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FURTHER STUDIES ON THE INTERACTION OF PROTEIN KINASE C WITH PHOSPHOLIPID, DIACYLGLYCEROL, AND PHORBOL ESTER

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INDEXING WORDS

protein kinase C, phorbol ester, diacylglycerol, phospholipid

SYNOPSIS

It is generally accepted that the receptor-mediated hydrolysis of inositol phospholipids produces diacylglycerol for the activation of protein kinase C to modulate many Ca^{2+}-dependent processes. Phospholipids and Ca^{2+} are essential for the activation of protein kinase C, and diacylglycerol increases the affinity of this enzyme for phospholipid and this divalent cation. Tumor-promoting phorbol esters substitute for diacylglycerol to activate this protein kinase, and pleiotropic actions of the phorbol ester are, if not all, believed to be mediated through the protein phosphorylation by this protein kinase. It has been also clarified that protein kinase C is activated by 1,2-*sn*-diacylglycerol but not by 2,3-*sn*- nor 1,3-diacylglycerol. However, the precise biochemical mechanism in which this enzyme is activated by these substances has not well been elucidated. In this study, interaction of protein kinase C with phospholipid was examined using a

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Abbreviations used are : EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

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phospholipid monolayer technique as a model of the cell membrane. Protein kinase C was activated by phorbol ester in the presence of a monolayer of phosphatidylserine, and the potency of the phospholipid for the activation of protein kinase C was proportional to the surface pressure of the monolayer film. Even in the presence of phorbol ester, protein kinase C did not form a rigid complex with phospholipid monolayer, and a part of the enzyme activity was shown to remain in the solution beneath the monolayer. Kinetic studies for the specificity of diacylglycerol was also made using 1,2-diacylglycerol free from 1,3-isomer in the liposome of phosphatidylserine. 1-Stearoyl-2-arachidonylglycerol, a main species of diacylglycerol generated from the receptor-linked hydrolysis of inositol phospholipids, was most active, although many other diacylglycerols having at least one unsaturated fatty acyl moiety were almost equally active. 1,2-Diacylglycerols having saturated short carbon chains, which are permeable to intact cell membranes to induce the protein phosporylation by protein kinase C, showed the Ka values for this enzyme activation slightly larger than those of unsaturated diacylglycerols. These results suggest the specific interaction of protein kinase C with phospholipid, diacylglycerol, and phorbol ester.

INTRODUCTION

A great deal of interest has been focused on the role of Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) in the regulation of cellular functions.[23, 24] Under physiological conditions, protein kinase C is activated by diacylglycerol, which is derived from the receptor-mediated hydrolysis of inositol phospholipids, in the presence of Ca^{2+} and phospholipid.[14, 32] The physiological picture as well as the biochemical mechanism of this enzyme activation, however, has not yet been fully understood. Although 1-stearoyl-2-arachidonylglycerol is a main product among the diacylglycerols produced from inositol phospholipids, other unsaturated diacylglycerols such as 1,2-dioleoylglycerol have been demonstrated to be also effective for this enzyme activation *in vitro*.[14] Subsequently, permeable 1,2-diacylglycerols with one acetyl moiety or with two relatively short saturated fatty acyl chains were found to be effective in both purified enzyme and intact cell systems,[6, 8, 17] although

such diacylglycerols do not appear in normal cells and tissues. Later, tumor-promoting phorbol esters such as TPA were shown to substitute for diacylglycerol and activate protein kinase C.[4, 35] Diacylglycerols and phorbol esters presumably produce a quarternary complex with Ca^{2+}, phospholipid, and this enzyme[12] but the precise nature of the interaction of protein kinase C with phospholipid, Ca^{2+}, and diacylglycerol or TPA remains unknown. Recently, stereospecificity of diacylglycerol for protein kinase C activation has been reported, and the effective diacylglycerols in this enzyme activation have 1,2-*sn*- but not 2,3-*sn*-nor 1,3-configuration,[3, 25, 30] although the results described thus far were obtained with a mixture of three possible isomers of diacylglycerol.[14, 32] Protein kinase C was once regarded as a single entity, but recent sequence analysis of its complementary DNA clones has revealed that this enzyme exists as a family of multiple subspecies with closely related structures.[5, 11, 15, 21, 26, 27, 28, 29] The present studies were undertaken to investigate some aspects of the interaction of protein kinase C with phospholipid and TPA for this enzyme activation, by using a monolayer of phospholipid and TPA as a model of the cell membrane. The specificity of diacylglycerol was also examined both *in vitro* and *in vivo*, with various 1,2-*rac*-diacylglycerols free from 1,3-isomers.

