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A PHYSICAL MODE OF INHIBITORY ACTION OF PHOSPHOLIPID-INTERACTING COMPOUNDS ON THE ACTIVATION OF CELLULAR PROTEIN KINASE C

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SUMMARY

A relationship between the inhibition of protein kinase C activation by phospholipid -interacting compounds and the associated change in the physical properties of phospholipid vesicle membranes was studied using physically characterizable phosphatidylserine -phosphatidylcholine-diacylglycerol (3:6:1) mixture vesicle membranes. The vesicles supported protein kinase C activation as well as phosphatidylserine-diacylglycerol (9:1) vesicles. Under the same enzyme assay conditions, trifluoperazine and to a lesser degree chlorpromazine inhibited the enzyme activation and increased the fluorescence polarization of 1,6-diphynylhexatriene in the vesicle membranes in a dose-related fashion. At the concentration of 10^{-4} M, n-dibutyltin dichloride also showed a slight tendency to inhibit the enzyme activation and to increase the polarization whereas dibucaine scarcely showed any significant effect. At least DBC of more than 5×10^{-4} M was required for manifestation of the detectable effect. There was a good correlation between the relative order of potency of these compounds for the inhibition of the enzyme activation and for the ordering of the membranes. Moreover, the compounds inhibited the enzyme and substrate binding to the vesicle membranes in a dose-related fashion. These results shows that the phospholipids -interacting compounds such as trifluoperazine and chlorpromazine may exert their inhibitory action on the enzyme activation by ordering the vesicle membranes or by the change in phase separation.

Key words: Protein kinase C; Activation; Phospholipid-interacting compounds; Phospholipid vesicles; Membrane order; Fluorescence polarization

Protein kinase C (PKC) is a Ca^{2+}-and phospholipid-dependent enzyme that is activated by diacylglycerol[1-3]. Although the enzyme is independent of calmodulin, it is intensively inhibited by various phospholipid-interacting compounds such as chlorpromazine, trifluoperazine and to a lesser degree dibucaine, which are known to be calmodulin inhibitors[4, 5]. This inhibitory action has been shown to be due to the inhibition of the activation process but not to the interaction with the active site of enzyme by the kinetic analysis[4, 7]. Moreover, the suggestion has been made that the inhibitory action of these compounds may be mediated through a competitive inhibition of enzyme activation by interacting with phospholipid[4, 6], and that none of these compounds appears to compete with Ca^{2+} or to counteraction the unigue effect of unsaturated diacylglycerol[4]. However, little has been done on the study of the physical mode of the inhibitory action of these compounds against the activation of PKC. For the activation of PKC, phosphatidylserine is indispensable and other phospholipids show positive or negative cooperation with phosphatidylserine[8]. At high cation concentrations, such as those used in the enzyme assay, phosphatidylserine forms highly dehydrated bilayers[9] and does not form typical fluid bilayer structures[10]. Moreover, the sonicated phos-

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phatidylserine vesicles are liable to fuse spontaneously[11]. High cation concentrations are also known to cause phospholipid mixtures containing phosphatidylserine to induce phase separation[12-14]. Therefore, a more stable and characterizable membrane system is necessary to clarify the physical mode of the inhibitory action of phospholipid-interacting compounds on the enzyme activation.

In this study, bilayer membranes formed from phosphatidylserine-phosphatidylcholine (1:2) mixtures dared to be used as the characterizable bilayer vesicles. In this mixture vesicles, a rapid and reversible two-dimensional phase separation is known to be induced by calcium ion[15] and proton[16]. Using this vesicles, the protein kinase activity and the fluorescence polarization of diphynylhexatriene in the vesicle membranes were measured to explore whether or not there is a clearcut quantitative correlation between this reversible enzyme activation and changes in the surrounding phospholipid membrane structure containing the membrane order and the possible phase separation in the presence of phospholipid-interacting compounds. A number of factors appears to be required for expression of enzyme activity. A possible requirement may be the enzyme and substrate binding to the vesicle membranes. Therefore, the effect of these compounds on the binding was also studied by the fluorescence energy transfer measurements using the phospholipidvesicles containing dansyl-phosphatidylethanolamine.

