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A POSSIBLE ROLE OF CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN PLATELET ACTIVATION*

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INDEXING WORDS

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SYNOPSIS

Human platelets contain a large quantity of Ca^{2+}-activated, phospholipid-dependent protein kinase originally found in mammalian brain (Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. J. Biol. Chem. 1979. 254. 3692/3695). At the micromolar range of Ca^{2+} concentrations this enzyme almost absolutely requires a small amount of unsaturated diacylglycerol, which may result from the thrombin-induced hydrolysis of phosphatidylinositol. The protein kinase thus activated is able to phosphorylate in vitro endogenous proteins, most predominantly one polypeptide having a molecular weight of approximately 40,000 (40K protein). This protein is phosphorylated rapidly in platelets stimulated by thrombin as well as by exogenous phospholipase C in vivo. This 40K protein phosphorylation in vivo appears to be preceded by diacylglycerol formation and followed by serotonin release reaction. These three reactions are inhibited to the same extent by prostaglandin E1. The phosphorylation of 40K protein in vivo induced by thrombin as well as the

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** Abbreviations used are: SDS, sodium dodecyl sulfate; and EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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activation of this kinase in vitro is profoundly inhibited in parallel manner by phospholipid-interacting drugs such as dibucaine and chlorpromazine, which are known to inhibit platelet activation. In human platelets cyclic nucleotide-dependent protein kinases are present in small quantities, and the enzymes are not susceptible to these drugs. The results suggest that Ca^{2+} -activated, phospholipid-dependent protein kinase may be directly involved in platelet activation through the phosphorylation of 40K protein.

INTRODUCTION

Platelets are known to contain several protein kinases such as cyclic AMP-dependent protein kinase,[14] phosphorylase kinase[7] and myosin light chain kinase,[5] and phosphorylation of endogenous platelet proteins is thought to play an important role in controlling platelet activation. In fact, it has been reported that a number of platelet proteins become increasingly phosphorylated after stimulation by thrombin, collagen and the divalent cation ionophore A23187.[11, 18, 23] In particular, two proteins having 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 these proteins is proposed to be intimately related to release reaction.[6, 11, 18, 28] Although 20K protein is considered to be a light chain of myosin and phosphorylated by myosin light chain kinase,[6] the exact properties as well as the regulatory mechanisms of the enzyme involved in the phosphorylation of 40K protein have not yet been defined. Recent reports presented by Rittenhouse-Simmons[20] and Bell and Majerus[3] have shown that diacylglycerol is produced as a primary product from phosphatidylinositol turnover which is provoked by thrombin. Takai et al.[15, 26] have recently described that Ca^{2+}-activated, phospholipid-dependent protein kinase found in many mammalian tissues may be activated by unsaturated diacylglycerol which results from phosphatidylinositol hydrolysis. This paper will describe that human platelets contain this unique protein kinase and the phosphorylation of 40K protein in vivo is catalyzed by this enzyme which is activated by diacylglycerol produced from the thrombin-induced hydrolysis of phosphatidylinositol. The protein kinase under discussion will be tentatively referred to as protein kinase C, and cyclic AMP-dependent and cyclic GMP-dependent protein kinases will be designated as protein kinases A and G, respectively.

EXPERIMENTAL PROCEDURES

Materials and chemicals

Washed human platelets were prepared by the method of Baenziger and Majerus.[1] Phospholipid was prepared from bovine brain by extraction with chloroform/methanol (2:1) followed by silicic acid column chromatography as described previously.[24] Synthetic diolein was purchased from Nakarai Chemicals. Calf thymus H1 histone was prepared as described previously.[9] [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell.[8] Bovine thrombin and Clostridium perfringens phospholipase C were the products of Mochida Pharmaceutical Co. and Sigma, respectively. Chlorpromazine hydrochloride and dibucaine hydrochloride were donated by Shionogi Research Laboratory and Teikoku Chemical Industry, respectively.

