaturated diacylglycerol which may result from phosphatidylinositol hydrolysis (8-11). This communication describes that the phosphorylation of 40K protein is catalyzed by protein kinase C, and that this enzyme may be directly involved in the transmembrane control of protein phosphorylation during platelet activation.

## EXPERIMENTAL PROCEDURES

Washed human platelets were prepared by the method of Baenziger and Majerus (12). Phospholipid was prepared from bovine brain as described previously (8). Calf thymus H1 histone was prepared as described earlier (13). [$\gamma$-$^{32}$P]ATP was prepared by the method of Glynn and Chappell (14). Bovine thrombin and Clostridium perfringens phospholipase C were the products of Mochida Pharmaceutical Co. and Sigma, respectively. Other chemicals were obtained from commercial sources. Protein kinases C, A and G[1] were assayed by measuring the incorporation of $^{32}P_i$ into H1 histone from [$\gamma$-$^{32}$P]ATP. Other details are specified in the legends to Figures. Labelling of platelets with [$^3$H]arachidonic acid and quantitation of diacylglycerol produced were carried out as described by Rittenhouse-Simmons (6). Labelling of platelets with $^{32}P_i$ was carried out as described by Lyons et al. (2). The platelet proteins labelled with $^{32}P_i$ were analyzed by SDS-polyacrylamide slab gel electrophoresis under the conditions described by Laemmli (15). The separating gel consisted of a 5 to 18% linear acrylamide gradient, and the stacking gel contained 3% acrylamide. After electrophoresis each slab gel was stained with Coomassie brilliant blue, dried on a filter paper and exposed to Kodak Royal X-Omat film to prepare autoradiogram. The relative intensity of each band was quantitated by densitometric tracing of the autoradiogram at 430 nm using a Shimadzu dual-wavelength chromatogram scanner, Model CS-910. Protein was determined by the method of Lowry et al. (16).

## RESULTS AND DISCUSSION

When platelets were disrupted in a buffer medium containing EGTA, protein kinases were recovered mostly in soluble fraction. Fig. 1 shows the chromatographic pattern of protein kinases A and C which were developed on a DEAE-cellulose (DE-52) column. With H1 histone as phosphate acceptor protein kinase C was 20 times more active than protein kinase A. Protein kinase G was extremely low in activity, and no peak was detected under comparable conditions. Protein kinase C (Fractions 12 through 22) was subjected further to gel filtration on a Sephadex G-150 column and employed for the present studies.[2/] The enzyme showed physical and kinetic properties which were indistinguishable from those of brain protein kinase C previously described (8,9,17,18). The enzyme was activated in a reversible manner by association with membrane phospholipid. $Ca^{2+}$ was indispensable for this activation. Among various