MATERIALS AND METHODS

L-α-Phosphatidylserine (PS, from bovine brain), L-α-phosphatidylcholine (PC, from frozen egg yolk), 1,2-dioleoyl-sn-glycerol (DAG), L-α-phosphatidylinositol (PI, from soybean), L-α-phosphatidylethanolamine dipalmitoyl-N-Dansyl (Dansyl-PE), and lysine-rich histone (type III-S, from Calf Thymus) were purchased from Sigma Chemical Company (St.Louis, MO, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from the Aldrige Chemical Company (Milwaukee, WI, USA). [γ-^{32}P]ATP (3 Ci/mmol) was obtained from New England Nuclear (Boston, MA), DEAE-cellulose (DE-52) was obtained from Whatman (Clifton, NJ). Phenyl-Sepharose CL-4B and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. Chlorpromazine hydrochloride (CPZ), trifluoperazine dihydrochlorides (TEP) and dibucaine hydrochloride (DBC) were purchased from Sigma Chemical Company. n-Dibutyltin dichloride (Bu_2SnCl_2) was obtained form K&K Laboratories (Plainview, NY, USA).

CPZ, TFP and DBC were solubilized in water. Bu_2SnCl_2 was dissolved in ethanol. The final concentration of ethanol in the reaction mixture should be less than 0.5 %, the concentration that did no affect either the inhibition of PKC activation by the compound or the fluorescence polarization of DPH in phospholipid vesicles.

Phospholipid vesicles were prepared by the following procedure. Phospholipids (1 mg) dissolved in chloroform-methanol were mixed at the desired composition and dried under a stream of nitrogen, followed by high vacuum pumping for 2 hr. They were then suspended in 5 ml of 20 mM Tris-HCl buffer (pH 7.5, containing 100 mM NaCl) and briefly sonicated at 0℃ for 1 min in a bath type ultrasonicator, followed by vigorous vortexing. Small unilameller vesicles were prepared by further sonication at 0℃ until the suspension became clear (for about 3 min). This vesicles were characterized by elution through a Sepharose 4B column.

For PKC activity measurements, the phospholipids were suspended in 20 mM Tris-HCl buffer (pH7.5) containing no 100 mM NaCl. Protein kinase C (PKC) was purified from fresh mouse brain soluble fraction by DEAE-cellulose (DE-52) and phenyl Sepharose CL-4B column chromatography[17], followed by Sephacryl S-200 gel filtration chromatography[18, 19]. The buffer solutions used for the purification were : 1) 2 mM EDTA, 10 mM EGTA, 10 % glycerol, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 0.2 mM leupeptin and 50 mM Tris-HCl for the preparation of crude extract; 2) 2 mM EDTA, 2 mM EGTA, 10 % glycerol, 50 mM 2-mercaptoethanol and 20 mM Tris-HCl for DE-52 column chromatography; 3) 2 mM EDTA, 2 mM EGTA, 10 % glycerol, 50 mM 2-mercaptoethanol, 0.1 M NaCl and 50

mM Tris-HCl for phenyl Sepharose CL-4B column chromatography and Sephacryl S-200 gel filtration chromatography. All buffers were adjusted to pH 8.0 at 25℃. The purified PKC represented a 275-fold purification. The specific binding activity of [20-^{3}H]phorbol 12,13 -dibutyrate (10 Ci/mmol) was 6200 pmol/mg of protein. The specific enzymatic activity was 1.6 μmol/mg/min.