Protein kinase assay

Protein kinase C was assayed by measuring the incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP. The reaction mixture (0.25 ml) contained 5 µmol of Tris/HCl at pH 7.5, 1.25 µmol of magnesium acetate, 50 µg of H1 histone, 2.5 nmol of [γ-^{32}P]ATP (5 to 10 x 10^4 cpm/nmol), 125 nmol of $CaCl_2$, 20 µg of phospholipid, 0.6 µg of diolein, and protein kinase C to be assayed. Phosphatidylserine was first mixed with diolein in a small volume of chloroform. After chloroform was removed in vacuo, the residue was suspended in 20 mM Tris/HCl at pH 7.5 by sonication as described previously.[26] The incubation was carried out for 3 min at 30°C. The acid-precipitable radioactivity was determined as described earlier.[24] Protein kinases A and G were similarly assayed except that, instead of $CaCl_2$, phospholipid, and diolein, 250 pmol of either cyclic AMP (for protein kinase A) or cyclic GMP (for protein kinase G) was added.

Labelling of platelets with [^{3}H]Arachidonic Acid and Quantification of Diacylglycerol

Platelet-rich plasma (90 ml) was incubated with 80 µCi of [^{3}H] arachidonic acid (62.2 Ci/mmol, New England Nuclear) for 60 min at 37°C by the method of Rittenhouse-Simmons.[20] The labelled platelets were incubated as specified in each experiment. The radioactive diacylglycerol produced was extracted with chloroform/methanol (2:1), isolated by silicic acid column chromatography followed by silica plate thin layer chromatography, and quantified as described by Rittenhouse-Simmons.[20]

Labelling of platelets with ^{32}Pi and analysis of phosphorylated proteins

Washed platelets (1×10^{10}) were suspended in 20 ml of Buffer A (15 mM Tris/HCl at pH 7.4 containing 140 mM NaCl and 5.5 mM Glucose), and incubated for 15 min to deplete them of phosphate. The platelets were sedimented at 2,250 x g for 15 min and resuspended in 20 ml of Buffer A. This procedure was repeated again, and the platelets, which were finally suspended in 5 ml of Buffer A, were incubated for 60 min with 2.5 mCi of ^{32}Pi (carrier free). The labelled platelets were washed twice with 5 ml of Buffer A. These procedures were carried out at room temperature. The platelet proteins labelled with ^{32}Pi were analyzed by SDS**-polyacrylamide gel electrophoresis with 2 mm thick slab gels under the conditions described by Laemmli.[16] 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 Whatman filter paper, No. 1, and exposed to Kodak Royal X-Omat film to prepare an autoradiograph. The relative intensity of each band was quantified by densitometric tracing of the autoradiograph at 430 nm using a Shimadzu dual-wavelength chromatogram scanner, Model CS-910.

Quantification of release reaction

Release reaction was quantified by the radioactive material released from the platelets which were preloaded with [2-^{14}C]serotonin (58 mCi/mmol New England Nuclear) by the method of Haslam et al.[10]

Determinations

Protein was determined by the method of Lowry et al.[17] with bovine serum albumin as a standard.

RESULTS

Partial purification and characterization of platelet protein kinase C

When washed platelets were disrupted in a Ca^{2+}-free medium as described in the legend to Fig. 1, protein kinases were recovered mostly in the soluble fraction. The soluble fraction obtained in this way was chromatographed on a DEAE-cellulose (DE-52) column under the conditions given in Fig. 1. With H1 histone as phosphate acceptor, the activity of protein kinase C far exceeded that of protein kinase A. Protein kinase G had

