Activation of Protein Kinase C with Cardiolipin-Containing Liposomes in Relation to Membrane-Protein Interaction

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Abstract. Many cytoplasmic proteins, including Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase (protein kinase C) of polymorphonuclear leukocytes (PMNs) associate in Ca\textsuperscript{2+}-dependent manner with phospholipid liposomes containing cardiolipin (CL), as in the case of phosphatidylserine (PS)-containing liposomes. A crude protein kinase C fraction was purified by association of the enzyme with CL-containing liposomes (flotation method). The partially purified protein kinase C from rat brain or guinea pig PMN was activated by the CL-containing liposomes in the presence of dioleoylglycerol (DG) and Ca\textsuperscript{2+}. This activation was analogous to that of PS. The half maximum activity was obtained with 20 μM CL in the presence of 1 μM Ca\textsuperscript{2+} and 5 μM DG. Many of the cytoplasmic proteins which associate with CL-containing liposomes were preferentially phosphorylated by membrane-associated protein kinase C in the presence of DG and Ca\textsuperscript{2+}. These results suggest that the association of cytoplasmic protein kinase C with the membrane has an important role in regulation of protein kinase C activity in relation to the association of other cytoplasmic proteins to the membrane.

Various extracellular information appears to be passed into the cell by various mechanisms of transmembrane signaling (1). As the inner surface of the plasma membrane is always in contact with cytoplasmic proteins, it has been expected that there must be some sort of direct interaction between them. Recently we have reported that several cytoplasmic proteins associate with phospholipid vesicles (liposomes) (23) and that some cytoplasmic proteins, such as the 33 kDa protein, from PMNs associated with PS-containing liposomal membrane in a calcium-dependent manner (20, 23, 24). This membrane-protein interaction is quite similar to that between Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase (protein kinase C) and the plasma membrane (17, 18). Furthermore, it is reported that the association of protein kinase C with the plasma membrane results in activation of the enzyme (6, 16, 25), and the characteristic structure of vesicles formed from various phospholipids affects protein kinase C activity (2). It was also found by an immunocytochemical method (20) that

Abbreviation used: DPPC, dipalmitoylphosphatidylcholine; C, cholesterol; DG, dioleoylglycerol; eggPC, egg phosphatidylcholine; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N', N'-tetraacetic acid; FMLP, formylmethionyl-leucyl-phenylalanine; HEPES, N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid; KRP, Krebs-Ringer-phosphate solution; PMN, polymorphonuclear leukocyte; PMSF, phenyl-methylsulfonyl fluoride; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.
tumor-promoting 12-O-tetradecanoyl-phorbol-13-acetate (TPA) caused a rapid translocation of protein kinase C from the cytoplasm to plasma membrane. Similarly, translocation of the 33 kDa protein from the cytoplasm to plasma membrane was caused by formylmethionyl-leucyl-phenylalanine (FMLP) (20). These results suggest that the interaction between cytoplasmic proteins and the inner surface of the plasma membrane may participate in regulating cellular signal transduction. Moreover, Ca\(^{2+}\)-dependent association of these cytoplasmic proteins similar to that with PS-containing liposomes was observed with CL-containing liposomes (25). Therefore, it is proposed that the activation of protein kinase C may be caused by the association of this enzyme with CL-containing phospholipid liposomes. Similar phospholipid dependencies of TPA binding and of the activation of the enzyme have been observed with various anionic phospholipids (PS, CL, phosphatidic acid) (7, 8). However, very little has been known about the detailed biochemical mechanism of CL-induced enzyme activation.

In this paper we described the interaction of CL-activated protein kinase C with the membrane and report that the membrane-associated cytoplasmic proteins were phosphorylated preferentially by the membrane-bound enzyme.