Protein kinase C was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus H1 histone (lysine-rich, type III-s). The assay conditions were similar to those reported by Leach and Blumberg[20]. The typical reaction mixture (300 μL) consisted of 20 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 μM [γ-^{32}P]ATP (10^6 cpm[γ-^{32}P]ATP per ml), designed phospholipid vesicles (20 μg/ml), DAG (2 μg/ml), histone (200 μg/ml) and enzyme preparation (5-8 μg/ml). The reaction was carried out for 3 min at 30℃. Various phospholipid-interacting compounds were added as indicated in each experiment. In the inhibition test of PKC activation by these compounds, the IC_{50} was defined as that compound concentration which produced 50 % inhibition of the maximally stimulated enzyme activity.

Fluorescence polarization was measured at 25℃ by a Parkin-Elmer Model MPF-44B fluorescence spectrophotometer using DPH as a fluorophore. The fluorescence was excited at 365 nm and detected at 425 nm. Lipid concentrations were 20 μg/ml (approximately 21-23 nmol/ml) and the ratio of DPH to lipid was 1/200 to 1/100. Fluorescence data were presented by either $I_{\parallel}/I_{\perp}$, $p=(I_{\parallel}/I_{\perp}-1)/(I_{\parallel}/_{\perp}+1)$ as the degree of fluorescence polarization, or $r=(I_{\parallel}/I_{\perp}-1)/(I_{\parallel}/I_{\perp}+2)$ as the fluorescence anisotropy, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured at parallel and perpendicular to the direction of polarization of the exciting beam, respectively. Results were expressed as a percentage change in the degree of fluorescence polarization, $100(P-P_0)/P_0$.

Fluorescence energy transfer was used to measure protein-membrane binding. The phospholipid vesicles (22 μg/ml) composed of PS-PC-DAG-dansyl-PE (3:6:1:1) were used. The excitation and the emission wave lengths were 284 and 512 nm, respectively. A 340 nm cut off filter was placed in front of the emission monochromater. The temperature was maintained at 25 ℃. Fluorescence energy transfer was expressed as a change in emission intensity, $\triangle I=I-I_0$ or as a percentage of the maximum change observed, $(100\times\triangle I/\triangle I_{max})$. In these equations, I is the fluorescence intensity of the protein-lipid complex, and I0 is the intensity of the phospholipid alone.

RESULTS AND DISCUSSION

Figure 1 shows the inhibitory effects of phospholipid-interacting compounds on the activation of PKC in the presence of specified phospholipid vesicles (PS-PC-DAG, 3:6:1). Of the compounds tested, TFP was the most potent inhibitor, with an IC_{50} of $2.5-3.5\times10^{-5}$ M under the assay conditions employed. CPZ was less potent, with IC_{50} value of $9.5-10.5\times10^{-5}$ M. At the concentration of 10^{-4} M, Bu_2SnCl_2 showed a slight tendency to inhibit the enzyme activation whereas DBC scarcely showed a significant effect. At least DBC of more than 5×10^{-4} M was required for expression of the detectable inhibition. IC_{50} values of Bu_2SnCl_2 and DBC were approximately 26-28 and 34-37 times greater than those for TFP, respectively. These results were similar to those obtained with PS-DAG (9:1) mixture vesicles. These results were also consistent with the data discussed at the high concentration range from 10^{-4} to 10^{-3} M by others[4, 6]. Thus, PS-PC-DAG (3:6:1) vesicles were found to support PKC activation as well as PS-DAG (9:1) vesicles, although the addition of PC diminished somewhat the ability of PS to activate the enzyme as compared with only pure PS.