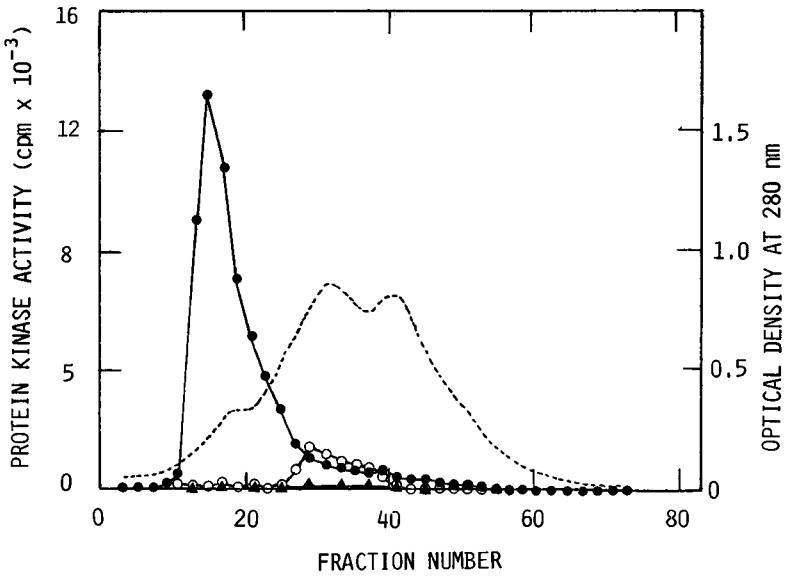

Fig. 1 DE-52 column chromatography of platelet protein kinases. Platelets (5 x 10^{10}) were suspended in 10 ml of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose, 10 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA, 0.01% leupeptin, and 2 mM phenylmethylsulfonylfluoride, and sonicated twice for 15 sec with an interval of 10 sec at 0°C with a Kontes sonifier, Model K-881440. The sonicate was centrifuged for 60 min at 100,000 x g at 4°C. The supernatant was employed as a soluble fraction. The soluble fraction (11 ml, 33 mg of protein) was subjected to a DE-52 column (5 x 1.4 cm) which was equilibrated with Buffer B (20 mM Tris/HCl at pH 7.5 containing 10 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, and 0.001% leupeptin). After the column was washed with 80 ml of Buffer B, elution was carried out with a 96-ml linear concentration gradient of NaCl (0 to 0.4 M) in the same buffer. Fractions of 1.3 ml each were collected. A 10 µl-aliquot of each fraction was assayed for protein kinase under the standard conditions. (●——●), with 0.5 mM $CaCl_2$, 20 µg of phospholipid, and 0.6 µg of diolein; (○——○), with 1 mM cyclic AMP; and (▲——▲), with 0.1 mM EGTA. (-------), optical density at 280 nm.

extremely low activity and no peak was detected under comparable conditions. The major peak of protein kinase C (Fractions 12 through 22) was concentrated to about 3 ml with Amicon ultrafiltration apparatus equipped with PM-10 membrane filter and subjected to a Sephadex G-150 column (80 x 1.6 cm) which was equilibrated with 20 mM Tris/HCl at pH 7.5 containing 50 mM 2-mercaptoethanol, 0.2 mM EDTA and 0.2 mM EGTA. Elution was carried out with the same buffer. Fractions of 2 ml each were collected. When each fraction was assayed for protein kinase C, a single peak appeared in Fractions 30 through 32. Active fractions were employed for subsequent studies. The enzyme preparation thus obtained was free of other protein kinases, calmodulin, interfering enzymes, and endogenous phosphate acceptor proteins. The enzyme showed physical and kinetic properties which were apparently indistinguishable from those of the brain enzyme previously described; [13, 24, 25, 27] the molecular weight was about 77,000, the pI value was pH 5.6, the optimum pH was 7.5 to 8.0 and the Km value for ATP was 7×10^{-6} M. GTP did not serve as phosphate donor. It was also noted that the mode of activation of platelet protein kinase C was similar to that found for the brain enzyme. [15, 24, 25, 26] In short, the enzyme was activated in a reversible manner by association with phospholipid and Ca^{2+} was indispensable for this activation. Among various phospholipids tested phosphatidylserine was the most effective. In addition, a small amount of unsaturated diacylglycerol markedly increased the affinity of enzyme for phospholipid. Concomitantly, the Ka value for Ca^{2+} was decreased dramatically to less than the micromolar range and, thus, the enzyme was fully active at physiologically lower concentrations of this cation. This unique effect of neutral lipid was very specific for diacylglycerol which possessed unsaturated fatty acid at least at position 2. Since phosphatidylinositol in mammalian tissues is composed of unsaturated fatty acid, mostly arachidonic acid, particularly at position 2, [12] it is possible that thrombin induces hydrolysis of phosphatidylinositol to produce unsaturated diacylglycerol, which in turn serves as a second messenger for the selective activation of protein kinase C.

Possible role of protein kinase C in platelet activation

In confirmation of the recent reports by Rittenhouse-Simmons [20] and Bell and Majerus, [3] diacylglycerol was rapidly produced in platelets which were stimulated by thrombin as shown in Fig. 2A. This reaction appeared to be followed by immediate phosphorylation of 40K protein and serotonin release reaction. It was suggested that this diacylglycerol was produced