**MATERIALS AND METHODS**

*Chemicals.* Aprotinin, dipalmytoylphosphatidylcholine (DPPC), cholesterol (C), phosphatidyl serine (PS), dioleoylglycerol (DG), calf thymus H\(_1\) histone (type IIIS), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemicals (St. Louis, Mo., USA). Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Nutrose was purchased from Eastman Kodak Co. (Rochester, N.Y., USA). DEAE-cellulose (DE52) was obtained from Whatman (Maidstone, England), and TSK-G 3000 SW was from LKB-Produkter AB, (Bromma, Sweden). \([^{32P}]\)ATP was obtained from ICN Radiochemicals (Irvine, Calif, USA). Leupeptin and other chemicals were obtained from Nakarai Chemicals (Kyoto, Japan). Glass filters were obtained from GF/C, Whatman Ltd. (Maidstone, England).

*Preparation of PMNs and proteins of cytoplasmic supernatant.* PMNs were obtained from male guinea pig after an intraperitoneal injection of 2% Nutrose as described previously (22, 23). The cells were washed 3 times by centrifugation and suspended in calcium-free Krebs-Ringer-phosphate solution (KRP). PMNs were homogenized at 0°C in the medium of 0.15 M KCl, 4 mM iodoacetate, 1 mM vanadate, 10 mM \(\beta\)-glycerophosphate, 1 mM \(\beta\)-mercaptoethanol, 1 mM PMSF, 1 \(\mu\)g/ml aprotinin, 0.01 % leupeptin, and 20 mM HEPES (pH 7.4). The cytoplasmic supernatant was obtained by ultracentrifugation of this homogenate at 100,000 \(\times\) g for 60 min at 4°C. The supernatant fraction was applied to the column of Sephadex G25 (0.5 \(\times\) 10 cm) for the separation of proteins from low molecular weight substances, including nucleotides.

*Preparation of protein kinase C.* A crude protein kinase C fraction from cerebral tissues of mail-Dawley rats, weighing 200 to 250 g, was obtained by the method of Kikkawa et al. (12). Rat brains were homogenized in the medium of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 0.01 % leupeptin, 10 mM \(\beta\)-mercaptoethanol, and 0.1 % Triton X-100 at 4°C, and then centrifuged for 60 min at 100,000 \(\times\) g. The supernatant was applied to a DE52 column (0.7 \(\times\) 7 cm) equilibrated with 30 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 6 mM \(\beta\)-mercaptoethanol, 1 mM PMSF, and 0.01 % leupeptin (Buffer A). The enzyme was eluted
from the column by the application of one step gradient of NaCl (30 mM to 100 mM) using buffer A containing 100 mM NaCl instead of 30 mM NaCl (Buffer B). The 0.2 ml fraction was then applied to a TSK-G 3000 SW column (1 × 30 cm) and the enzyme was eluted with Buffer C: 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, and 6 mM β-mercaptoethanol. Each fraction was assayed for the protein kinase activity in the presence of Ca²⁺, PS or CL, and DG. This method was applied for the preparation of protein kinase C from rat liver mitochondria and E. coli. Rat liver mitochondria were isolated by the modified method of Hogeboom-Schneider (22) and the supernatant was obtained after hypotonic treatment.

Assay of protein kinase C. The activity of protein kinase C was routinely assayed by measuring the incorporation of ³²P from [γ-³²P]ATP into calf thymus H1 histone (type at 30°C [2]. The reaction mixtures (250 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM Mg acetate, 0.3 mM CaCl₂, 100 µM phospholipids (PS/PC, 4/1), 5 µM DG or 100 nM TPA, and 10 µM (20–30 Ci/mmol) [γ-³²P]ATP in the presence or absence of 0.2 mg/ml of the histone. Phospholipid and DG were first mixed in a small volume of chloroform and evaporated in vacuo. The residue was suspended in 20 mM Tris-HCl at pH 7.5 by sonication at 4°C for 30 min under nitrogen gas. After incubation in the reaction mixture at 30°C for 3 min, this reaction was stopped by the addition of 25% trichloroacetic acid. The acid-precipitable materials were collected on a glass filter (GF/C, Whatman Ltd.). When necessary, SDS polyacrylamide gel electrophoresis (PAGE) (13) of the reaction mixture was employed; gels were stained with Coomassie brilliant blue R250 or by the silver staining method and then autoradiograms of the ³²P-labelled proteins were made.

