Cooperative Roles of Various Membrane Phospholipids in the Activation of Calcium-activated, Phospholipid-dependent Protein Kinase*

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Although phosphatidylserine is the sole phospholipid effective for the activation of Ca$^{2+}$-activated, phospholipid-dependent protein kinase in the presence of a small amount of unsaturated diacylglycerol and micromolar concentrations of Ca$^{2+}$ (Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218-1224), other species of phospholipids modulate the activation of enzyme considerably. When phosphatidylserine is supplemented with phosphatidylethanolamine, further enhancement of the enzymatic activity is observed. Inversely, the addition of phosphatidylethanolamine or sphingomyelin markedly diminishes the enzyme activation by phosphatidylserine. Phosphatidylinositol, which serves as the source of unsaturated diacylglycerol, and phosphatidic acid do not show significant effects. Kinetic analysis has indicated that phosphatidylethanolamine enhances the enzyme activation by marked increase in the affinity of enzyme for Ca$^{2+}$ and also by slight increase in the affinity for phosphatidylserine as well as for unsaturated diacylglycerol without affecting the maximum reaction velocity. Phosphatidylethanolamine and sphingomyelin diminish the enzyme activation in an uncompetitive manner with respect to Ca$^{2+}$ and in a competitive manner with respect to both phosphatidylserine and unsaturated diacylglycerol. These results suggest that each species of the various membrane phospholipids plays a specific role with positive or negative cooperativity in the activation of this unique protein kinase.

The membrane lipid bilayer usually contains several chemically distinct phospholipids. Recently, considerable attention has been paid to the phospholipid metabolism, which is affected by many extracellular messengers such as hormones and neurotransmitters. In particular, phosphatidylinositol turnover has been most extensively investigated (for reviews, see Refs. 1-4). This phospholipid turnover, first recognized by Hokin and Hokin (5) in 1955 in pigeon pancreas and guinea pig brain stimulated by acetylcholine, was subsequently recognized by many investigators in virtually all types of tissues that are activated by a wide variety of extracellular messengers including α-adrenergic and muscarinic cholinergic neurotransmitters, peptide hormones, and many other biologically active substances (1-4). A series of recent studies in this laboratory has uncovered a new species of cyclic nucleotide-independent protein kinase in most mammalian tissues, which is normally present as an inactive form but is reversibly activated by the simultaneous presence of Ca$^{2+}$ and membranes (6, 7). The active component associated with membranes has been identified as phospholipid (6, 7). Among various phospholipids tested, only phosphatidylserine is effective, particularly at lower concentrations of Ca$^{2+}$. A small amount of unsaturated diacylglycerol markedly increases the affinity of enzyme for Ca$^{2+}$ to less than the micromolar range and such a dramatic effect is observed only in the presence of phosphatidylserine and not other phospholipids (8). Thus, unsaturated diacylglycerol derived from the phosphatidylinositol turnover mentioned above may serve as a second messenger for the selective activation of this protein kinase (8, 9). Recent reports from this laboratory (10, 11) have presented evidence that such a protein kinase system indeed operates in vivo in human platelets during activation by thrombin. The present paper will describe that various other phospholipids are inactive by themselves but nevertheless show positive or negative cooperative roles in the activation of this protein kinase. The Ca$^{2+}$-activated, phospholipid-dependent protein kinase will be referred to tentatively as protein kinase C.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—Protein kinase C and Ca$^{2+}$-dependent protease were prepared from rat brain as described previously (12). The catalytically active fragment of protein kinase C was prepared from the native enzyme by limited proteolysis with Ca$^{2+}$-dependent protease as specified earlier (12). These protein kinase preparations were practically free of endogenous phosphate acceptor proteins. Histone was prepared from calf thymus as described previously (13). Phosphatidylinositol (bovine brain) and phosphatidic acid (egg yolk), purchased from Sigma, were purified by two-dimensional silica plate thin layer chromatography under the conditions described earlier (6). Phosphatidylethanolamine (bovine brain), phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin (human erythrocytes) were generous gifts from Drs. T. Fujii and A. Tamura, Kyoto College of Pharmacy. These phospholipid preparations were chromatographically pure. Synthetic diolein was purchased from Nakarai Chemicals. [$\gamma$-32P]ATP was prepared by the method of Glyn and Chappell (14). All materials...
RESULTS AND DISCUSSION