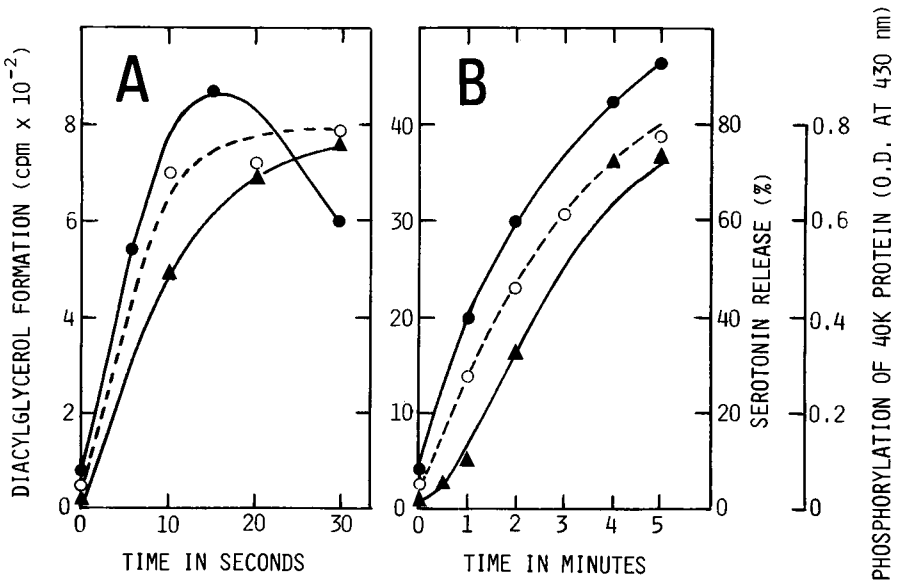

Fig. 2 Stimulation of diacylglycerol formation, phosphorylation of 40K protein and serotonin release by thrombin and phospholipase C. Samples of platelets labelled with [^{3}H]arachidonic acid, ^{32}Pi and [^{14}C] serotonin were stimulated by thrombin (0.04 unit per 1 x 10^{8} platelets) or by phospholipase C (0.3 unit per 1 x 10^{8} platelets). Diacylglycerol formation, phosphorylation of 40K protein and serotonin release were estimated as described under EXPERIMENTAL PROCEDURES. (A), with thrombin; (B), with phospholipase C; (●——●), diacylglycerol formation; (O-----O), phosphorylation of 40K protein; and (▲——▲), serotonin release.

at the expense of phosphatidylinositol and the rapid disappearance of this lipid was presumably due to further degradation and also 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.[4, 22] Fig. 2B shows that phosphorylation of 40K protein and serotonin release reaction were induced upon treatment with Clostridium perfringens phospholipase C and these reactions were roughly parallel with diacylglycerol formation. So far, diacylglycerol formation always appeared to accompany 40K protein phosphorylation and serotonin release reaction. Moreover, these reactions were equally inhibited to the same extent by various concentrations of prostaglandin E1 which

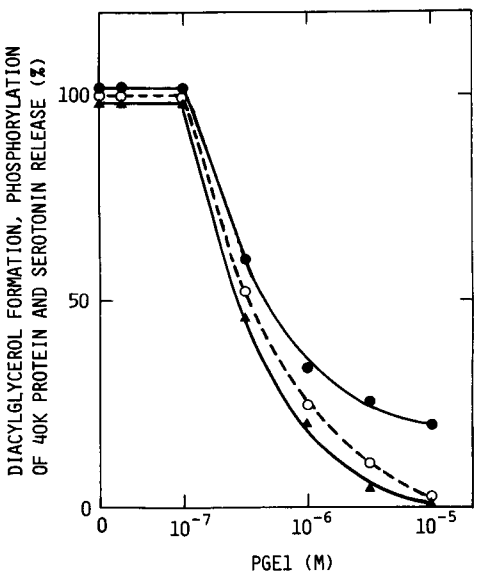

Fig. 3
Parallel inhibition by PGE1 of thrombin-induced diacylglycerol formation, phosphorylation of 40K protein and serotonin release. Diacylglycerol formation, phosphorylation of 40K protein and serotonin release were estimated as described in the legend to Fig. 2 except that various concentrations of PGE1 were added as indicated. (●——●), diacylglycerol formation; (O-----O), phosphorylation of 40K protein; and (▲——▲), serotonin release.

is known to inhibit platelet activation (Fig. 3). In order to obtain evidence for coupling of diacylglycerol formation and 40K protein phosphorylation, the next experiment given in Fig. 4 was designed to show that protein kinase C was indeed responsible for the phosphorylation of 40K protein in vitro. When the crude extract was incubated with Ca^{2+} and radioactive ATP, 40K protein was phosphorylated by endogenous protein kinase to some extent (Fig. 4A). The phosphorylation of this protein was enhanced by the addition of exogenous protein kinase C (Fig. 4B) and this enhancement was not evident in the presence of EGTA. In agreement with the previous observation,[6, 11, 18, 28] the phosphorylation of this protein appeared to be remarkably enhanced in vivo upon stimulation by thrombin (Fig. 4C).