Interaction of cytoplasmic proteins with phospholipid vesicles. The supernatant of PMN homogenate or crude protein kinase C was incubated with 15 mM phospholipid liposomes (DPPC/cholesterol/CL, 0.8/1/0.2, M/M or DPPC/cholesterol, 1/1, M/M) at 4 or 20°C for 10 min in the medium of 0.15 M KCl and 20 mM HEPES (pH 7.4). The liposome-associated proteins were separated from non-associated proteins by discontinuous Ficoll density gradient (flotation method) as described in previous papers (23, 24). After separation of the liposome-associated proteins from non-liposome-associated ones, the activity of protein kinase C in both fractions was assayed. Protein content was determined by the method of Lowry et al. (14) and lipid content by that of Lowry et al. (15).

RESULTS AND DISCUSSION

Association of C-kinase with CL-containing liposomes. It was recently demonstrated that multifunctional Ca²⁺-binding proteins undergo a substantial conformational change upon binding to Ca²⁺, exposing hydrophobic sites (4, 5, 25). The binding of protein kinase C to a specific site in biological membrane, such as PS, was induced by micromolar concentrations of Ca²⁺ and resulted in activation of the enzyme (6, 25). As reported in previous papers (20, 23), when the PMN supernatant was incubated with the phospholipid liposomes which contain CL, several proteins, including the 33 kDa protein, associated with the liposomes in a calcium-dependent manner, and then were separated from non-associated proteins by using the flotation method (Fig. 1) (22). In this case, only the liposomal fraction (Fig. 2a, L) showed the activity of protein kinase C, and no enzyme activity was observed in the non-associated fraction in the absence of exogenous substrate (H₁ histone) (Fig. 2A, R). Furthermore, the phosphorylation of the liposome-associated proteins was inhibited by mixing with the non-associated fraction (Fig. 2A, L+R); this may be due to the
presence of endogenous cytoplasmic inhibitors (3, 9, 16). The association of total cytoplasmic protein kinase C with the liposomes was proved by the addition of partially purified rat brain protein kinase C fraction to the heat-denatured L and R fractions. The enzyme in the liposome-associated fraction was inactivated by heat treatment at 55°C for 3 min (Fig. 2B). The endogenous substrate in both liposome-associated and non-associated fractions was phosphorylated by the addition of the partially purified rat brain protein kinase C fraction (Fig. 2C). These results indicate that all cytoplasmic protein kinase C associates with CL-containing liposomes; moreover, liposome-associated proteins were more preferentially phosphorylated than non-associated proteins. The enzyme had almost no affinity for DPPC-liposomes and the enzyme activity in the DPPC-liposome fraction containing cholesterol was very low (Fig. 2D).

**Requirements and concentration dependency of CL-activated protein kinase C.** Figure 3 shows the effect of various stimulators on the activity of partially purified protein kinase C of rat brain. In the absence of Ca²⁺, no phosphorylation was induced by DG, PS or CL. In the presence of 0.3 mM Ca²⁺, phosphorylation independent of protein kinase C was observed, and higher activities of the enzyme were induced by the association of the enzyme with PS- or CL-containing liposomes. The highest enzyme activation occurred when DG was also present; under this condition, no difference was observed between PS and CL.

The maximum enhancement of protein kinase C activity by CL was observed at a molar ratio of CL/eggPC of 1/4 (Fig. 4). The concentration dependency of protein kinase C activation on CL is shown in Fig. 5. The half maximum requirement of
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CL was about 10 μM. The dependence of the enzyme activation on Ca²⁺ concentration in the presence of CL or PS is shown in Fig. 6. The half maximum requirement of Ca²⁺ was about 10 μl for the CL-activated enzyme, which was quite similar to that of PS.