Protein kinase C was normally inactive but was activated by the simultaneous addition of Ca²⁺, phospholipid, and unsaturated diacylglycerol (6-9). Among various phospholipids tested, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid were active in support of enzyme activation to variable extents at relatively higher concentrations of Ca²⁺ (10⁻²-10⁻³ M), but only phosphatidylserine was effective at the micromolar range of Ca²⁺ concentrations as shown in Table I. In this experiment, synthetic diolen was employed as an unsaturated diacylglycerol. Phosphatidylcholine and sphingomyelin were practically inactive over a wide range of Ca²⁺ concentrations. However, some of these inactive phospholipids showed marked effects on this enzyme activity in the presence of phosphatidylserine, unsaturated diacylglycerol, and micromolar concentrations of Ca²⁺.

Fig. 1 shows the effects of various phospholipids in the presence of a fixed amount of phosphatidylserine at two different concentrations of Ca²⁺. Further enhancement of the enzymatic activity was observed with phosphatidylethanolamine as a supplement to phosphatidylserine. Inversely, the enzymatic activity was markedly and progressively inhibited by the addition of increasing amounts of either phosphatidylcholine or sphingomyelin. Phosphatidylinositol and phosphatidic acid showed practically no effect. Unsaturated diacylglycerol was always indispensable at micromolar concentrations of Ca²⁺.

TABLE I

| Phospholipid added | Concentration added | Reaction velocity (cpm) |
|--------------------|---------------------|-------------------------|
| Phosphatidylserine| 20 µg/ml            | 13,520                  |
| + Phosphatidylethanolamine| 20 µg/ml | 13,570                  |
| + Phosphatidylcholine| 40 µg/ml          | 13,480                  |
| + Phosphatidylcholine| 40 µg/ml          | 13,550                  |
| Sphingomyelin      | 10 µg/ml            | 4,500                   |

Neither phosphatidylethanolamine, phosphatidylcholine, nor sphingomyelin appeared to interact directly with the catalytic site of the enzyme, since none of these phospholipids activated or inhibited the catalytically active fragment of enzyme which was obtained by limited proteolysis with Ca²⁺-dependent protease, as shown in Table II. This fragment was fully active in the absence of Ca²⁺, phosphatidylcholine, and unsaturated diacylglycerol as described earlier (6, 12, 16). These results indicate that the various phospholipids modulate the activation of enzyme rather than the catalytic process itself.