Mori et al.[19] have described that several phospholipid-interacting drugs such as chlorpromazine and dibucaine inhibit the activation process of protein kinase C by competing with phospholipid although these drugs do not interact with the active center of enzyme. Neither protein kinase A nor G is susceptible to these drugs. Indeed, the radioactive band corresponding to 40K protein did not appear when platelets were stimulated in the presence of these drugs and the quantitative analysis shown in Fig. 5 indicates that the in vivo phosphorylation of 40K protein induced

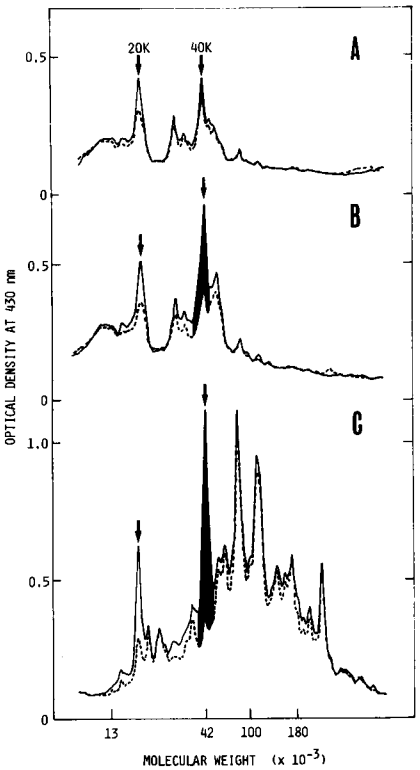

Fig. 4

Phosphorylation of 40K protein by exogenous protein kinase C in vitro and upon stimulation of platelets by thrombin. (A), phosphorylation of platelet proteins by endogenous protein kinase. The isolated platelets (1×10^{10}) were suspended in 2 ml of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose, and sonicated twice for 15 sec with an interval of 10 sec at 0 °C. The sonicate was directly employed as a crude extract. The crude extract (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 [γ-^{32}P]ATP (1×10^6 cpm/nmol). The incubation was carried out for 30 sec at 30°C. The reaction was terminated by the addition of 50 µl of SDS-stop solution which contained 9% SDS, 6% 2-mercaptoethanol, 15% glycerol, and 0.186 M Tris/HCl at pH 6.7 and placed in a boiling water bath for 3 min. Each aliquot (30 µg of protein) was subjected to SDS-polyacrylamide slab gel electrophoresis. Gel electrophoresis and densitometric tracing were performed as described under EXPERIMENTAL PROCEDURES. (———), with $CaCl_2$; and (-------), with EGTA. (B), phosphorylation of platelet proteins by the addition of exogenous protein kinase C. The crude extract was made as described above. The extract was incubated with 0.2 mM $CaCl_2$ or 0.2 mM EGTA under the conditions described above except that the purified platelet protein kinase C (2 µg of protein) was exogenously added. (———), with $CaCl_2$; and (-------), with EGTA. (C), phosphorylation of platelet proteins upon stimulation of platelets by thrombin. Samples of washed platelets (0.1 ml, 2×10^8) which were labelled with ^{32}Pi were incubated with or without 0.2 unit of thrombin for 30 sec at 37 °C. The radioactive proteins were analyzed as described under EXPERIMENTAL PROCEDURES. (———), with thrombin; and (-------), without thrombin.