As for the mechanism of the enzyme activation by DG, DG apparently binds to the enzyme, increasing its affinity for Ca²⁺ at the physiological concentration range (approximately micromolar), exposing the hydrophobic domain of the enzyme for association with membrane (11). Both PS and CL have the ability to bind Ca²⁺ in the membrane, and the Ca²⁺-binding forms of these phospholipids may participate in the association and activation of the enzyme in membrane.

Purification of protein kinase C based on the association with CL-liposomes. SDS-PAGE patterns of proteins in each purification step are shown in Fig. 7. The rat brain supernatant was prepared in the medium containing relatively high concentrations of EDTA and EGTA to prevent the association of protein kinase C with membrane, and to protect the proteolytic enzyme activity (Fig. 7, 1: 100 k × g. Sup). The supernatant was applied to a DE52 column (0.7 × 7 cm) equilibrated with Buffer A. After washing with Buffer A, the enzyme was eluted with Buffer B (Fig. 7. DE52). The DE52 fraction was applied to a TSK-G 3000SW column (1 × 30 cm) and eluted with Buffer C. When each fraction was assayed for protein kinase C by the method of Kikkawa et al. (12), a single peak appeared in fraction 14 (Fig. 7, 1) (TSK-G

Fig. 2. Association of protein kinase C with cholesterol- and CL-containing liposomes. The supernatant fraction of PMN homogenate (10 mg protein/ml) was incubated with DPPC/cholesterol/CL liposomes (15 μmol/ml phospholipid 0.8/1/0.2 M/M) at 4°C for 10 min in 0.15 M KCl medium containing 1 mM CaCl₂. Protein associated liposomes were separated by the flotation method. The proteins of B and C (denatured) were treated at 55°C for 3 min for the inactivation of protein kinase C. Protein kinase C (PKC) was partially purified from rat brain by the method of Kikkawa et al. (12). Protein kinase activity was assayed in the incubation mixture of 0.3 mM CaCl₂, DPPC/cholesterol/CL (600/750/150 μM), and 5 μM DG to make the concentration of phospholipids identical in all cases. PC/Chol/CL, DPPC/cholesterol/cardiolipin liposomes; PC/Chol, DPPC/cholesterol liposomes; denatured, denatured by heat treatment; PKC, partially purified rat brain protein kinase C L, liposome-associated protein fraction; R, non-associated fraction.
Fig. 3. Requirements for activation of protein kinase C in the presence or absence of CL. Partially purified protein kinase C was obtained from rat brain by ion exchange column chromatography (DE 52) and gel filtration column chromatography (TSK-G 300SW), and C-kinase was assayed by measuring the incorporation of $^{32}\text{P}$ from $[^{32}\text{P}]\text{ATP}$ into histone at 30°C for 3 min. 0.25 ml of incubation mixture contained 0.075 μmoles CaCl$_2$, 1.25 nmoles of DG and, 5/20 nmoles of CL/ or PS/egg PC. Other incubation conditions were described in MATERIALS AND METHODS.

Fig. 4. Effect of cardiolipin concentration on the activation of protein kinase C. Protein kinase C activity of rat brain was assayed at various concentrations of cardiolipin. The total phospholipid concentration was 100 μM; the cardiolipin concentration was changed with the change of egg phosphatidylcholine. The other experimental conditions were the same as described in Fig. 3.
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Fig. 5. Concentration dependency of protein kinase C on cardiolipin or phosphatidylserine. Protein kinase C activity of rat brain was assayed at various concentrations of cardiolipin (CL) or phosphatidylserine (PS). The molar ratio of CL to egg PC or PS to egg PC was fixed at 1/4. Incubation was carried out in the medium containing 0.3 mM CaCl₂ and 5 μM DG. The other conditions were the same as described in Fig. 3. DG only, in the absence of phospholipids; egg PC only, in the absence of DG.