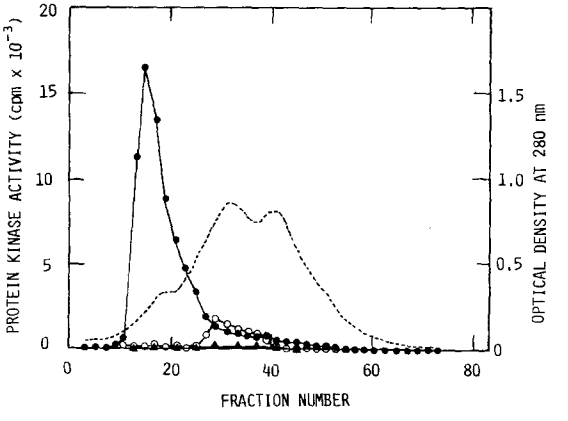


Fig. 1. DE-52 column chromatography of platelet protein kinases. Platelets (5 x $10^{10}$) were disrupted by sonication in 10 ml of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose, 10 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA, 0.01% leupeptin and 2 mM phenylmethylsulfonylfluoride. The 100,000 x g supernatant (10 ml, 33 mg of protein) was subjected to a DE-52 column (5 x 1.4 cm) equilibrated with 20 mM Tris/HCl at pH 7.5 containing 10 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA and 0.001% leupeptin. Elution was carried out with a 96-ml linear concentration gradient of NaCl (0 to 0.4 M) in the same buffer. Protein kinases were assayed in the presence of 0.5 mM $CaCl_2$, 20 µg of phospholipid, 0.6 µg of diolein, 1 µM cyclic AMP and 0.1 mM EGTA as indicated under the conditions described earlier (10). (●——●), with $CaCl_2$, phospholipid and diolein; (o——o), with cyclic AMP; (▲——▲), with EGTA; (------), optical density at 280 nm.

2/ The enzyme preparation thus obtained was free of other protein kinases, calmodulin, interfering enzymes and endogenous phosphate acceptor proteins.

phospholipids tested phosphatidylserine was most effective. In addition, a small amount of unsaturated diacylglycerol markedly increased the affinity of enzyme for phospholipid as well as for $Ca^{2+}$, and allowed enzyme fully active at the micromolar range of this divalent cation. Since phosphatidylinositol in mammalian tissues is composed of unsaturated fatty acid, mostly arachidonic acid, particularly at position 2 (19), it is possible that thrombin induces the specific hydrolysis of phosphatidylinositol to produce unsaturated diacylglycerol, which in turn serves as an initiator for the selective activation of protein kinase C.

The experiment given in Fig. 2A shows that, in confirmation of the previous observations by Lyons et al. (2), 40K protein was rapidly phosphorylated in platelets upon stimulation by thrombin. Quantitative results given in Fig. 3A show that diacylglycerol was even more rapidly produced, and that this reaction appeared to be immediately followed

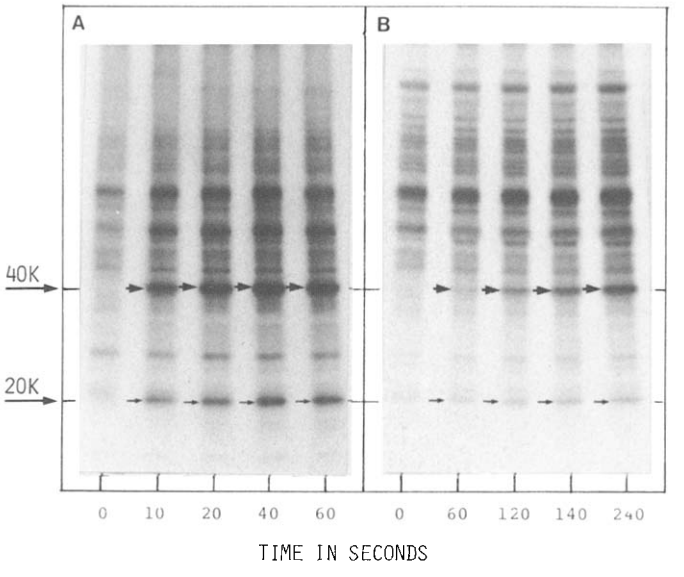


Fig. 2. Stimulation of 40K protein phosphorylation by thrombin and phospholipase C. Samples of platelets which were labelled with $^{32}P_i$ were stimulated by thrombin (0.04 unit/1 x $10^8$ platelets) or by phospholipase C (0.3 unit/1 x $10^8$ platelets) at 37°C. Radioactive proteins were analyzed as described under "EXPERIMENTAL PROCEDURES". A, with thrombin; B, with phospholipase C.