EXPERIMENTAL PROCEDURES

Materials and chemicals

Protein kinase C was purified from rat brain by DE-52 (Whatman), threonine-Sepharose, and TSK phenyl-5PW (Toyo Soda) column chromatographies as described previously.[13] Purified enzyme was stored at 0C and used within 3 days after preparation. Calf thymus H1 histone was prepared as described.[10] 1-Stearoyl-2-arachidonylglycerol and 1-palmitoyl-2-arachidonylglycerol were obtained from Prof. B. W. Agranoff. 1,2-Dioctanoylglycerol and 1,2-didecanoylglycerol were prepared as described.[25] Phosphatidylserine (bovine brain) and other diacylglycerols were purchased from Serdary Research Laboratories or Avanti Polar Lipids. TPA was obtained from Chemicals for Cancer Research. All reagents employed for the present studies were taken up in water which was

prepared by a double distillation apparatus followed by passing through a Chelex-100 column to remove as much Ca^{2+} as possible as described previously.[14]

Preparation of a monolayer of phospholipid

The surface tension was measured using a modified method of Bangham and Dawson.[1] A platinum plate (0.5 x 0.5 x 0.1 cm) of surface tensometer ST-1 (Shimadzu) was dipped into the reaction solution of protein kinase C in a trough (2.0 cm diameter x 0.7 cm deep). To form a lipid film, phosphatidylserine dissolved in petroleum ether was spread using a microsyringe on the reaction solution to assay protein kinase C as described below. The reaction solution was kept at 30C and stirred continuously by a magnetic stirrer. The surface pressure of phosphatidylserine film was defined as the difference between the surface tensions obtained before and after formation of phosphatidylserine film.[7] This surface pressure was calculated using methanol as a standard solution.

Assay of protein kinase C

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone. When protein kinase C activity was measured using the monolayer of phosphatidylserine, the reaction solution (2 ml) in the trough contained 20 *mM* Tris/HCl at pH 7.5, 200 μg/ml H1 histone, 10 μ*M* [γ-^{32}P]ATP (50 to 100 cpm/pmol), 5 *mM* magnesium acetate, 1 *mM* EGTA, and 0.988 *mM* $CaCl_2$. Free Ca^{2+} concentration was calculated to be 10 μ*M* using Ca^{2+}_2-EGTA buffer.[2] Various amount of phosphatidylserine dissolved solved in petroleum ether were added on the surface of the reaction solution in the trough as described above, and the reaction solution was kept at 30C with continuous stirring. Where an appropriate amount of TPA dissolved in petroleum ether was mixed with the solution of phosphatidylserine before the preparation of the monolayer. After evaporation of petroleum ether, the reaction was started by injecting 0.5-1.0 μg of protein kinase C from a side hole of the trough. After 10 min at 30C, a 500-μl aliquot of reaction solution was removed from another side hole of the trough and pipetted into a plastic tube containing 25% trichloroacetic acid. Acid-precipitable materials were collected

on a nitrocelulose filter, and the radioactivity was determined. When liposome of phosphatidylserine was used for assay, protein kinase C activity was measured as described previously.[14] The reaction mixture (0.25 ml) contained 20 *mM* Tris/HCl at pH 7.5, 200 μg/ml H1 histone, 10 μ*M* [γ-^{32}P]ATP (50 to 100 cpm/pmol), 5 *mM* magnesium acetate, 1 *mM* EGTA, 0.5-1.0 μg of protein kinase C, and various amount of phosphatidylserine, diacylglycerols, TPA, and $CaCl_2$ as indicated in each experiment. When diacylglycerol was used, diacylglycerol and phosphatidylserine were first mixed in a small volume of chloroform. After the chloroform was removed under N_2 stream, the residue was suspended in 20 *mM* Tris/HCl at pH 7.5 by sonication for 5 min at 0C.