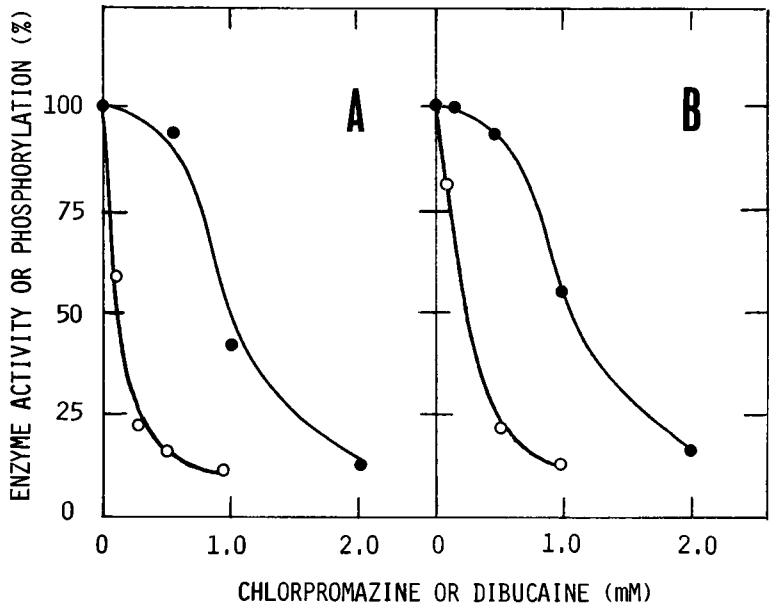

Fig. 5 Parallel inhibition by chlorpromazine and dibucaine of protein kinase C activation and thrombin-induced in vivo phosphorylation of 40K protein. (A), inhibition of protein kinase C activation. Protein kinase C was assayed under the standard conditions with 4 μg of phospholipid, 0.2 μg of diolein, and 0.5 μg of purified platelet protein kinase C. Chlorpromazine or dibucaine was added as indicated. (B), inhibition of thrombin-induced in vivo phosphorylation of 40K protein. Samples of wahsed platelets (0.1 ml, 2×10^8) which were labelled with ^{32}Pi were preincubated for 2 min at 30°C with or without a drug as indicated. Then, either 0.2 unit of thrombin or saline was added and the incubation was continued for another 1 min. The reaction was terminated as described in the legend to Fig. 4. The radioactive proteins were analyzed as described under EXPERIMENTAL PROCEDURES. (○——○), with chlorpromazine; and (●——●), with dibucaine.

by thrombin and the enzymatic activity of protein kinase C assayed in vitro were profoundly inhibited in parallel manners by increasing concentrations of dibucaine and chlorpromazine.

DISCUSSION

The experimental results presented above suggested strongly that protein kinase C which is present in large quantities in human platelets

is activated by diacylglycerol produced from thrombin-induced phosphatidylinositol hydrolysis and the enzyme thus activated is responsible for the phosphorylation of at least 40K protein which has been supposed to be directly related to release reaction.[6, 11, 18, 28] Since exogenously added protein kinase C could not significantly phosphorylate 20K protein in vitro (Fig. 4B), the phosphorylation of this protein appears to belong to another entity and is presumably catalyzed by myosin light chain kinase as proposed by Daniet et al.[6] Although 20K protein is thought to be a light chain of myosin and the phosphorylation of this protein has been shown to result in increased actin-activated myosin ATPase activity, the function of 40K protein and the physiological role of the phosphorylation of this protein have not yet been clarified. To understand more exactly the molecular mechanism of platelet activation, in particular release reaction, it seems necessary to purify the native 40K protein and clarify its function.

Although phosphatidylinositol hydrolysis stimulated by extracellular stimulators is observed in various tissues, the physiological significance of this phenomenon has not yet been clarified. Recently Rittenhouse-Simmons[21] has presented evidence that arachidonic acid, the precursor of prostaglandins, may be generated from diacylglycerol which is the primary product of phosphatidylinositol hydrolysis and such diacylglycerol lipase has been demonstrated in human platelets.[2] On the other hand, Takai et al.[15, 26] discovered that diacylglycerol especially with an unsaturated fatty acid composition, such as would be released during phosphatidylinositol hydrolysis, could remarkably activate protein kinase C in vitro. They have proposed that such protein kinase activation could occur in various tissues in vivo in response to extracellular stimulators which could provoke phosphatidylinositol hydrolysis. The results presented in this paper support strongly their fascinating idea as to a possible significance of phosphatidylinositol hydrolysis.