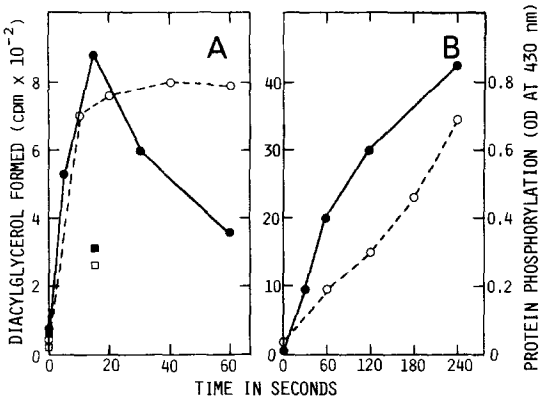


Fig. 3. Parallel stimulation of diacylglycerol formation and 40K protein phosphorylation by thrombin and phospholipase C. Samples of platelets which were labelled with [$^3$H]arachidonic acid or $^{32}P_i$ were stimulated by thrombin or phospholipase C as described in the legend to Fig. 2. In some experiments samples of platelets were preincubated with 10 μM prostaglandin E1 for 5 min at 37°C and then stimulated by thrombin. Diacylglycerol formation and 40K protein phosphorylation were quantitated as described under "EXPERIMENTAL PROCEDURES". Backgrounds for diacylglycerol formation and 40K protein phosphorylation, which were obtained in the absence of thrombin and phospholipase C, were about 250 cpm and 0.3 OD, respectively. These values were subtracted from each point. A, with thrombin; B, with phospholipase C. (●——●) and (■), diacylglycerol formation in the absence and presence of prostaglandin E1, respectively; (o-----o) and (□), 40K protein phosphorylation in the absence and presence of prostaglandin E1, respectively.

by 40K protein phosphorylation. It was suggested that this diacylglycerol was produced in the expense of phosphatidylinositol, and that the rapid disappearance of this lipid was presumably due to further degradation and also to conversion to phosphatidic acid (data not shown). If such diacylglycerol formation is a signal for 40K protein phosphorylation, then the exogenous addition of phospholipase C may induce platelet activation. In fact, bacterial phospholipase C has been shown to mimic thrombin action and induce irreversible aggregation and release reaction (20,21). Fig. 2B shows that 40K protein was indeed phosphorylated upon treatment with Clostridium perfringens phospholipase C, and Fig. 3B shows that the reaction was roughly parallel with diacylglycerol formation. So far, in all experiments, diacylglycerol formation always accompanied 40K protein phosphorylation, and both reactions were equally inhibited to same extent by prostaglandin E1, an antagonist of thrombin, as also shown in Fig. 3A.

The experiments described in Fig. 4 were designed to show that protein kinase C was indeed able to phosphorylate 40K protein *in vitro*. When the crude extract was incubated with $Ca^{2+}$ and radioactive ATP,