Purification of 1,2-diacylglycerols

1,2-*rac*-Diacylglycerols were separated from 1,3-isomers using HPLC by the method of Hamilton and Comai[9] with a modification. The mixture of 1,2-*rac*- and 1,3-diacylglycerols (1-2 mg) was applied to a silica 60 column (Toyo Soda, 0.46 x 25 cm) equipped with HLC-803D (Toyo Soda). The column was equilibrated and eluted with hexane/isopropanol/acetic acid (100:1:0.01, v/v/v) as a solvent at a flow rate of 1 ml/min. 1,3- and 1,2-*rac*-diacylglycerols were normally eluted at 21 to 25 min and at 35 to 40 min after injection, respectively. The lipid was detected by a differential refractometer (Toyo Soda RI-8000). The solvent was removed under N_2 stream, and the purity of each diacylglycerol was determined by thin layer chromatography on a silica gel 60 plate (Merck) using chloroform/acetone/methanol (94.5:5.0:0.5, v/v/v) as a solvent.

Measurement of 47kDa protein phosphorylation in platelets

Washed platelets (4 x 10^9 cells) were labeled with 1 mCi of carrier-free ^{32}Pi for 1 h at 37C as described by Lyons *et al.*[19] The radioactive platelets (1.2 x 10^8 cells) were stimulated for 1 min at 37C either with thrombin (3.3 unit/ml) or each saturated diacylglycerol (100 μ*M*). The radioactive 47kDa protein was separated by SDS-polyacrylamide slab gel electrophoresis. The gel was dried on a filter paper and exposed to an X-ray film. The relative intensity of the radioactive protein bands visualized by autoradiography was determined by densitometric tracing at 430 nm as described.[31]

Determination

Protein was determined by the method of Lowry *et al.*[18] with bovine serum albumin as a standard.

RESULTS

The interaction of protein kinase C with phospholipid was examined using a monolayer film of phosphatidylserine as a model of the cell membrane. As shown in Fig. 1, the surface pressure of the film of phosphatidylserine was dependent on the concentration of phosphatidylserine. The collapse pressure of phosphatidylserine film was 33 dyn/cm, which remained unchanged by the further increase of the amount of phosphatidylserine. Phosphatidylserine was supposed to be in a monolayer film when the surface pressure was lower than the collapse pressure. When higher concentrations of phosphatidylserine were used, the film appeared to produce in a multilayer state. Fig. 2 shows the effect of phosphatidylserine concentration on the activation of protein kinase C in the monolayer and liposome assays. As shown in Fig. 2A, in the presence of TPA the protein kinase C activity was dependent on the concentration of phosphatidylserine. The enzyme was not activated significantly in the absence of TPA. The enyme activity was not proportional to the concentration of phosphatidylserine above collapse pressure. However, even if phosphatidylserine and TPA exist in a multilayer state, a slight increase of protein kinase C activity was observed. The surface pressure of the film of phosphatidylserine and TPA was slightly higher than that of phosphatidylserine alone (data not shown). Compared to protein kinase C activity in a monolayer system, protein kinase C activity with a liposome system increased linearly with the concentration of phosphatidylserine in the presence of 0.162 μM TPA as shown in Fig. 2B. Protein kinase C was activated slightly by higher concentrations of phosphatidylserine in liposome even in the absence of TPA. At the concentration lower than 0.4 μM phosphatidylserine in the liposome form was slightly more effective than that in the monolayer form. Above 0.4 μM a significant difference of the enzyme activity was seen between the liposome and monolayer assays. Fig. 3 shows the effect of TPA on protein kinase C activation in monolayer and in liposome assays. In the monolayer assay,

TPA activated protein kinase C in a dose-dependent manner in the presence of 0.362 μ*M* phosphatidylserine and 10 μ*M* Ca^{2+} as shown in Fig. 3A. TPA alone did not activate the enzyme in the absence of phosphatidylserine. Under these conditions a part of protein kinase C presumably remained in the aqueous phase in the reaction mixture. On the other hand, protein kinase C showed nearly maximal enzymatic activity in the liposome assay at 0.0162 μ*M* TPA in the presence of 0.362 μ*M* phosphatidylserine and 10 μ*M* Ca^{2+}, as shown in Fig. 3B.