The next set of experiments was performed to explore the mode of action of the three species of phospholipids mentioned above. Since the activation of protein kinase C required Ca²⁺, phosphatidylserine, and unsaturated diacylglycerol, one of these activators was varied in the presence and absence of either phosphatidylethanolamine, phosphatidylcholine, or sphingomyelin. As shown in Fig. 2A, phosphatidylethanolamine appeared to decrease markedly the apparent Kₐ value for Ca²⁺, the concentration of Ca²⁺ needed for half-maximum...
activation. The double reciprocal plots indicated that this phospholipid decreased the $K_a$ value for Ca$^{2+}$ sharply to about one-tenth of the concentration needed in the presence of phosphatidylserine alone. The $V_{\text{max}}$ value was increased slightly. However, the enzymatic activity in this experiment was assayed in the presence of limited quantities of phosphatidylyserine and diolein. When these two activators were employed in sufficient and saturated quantities, phosphatidylethanolamine did not affect the $V_{\text{max}}$ value. In a marked contrast to phosphatidylethanolamine, both phosphatidylcholine and sphingomyelin appeared to inhibit enzyme activation over a wide range of Ca$^{2+}$ concentrations as shown in Fig. 2, B and C. The double reciprocal analysis of these results indicated that both phospholipids increased slightly the $K_a$ value for Ca$^{2+}$ and decreased the $V_{\text{max}}$ value, implying that these phospholipids inhibited the activation of protein kinase C in an uncompetitive manner with respect to Ca$^{2+}$. Fig. 3 shows the experiments in which phosphatidylyserine was varied in the presence and absence of one of the other three phospholipids. The double reciprocal plots obtained from these results indicated that phosphatidylethanolamine decreased the $K_a$ value for phosphatidylyserine. An apparent slight increase in the $V_{\text{max}}$ value shown in Fig. 4 again appeared to be due to this assay condition; here Ca$^{2+}$ and diolein were limited, i.e. when these two ingredients were sufficiently added, phosphatidylethanolamine did not show any effect on the $V_{\text{max}}$ value. Fig. 3, B and C, indicates that both phosphatidylcholine and sphingomyelin were inhibitory. The double reciprocal plots indicated that both phosphatidylcholine and sphingomyelin increased the $K_a$ value for phosphatidylserine and inhibited the enzyme activation competitively with respect to this phospholipid. Finally, in the experiment given in Fig. 4, unsaturated diacylglycerol, diolein in this case, was varied, and the result indicated that phosphatidylethanolamine again enhanced the enzyme activation by decreasing the $K_a$ value for diolein without affecting the $V_{\text{max}}$ value. Both phosphatidylcholine and sphingomyelin increased the $K_a$ value for diolein and inhibited the enzyme activation competitively with respect to this diacylglycerol. These kinetic results for the mode of activation and inhibition of protein kinase C are summarized in Table III.

The experimental results presented above together with those reported earlier (8) seem to indicate that the various phospholipids in membranes play cooperative roles in the
activation of protein kinase C. Upon stimulation by extracellular messengers, phosphatidylinositol produces unsaturated diacylglycerol which serves as a second messenger for the activation of protein kinase C. Phosphatidylserine plays an inevitable role during the enzyme activation. Phosphatidylethanolamine appears to facilitate this activation process, whereas phosphatidylcholine and sphingomyelin are inhibitory. However, it may be noted that interpretation of these results must be limited, since the various lipids added are all insoluble and, thus, the concentrations listed do not necessarily represent the actual physiological picture. Under these conditions, the phospholipids appear to form uni- or multilamellar systems and to be suspended in water to different degrees. Mixed lipid systems seem to form mixed lamellae, and phase separations may be anticipated. It would also be expected that Ca$^{2+}$ may affect the lamellae structures and diacylglycerol may intercalate between the lamellae. Presumably, protein kinase C may bind variably to the lamellae formed. Much work remains to be done to resolve the complex role of Ca$^{2+}$, diacylglycerol, and each phospholipid in the activation of this unique protein kinase.

It has been well established that, in erythrocytes and platelets, most of the phosphatidylcholine and sphingomyelin is located in the outer monolayer, whereas phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine are largely located in the inner monolayer of membranes (for reviews, see Refs. 17-19). Although it is not known whether such asymmetric distribution of the various phospholipids is favorable for the activation of protein kinase C, it is possible to assume that each species of the various membrane phospholipids displays a specific role with positive or negative cooperativity in the activation of this unique protein kinase. On the other hand, Hirata et al. (20-23) have recently proposed that methylation of phosphatidylethanolamine to produce phosphatidylcholine may be stimulated by some extracellular messengers such as β-adrenergic agonists, concana-valin A, and chemotactic peptides. Since phosphatidylserine is well known to serve as precursor to phosphatidylethanolamine (24), it is possible that this decarboxylation reaction is stimulated by some extracellular messengers. In any case, it is conceivable that the activation of protein kinase C is intimately related to the metabolism of not only phosphatidylinositol but also of other phospholipids, since various phospholipids, particularly phosphatidylethanolamine, and phosphatidylcholine modulate the activation of protein kinase C as described in this paper.

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