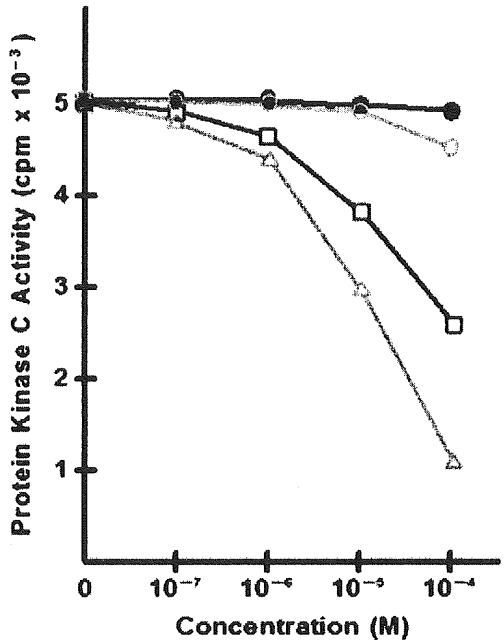

Fig. 1 Inhibitory effects of phospholipid-interacting compounds on the activation of protein kinase C. Conditions were as described under "Material and Methods" with various concentrations of compounds added as indicated. Phospholipid vesicles composed of PS-PC-DAG (3:6:1) were used.
TFP(△), CPZ(□), DBC(●), Bu_2SnCl_2(○).

Under the same conditions as employed in this PKC assay, the associated change in the physical properties of phospholipids vesicle membranes was studied by equilibrium fluorescence polarization of hydrophobic fluorophore, DPH (Fig. 2). TFP and to a lesser degree CPZ increased in a dose-related fashion the fluorescence polarization. Bu_2SnCl_2 showed a slight tendency to increase the polarization at the concentration of 10^{-4} M. DBC did not cause any change in the polarization at the same concentration, and at least more than 5×10^{-4} M was required for expression of the detectable changes. However, unfortunately at the concentration of more than 5×10^{-4} M, TFP, CPZ and Bu_2SnCl_2 produced a considerable precipitation, while DBC prevented the fluorescence measurements because of its own strong fluorescence. At the concentration of less than 10^{-4} M, there was a good correlation between the relative order of potency of the phospholipid-interacting compounds for the inhibition of PKC (Fig. 1) and for the ordering of the membranes (Fig. 2). These results with PS-PC-DAG (3:6:1) vesicles was similar to those obtained with PS-DAG (9:1) vesicles. However, in either case, the polarization values obtained under the complete enzyme assay conditions were all excessivelly high. This is perhaps due to the formation of highly dehydrated bilayer by high cation concentration.

Figure 3 shows the effect of phospholipid-interacting compounds on fluorescence polarization of DPH in PS-PC-DAG (3:6:1) vesicles suspended in 20 mM Tris-HCl buffer (pH 7.5, containing 100 mM NaCl) alone. In the same manner as obtained under the complete enzyme assay conditions, TFP and to a lesser degree CPZ increased in a dose-related fashion the fluorescence polarization of DPH. At the concentration of 10^{-4} M, Bu_2SnCl_2 also showed a tendency to increase the polarization whereas DBC scarcely caused any significant change in the polarization. These results also showed that there was a good correlation between the relative potency order of the compounds for the inhibition of PKC and for the ordering of the membranes. These findings suggest that the inhibitory mechanism of