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REFERENCES

1. Baenziger, N.L., and Majerus, P.W. Methods Enzymol. 1974. 31. 149/155. Isolation of human platelets and platelet surface membranes.
2. Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. Proc. Natl. Acad. Sci. USA. 1979. 76. 3238/3241. Diglyceride lipase: A pathway for arachidonate release from human platelets.
3. Bell, R.L., and Majerus, P.W. J. Biol. Chem. 1980. 255. 1790/1792. Thrombin-induced hydrolysis of phosphatidylinositol in human platelets.
4. Chap, H.J., Zwaal, R.F.A., and Van Deenen, L.L.M. Biochim. Biophys. Acta 1977. 467. 146/164. Action of highly purified phospholipases on blood platelets. Evidence for an asymmetric distribution of phospholipids in the surface membrane.
5. Daniel, J.L., and Adelstein, R.S. Biochemistry 1976. 15. 2370/2377. Isolation and properties of platelet myosin light chain kinase.
6. Daniel, J.L., Holmsen, H., and Adelstein, R.S. Thrombos. Haemostas. 1977. 38. 984/989. Thrombin-stimulated myosin phosphorylation in intact platelets and its possible involvement secretion.
7. Gear, A.R.L., and Schneider, W. Biochim. Biophys. Acta 1975. 392. 111/120. Control of platelet glycogenolysis activation of phosphorylase kinase by calcium.
8. Glynn, I.M., and Chappell, J.B. Biochem. J. 1964. 90. 147/149. A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity.
9. Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K., and Iwai, K. J. Biol. Chem. 1976. 251. 6287/6293. Studies on the sites in histones phosphorylated by adenosine 3':5'-monophosphate-dependent and guanosine 3':5'-monophosphate-dependent protein kinases.
10. Haslam, R.J., Davidson, M.M.L., and McClenaghan, M.D. Nature 1975. 253. 455/457. Cytochalasin B, the blood platelet release reaction and cyclic GMP.
11. Haslam, R.J., and Lynham, J.A. Biochem. Biophys. Res. Commun. 1977. 77. 714/722. Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents I. Effects of different aggregating agents.
12. Holub, B.J., Kuksis, A., and Thompson, W. J. Lipid. Res. 1970. 11. 558/564. Molecular species of mono-, di-, and triphospho-inositides of bovine brain.
13. Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. J. Biol. Chem. 1977. 252. 7610/7616. Studies on a cyclic nucleotide-independent

protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain.

14. Kaulen, H.D., and Gross, R. Hoppe-Seyler's Z Physiol. Chem. 1974. 355. 471/480. Purification and properties of a soluble cyclic AMP-dependent protein kinase from human platelets.
15. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. J. Biol. Chem. 1980. 255. 2273/2276. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover.
16. Laemmli, U.K. Nature 1970. 227. 680/685. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
17. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem. 1951. 193. 265/275. Protein measurement with the folin phenol reagent.
18. Lyons, R.M., Stanford, N., and Majerus, P.W. J. Clin. Invest. 1975. 56. 924/936. Thrombin-induced protein phosphorylation in human platelets.
19. Mori, T., Takai, Y., Minakuchi, R., Yu, B., and Nishizuka, Y. J. Biol. Chem. 1980. 255. 8378/8380. Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase.
20. Rittenhouse-Simmons, S. J. Clin. Invest. 1979. 63. 580/587. Production of diglyceride from phosphatidylinositol in activated human platelets.
21. Rittenhouse-Simmons, S. J. Biol. Chem. 1980. 255. 2259/2262. Indomethacin-induced accumulation of diglyceride in activated human platelets.
22. Schick, P.K., and Yu, B.P. J. Clin. Invest. 1974. 54. 1032/1039. The role of platelet membrane phospholipids in the platelet release reaction.
23. Steiner, M. Arch. Biochem. Biophys. 1975. 171. 245/254. Endogenous phosphorylation of platelet membrane proteins.
24. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. J. Biol. Chem. 1979. 254. 3692/3695. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids.
25. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y., Tamura, A., and Fujii, T. J. Biochem. 1979. 86. 575/578.

A role of membranes in the activation of a new multifunctional protein kinase system.

26. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. Biochem. Biophys. Res. Commun. 1979. 91. 1218/1224. Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system.
27. Takai, Y., Yamamoto, M., Inoue, M., Kishimoto, A., and Nishizuka, Y. Biochem. Biophys. Res. Commun. 1977. 77. 542/550. A proenzyme of cyclic nucleotide-independent protein kinase and its activation by calcium-dependent neutral protease from rat liver.
28. Wallace, W.C., and Bensusan, H.B. J. Biol. Chem. 1980. 255. 1932/1937. Protein phosphorylation in platelets stimulated by immobilized thrombin at 37°C and 4°C.