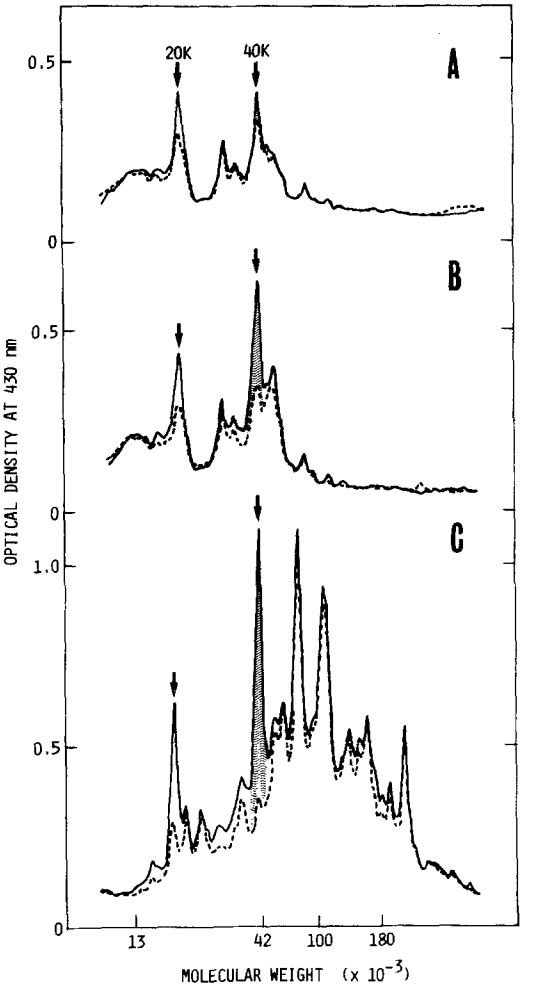


Fig. 4. Phosphorylation of 40K protein by exogenous protein kinase C *in vitro* and in platelets *in vivo* induced by thrombin. A, *In vitro* phosphorylation with endogenous protein kinase. The isolated platelets ($1 \times 10^{10}$) suspended in 2 ml of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose were disrupted by sonication. The sonicate (100 µg of protein) was incubated with 0.2 mM $CaCl_2$ or 0.2 mM EGTA in the reaction mixture (0.1 ml) containing 2 µmol of Tris/HCl at pH 7.5, 0.5 µmol of magnesium acetate and 5 nmol of [$\gamma$-$^{32}$P]ATP ($1 \times 10^6$ cpm/nmol). The incubation was carried out for 30 sec at 30°C. The radioactive proteins were analyzed as described under "EXPERIMENTAL PROCEDURES". (———), with $CaCl_2$; (------), with EGTA. B, *In vitro* phosphorylation by exogenous protein kinase C. The sonicate was incubated under the conditions described above except that the purified platelet protein kinase C (2 µg of protein) was exogenously added. (———), with $CaCl_2$; (------), with EGTA. C, *In vivo* phosphorylation. *In vivo* phosphorylation was carried out with or without thrombin for 30 sec as described in the legend to Fig. 2. (———), with thrombin; (------), without thrombin.

40K protein was phosphorylated by endogenous protein kinase to some extent (Fig. 4A). The phosphorylation of this protein was significantly enhanced by the addition of exogenous protein kinase C (Fig. 4B), and this enhancement was not evident in the presence of EGTA.[3/] The phosphorylation of this protein was remarkably enhanced *in vivo* upon stimulation by thrombin as described above (Fig. 4C). A preceding report from this laboratory (22) has described that several phospholipid-interacting drugs such as chlorpromazine and dibucaine specifically inhibit the activation of protein kinase C by competing with phospholipid, although these drugs do not interact with the catalytic site of enzyme. Neither protein kinase A nor G is susceptible to these drugs. In consistent with these observations the phosphorylation of 40K protein *in vivo* induced by thrombin was profoundly and selectively inhibited by these drugs as shown in Fig. 5.

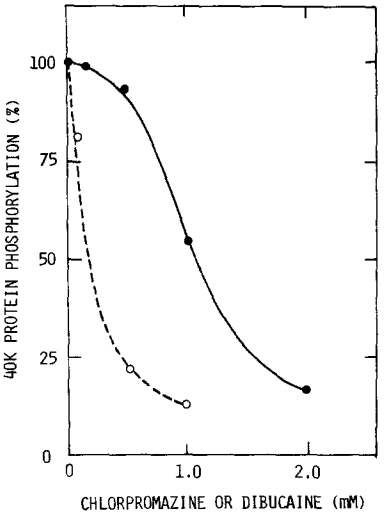


Fig. 5. Inhibition by chlorpromazine and dibucaine of phosphorylation of 40K protein *in vivo* induced by thrombin. *In vivo* phosphorylation was carried out with thrombin for 30 sec as described in the legend to Fig. 2. except that the incubation mixture contained chlorpromazine or dibucaine as indicated. (o-----o), with chlorpromazine; (●——●), with dibucaine.

3/ When the crude extract which was heated briefly (90°C for 2 min) to inactivate endogenous protein kinase was employed, 40K protein was more clearly shown to serve as phosphate acceptor for protein kinase C.

The results presented above suggest strongly that protein kinase C is activated in a manner directed by thrombin, and is responsible for the phosphorylation of at least 40K protein which has been proposed to be directly related to release reaction. Although the experimental support described above has resulted from a limited number of studies with human platelets, it is possible that phosphatidylinositol turnover provoked by a variety of extracellular messengers may be a signal for transmembrane control of protein phosphorylation.

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