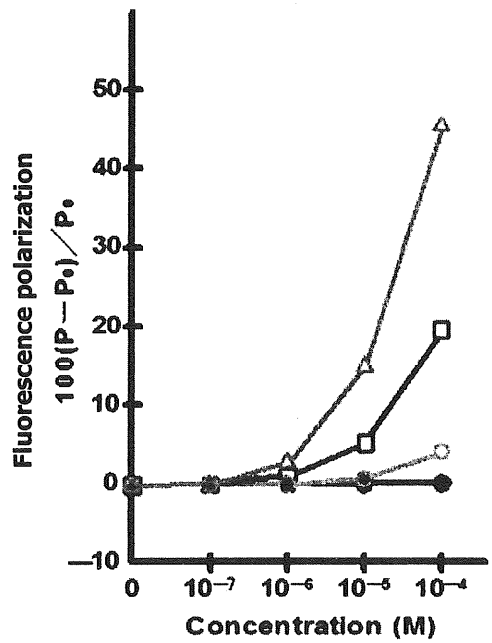

Fig. 2 Dose effects of phospholipid-interacting compounds on fluorescence polarization of DPH in PS-PC-DAG (3:6:1) vesicles under the same conditions as employed in PKC assay. Phospholipid vesicles contained 20 μ g/ml of total phospholipid. Fluorescence data were expressed as a percentage change in the degree of fluorescence polarization, $100(P-P_0)/P_0$. Each point is corrected for the value obtained with PS-PC-DAG (3:6:1) vesicles containing no fluorophore DPH.
TFP(△), CPZ(□), DBC(●), Bu_2SnCl_2(○).

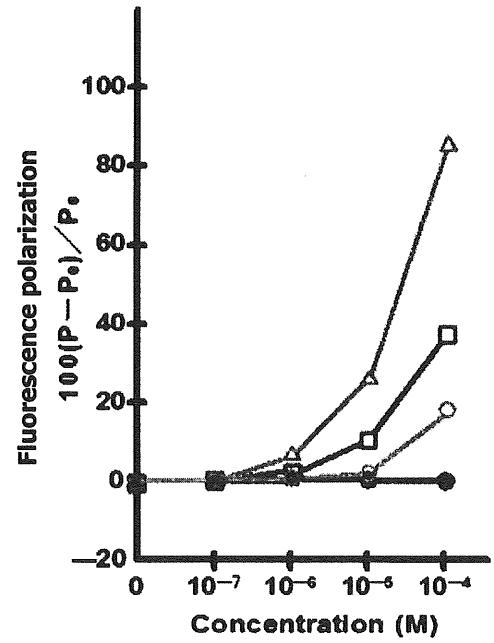

Fig. 3 Dose effects of phospholipid-interacting compounds on fluorescence polarization of DPH in PS-PC-DAG (3:6:1) vesicles suspended in 20 mM Tris-HCl buffer (pH 7.5, containing 100 mM NaCl) alone. Phospholipid vesicles contained 20 μ g/ml of total phospholipid. Fluorescence data were expressed as a percentage change in the degree of fluorescence polarization, $100(P-P_0)/P_0$. Each point is corrected for the value obtained with PS-PC-DAG (3:6:1) vesicles containing no fluorophore DPH.
TFP(△), CPZ(□), DBC(●), Bu_2SnCl_2(○).

these compounds on the PKC activation is associated with physical change of PS-PC-DAG (3:6:1) vesicles, especially with changes in membrane order and phase separation of the vesicles. As described in the introduction, in PS-PC (1:2) vesicles, 75 % of the vesicles is liquid-crystal state and 25 % of the vesicles is solid-gel state in the presence of Ca^{2+} at 23℃ [15]. Moreover, 64 % of the solid-gel state is PS. This Ca^{2+}-induced phase separation is rapidly and reversibly caused into a solid phase of Ca^{2+}-chelated PS aggregates and a liquid phase of neutral lipids such as PC. Therefore, the presence of DAG appears to be a great convenience to the Ca^{2+}-induced phase separation in PS-DAG vesicles, because DAG is unsaturated fatty acid and also is apt to hold liquid-crystal phase. When patch of solid-gel state is produced in liquid crystal phase, fusion and crystallization may occur one after the other in the vesicles, and consequently fluctuation of density of phospholipid may occur, and in turn disorder of the membranes may occur. Phospholipid-interacting compounds may exert their inhibitory action on the PKC activation by ordering the vesicle membranes or by the change in phase separation of the vesicles.

Figure 4 shows results of fluorescence energy transfer from tryptophan residues in the protein to dansyl-PE in the membranes. PKC bound in a dose-related fashion to the PS-PC-DAG-Dansyl-PE (3:6:1:1) vesicle membranes in the presence of histone (as the substrate) under the complete enzyme assay conditions as described in "Materials and Methods". The presence of histone was essential for this PKC-membrane binding, although histone alone bound in a dose-related fashion to the vesicle membranes.