Fig. 6. Dependency of the CL- or PS-activated protein kinase C on Ca²⁺ concentration. Protein kinase C activity was assayed in the medium containing 100 μM phospholipid (CL/egg PC or PS/egg PC, 20/80 μM) and 5 μM DG in the presence of various concentrations of Ca²⁺. The concentration of Ca²⁺ in the medium was changed by EGTA-Ca²⁺ buffer at 0.1 mM of EGTA.
The fraction was pooled and interacted with CL-containing liposomes. After the association of protein kinase C with the liposomes, the liposomes were isolated (Fig. 7, 2. L) from non-associated proteins (Fig. 7, 2. R) by the flotation method (23, 24). Protein kinase C in the liposome-associated fraction showed a fairly high purity, but the total recovery and enzyme activity were not calculated because of the high concentration of phospholipid (750 μM).

**Preferential phosphorylation of cytoplasmic proteins due to association with membrane.** Even in the absence of exogenous substrate (H1 histone), many membrane-associated cytoplasmic proteins are phosphorylated by membrane-bound protein kinase C (17, 19). Protein kinase C catalyzed the phosphorylation of polypeptides with apparent molecular weights of 150, 115, 80, 65, 43, 33, and 20K (Fig. 8). As expected, non-associated proteins were not phosphorylated. Furthermore, the phosphorylation of the liposome-associated proteins was not increased by mixing with non-associated fraction (Fig. 8A). Protein kinase C was inactivated by heat treatment at 55°C for 3 min. Therefore, little phosphorylation activity was observed in either the liposome-associated or non-associated fraction (Fig. 8B). Upon addition of partially purified protein kinase C from rat brain to the heat-treated fractions, however, both liposome-associated and non-associated proteins were similarly phosphorylated (Fig. 8C). The patterns of phosphorylated proteins in non-denatured liposome-associated fractions were quite similar to these induced by adding protein kinase C to the heat treated fractions (Fig. 8). These results indicate that the membrane-associated cytoplasmic proteins are more preferentially phosphorylated than non-membrane associated proteins.

Fig. 7. Changes in SDS-PAGE patterns of contaminated proteins during the purification steps using the flotation method. Experimental conditions were described in MATERIALS AND METHODS. 1) Purification by an ED52 anion exchange column and TSK-G 3000SW gel filtration; 2) Purification by the flotation method; L, Liposome associated proteins (protein kinase C); R, non-liposome associated proteins.
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C-kinase activity was detected in either rat liver mitochondria or *E. coli*. At present, there is no evidence of a role of CL in mitochondria and *E. coli* in relation to the enzyme activity in vivo (data not shown).

Stimulation of neutrophils by external agents elicits a variety of responses. It has been proposed that many of these responses are mediated by a series of events involving the tight binding of protein kinase C to the plasma membrane, resulting in its activation and phosphorylation of membrane proteins (17).

Together with previous results (19, 23), the data obtained in these experiments support the idea that the signal, which translocates protein kinase C from cytoplasm to plasma membrane by exposing its hydrophobic domain by PMA or mobilized intracellular calcium ions, would lead to phosphorylation of not only the membrane protein but also the transiently-associated cytoplasmic proteins which regulate the state of membrane lipids or the intracellular calcium ion concentration. Therefore, different cytoplasmic proteins may be phosphorylated by different stimuli (such as FMLP and PMA).

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Fig. 8. Autoradiographic expression of protein kinase C activity in SDS-PAGE of the liposome-associated or non-associated protein fraction of PMN supernatant. The experimental conditions were the same as described in Fig. 2. The 32P-labeled proteins were observed in SDS-PAGE (10% polyacrylamide gel) by autoradiography. A) Association with DPPC/Chol/CL liposomes; B) denatured protein after association with DPPC/Chol/CL liposomes; C) Rat brain protein kinase C was added to B; PKC, protein kinase C; L, liposome-associated fraction; R, non-liposome associated fraction.
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