Diacylglycerols were not as active as TPA in a monolayer system, probably because they significantly affected surface pressure, and disrupted the monolayer of phospholipid. Thus, the specificity of diacylglycerols for the activation of protein kinase C was investigated in a liposome system. 1,2-Diacylglycerol fractions obtained from commercial sources normally contained 1,3-diacylglycerol, since the isomerization from 1,2-*sn*- or 2,3-*sn*-diacylglycerol to 1,3-diacylglycerol occurs during storage. 1,2-*rac*-Diacylglycerol (a mixture of 1,2-*sn*- and 2,3-*sn*-enantiomers) was separated from the 1,3-isomer by HPLC, and employed for the kinetic analysis for the activation of protein kinase C. The purity of 1,2-diacylglycerols obtained by HPLC was determined by thin layer chromatography, and used immediately after preparation. Specificity of various diacylglycerols for protein kinase C activation was examined at low concentrations of phosphatidylserine (2 μg/ml) and Ca^{2+} (1 μ*M*). As shown in Fig. 4A, the enzyme was activated by diacylglycerols containing at least one unsaturated fatty acid such as 1-stearoyl-2-arachidonylglycerol. Although the 1-stearoyl-2-arachidonylglycerol is a main species among diacylglycerols derived from the receptor-mediated hydrolysis of inositol phospholipids under physiological conditions, there was no significant difference on the activation of protein kinase C between various 1,2-diacylglycerols having one unsaturated fatty acid and those having two unsaturated acyl moieties. Fig. 4B shows the effect of 1,2-diacylglycerols having two saturated fatty acids with various chain lengths. 1,2-Diacylglycerols containing saturated fatty acids with short chains, such as 1,2-dioctanoylglycerol and 1,2-didecanoylglycerol, were as effective as those having unsaturated ones, as described by Bell and co-workers.[17] In contrast, 1,2-diacylglycerols containing saturated long chains, such as 1,2-dipalmitoylglycerol and 1,2-

distearoylglycerol, were inactive. 1,2-Dimyristoylglycerol activated protein kinase C significantly. Apparent Ka and Vmax values of various diacylglycerols for protein kinase C activation are summarized in Table 1. The Vmax values of unsaturated diacylglycerols and saturated diacylglycerols with short acyl chains were similar, although Ka values for the formers were lower than those for the latters.

Table 1 Activation of protein kinase C by 1,2-diacylglcerols having various saturated and unsaturated fatty acyl moieties. Protein kinase C was assayed as described in the legend to Fig. 4. The Ka value for each diacylglycerol was the concentration giving the half maximum reaction velocity. The Vmax values are expressed as percentages, with that for 1-stearoyl-2-arachidonylglycerol as 100%. Under the given conditions the Vmax value for this diacylglycerol was 124 pmol/min/μg protein. The numbers indicate the average values of 4 determinations.

Diacylglycerols tested	Ka	Vmax
	(μM)	(%)
1) Unsaturated diacylglycerols		
1-Stearoyl-2-arachidonylglycerol	0.42	100
1-Palmitoyl-2-arachidonylglycerol	0.51	92.8
1-Stearoyl-2-oleoylglycerol	0.35	82.3
1-Stearoyl-2-linoleoylglycerol	0.43	82.6
1,2-Dioleoylglycerol	0.21	87.3
1,2-Diarachidonylglycerol	0.38	83.7
1,2-Dilinoleoylglycerol	0.23	90.7
2) Saturated diacylglycerols		
1,2-Dioctanoylglycerol	0.68	93.5
1,2-Didecanoylglycerol	0.75	88.8
1,2-Dimyristoylglycerol	0.43	40.0
1,2-Dipalmitoylglycerol	0.26	12.4
1,2-Distearoylglycerol	0.20	5.3

It has been previously shown that, in intact platelets, an endogenous protein with an approximate molecular weight of 47,000 (47kDa protein) serves as a specific substrate for protein kinase C, and that some diacylglycerols such as 1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol are intercalated into the membrane and activate protein kinase C directly. Although the rate of 47kDa protein phosphorylation induced by 1,2-diacylglycerols was slower than that induced by thrombin, the relative potencies of the saturated diacylglycerols to cause 47kDa protein phosphorylation was proportional to the activities of these diacylglycerols to activate protein kinase C *in vitro* (Table 2). 1,2-Dipalmitoylglycerol and 1,2-distearoylglycerol were not permeable to the membrane.

Table 2 Activation of protein kinase C in intact platelets by the addition of thrombin or diacylglycerol. The enzyme activation in intact platelets was estimated by the phosphorylation of the specific endogenous 47kDa substrate protein as described under "EXPERIMENTAL PROCEDURES."