As shown in Fig. 5, this PKC-membrane binding was in a dose-related fashion inhibited by phospholipid-interacting compounds as well as histone-membrane binding. Therefore, this result also shows that phospholipid-interacting compounds may inhibit the binding of PKC and substrate to the vesicle membrane by ordering the membranes.

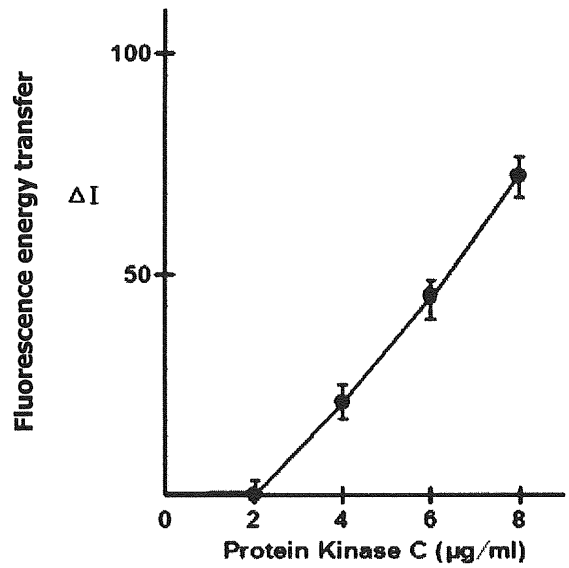

Fig. 4 PKC-histone binding to PS-PC-DAG (3:6:1) vesicles. The binding was measured by fluorescence energy transfer in 3 ml of 20 mM Tris-HCl buffer (pH 7.5) containing phospholipid vesicles (20 μg/ml), Ca^{2+} (0.2 mM), histone (200 μg/ml), Mg^{2+} (10 mM) and ATP (5 μM). Fluorescence energy transfer was expressed as a change in emission intensity, $\triangle I=I-I_0$. Vertical bars denote S.E. of the mean for five determinations.

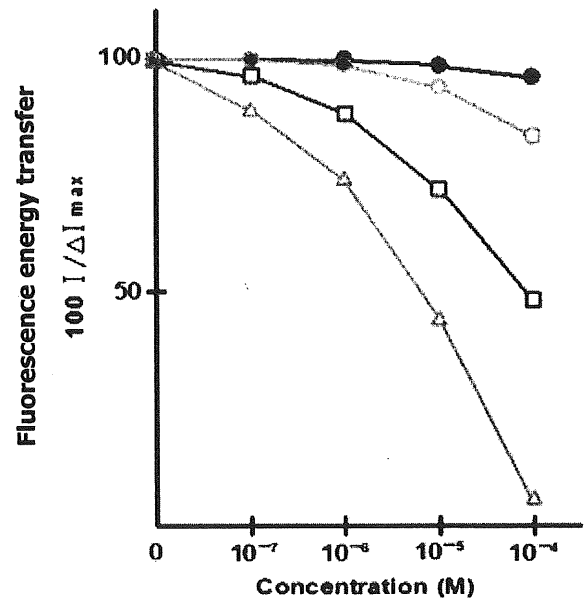

Fig. 5 Inhibitory effect of phospholipid-interacting compounds on the PKC-histone binding to PS-PC-DAG (3:6:1) vesicles. The binding was measured by fluorescence energy transfer in 3 ml of 20 mM Tris-HCl buffer (pH 7.5) containing phospholipid vesicle (20 μg/ml), Ca^{2+} (0.2 mM), PKC (8 μg/ml), histone (200 μg/ml), Mg^{2+} (10 mM), and ATP (5 μM). Fluorescence energy transfer was expressed as a percentage of the maximum change observed, (100 x $\triangle I/\triangle I_{max}$). Vertical bars indicate S.E. of the mean for five determinations.
TFP(△), CPZ(□), DBC(●), Bu_2SnCl_2(○).

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