Stimulant	Activation of protein kinase C (47kDa protein phosphorylation)	
	Arbitrary units	(%)
Thrombin	33,400	100
1,2-Dioctanoylglycerol	26,200	78
1,2-Didecanoylglycerol	5,800	17
1,2-Dimyristoylglycerol	1,200	4
1,2-Dipalmitoylglycerol	0	0
1,2-Distearoylglycerol	0	0

DISCUSSION

Since TPA may be intercalated into the membrane phospholipid bilayer where it activates protein kinase C by substituting for diacylglycerol, it has been postulated that the protein kinase C-phospholipid complex is most likely a receptor of the phorbol esters.[4, 22, 35] Thus, a monolayer technique was employed as a model of cell membrane to investigate the interaction of protein kinase C with phospholipid and diacylglycerol for this enzyme activation. As shown in Fig. 2A, assuming that phosphatidylserine

and TPA are present in a monolayer film, the efficiency of this membrane to activate protein kinase C was nearly equal as that of liposome of phosphatidylserine and TPA (Fig. 2B). Formation of a multilayer appeared to result in the decrease in the efficiency of phosphatidylserine and TPA for the enzyme activation. In short, it is plausible that the surface of phosphatidylserine and TPA membrane that is available for protein kinase C may be limited in a monolayer system. In the presence of low concentrations of TPA, as shown in Fig. 3A and 3B, the protein kinase C activity in a monolayer system was lower than that in a liposome system. Previously, Kraft and Anderson have demonstrated that the treatment of parietal yolk sacs cells with TPA induced a translocation of protein kinase C from a soluble to a membrane-bound compartment.[16] Recently, Cuatrecasas and co-workers[33, 34] have confirmed this enzyme-membrane interaction, using inside-out human erythrocyte vesicles. However, when protein kinase C showed submaximal enzyme activity in the presence of phosphatidyleserine and TPA in a monolyaer state, a part of protein kinase C appeared to remain in the aqueous phase of the reaction mixture (data not shown). Although the present monolayer system may not be equal to the cell membrane phospholipid bilayer, it is suggestive that protein kinase C may be activated by the lipid in monolayer. In this monolayer assay system, diacylglycerol seems to be inadequate, because a small amount diacylglycerol may produce higher surface pressure than 33 dyn/cm, which is the collapse pressure of phosphatidylserine film.

The specificity of diacylglycerol for protein kinase C activation, therefore, has been examined in a liposome system. Non-enzymatic racemization between 1,2-*sn*-and 2,3-*sn*-diacylglycerols is very rapid.[20] In addition, 1,2 (2,3)-*sn*-diacylglycerol isomerizes to 1,3-form, since the latter is thermodynamically more stable,[20] and thus diacylglycerol samples normally contain the three isomers at equilibrium. All samples of diacylglycerols employed in this study were purified just before use, and only 1,2-*rac*-diacylglycerols (mixtures of 1,2-*sn*-and 2,3-*sn*-isomers) were employed. These two enantiomers were not separable from each other. In the kinetic analysis presented above, each sample of the diacylglycerol tested was a racemic mixture of 1,2-*sn*- and 2,3-*sn*-isomers. Since diacylglycerols having 2,3-*sn*-configuration do not activate nor inhibit protein kinase C,[25] the effective Ka

values may be one half of those given in this paper. Among various diacylglycerols employed, 1-stearoyl-2-arachidonylglycerol is a major species of diacylglycerol derived from receptor-mediated hydrolysis of inositol phospholipids. This diacylglycerol was most effective for the activation of protein kinase C in *in vitro* study, but many other unsaturated diacylglycerols were found to be almost equally active in this role. It has been previously shown by Bell and co-workers[6, 17] that diacylglycerols having short saturated fatty acyl moieties, such as 1,2-dioctanoylglycerol and 1,2-didecanoylglycerol, can activate protein kinase C. The synthetic diacylglycerols gave different apparent Vmax values at their saturated concentrations (Fig. 4A, 4B). This may not be explained simply by differences of the affinites of these diacylglycerols for protein kinase C. The lipid-protein interaction may involve a specific diacylglycerol-induced perturbation of the structure of membrane phospholipid bilayer. Further study of interaction of protein kinase C with phospholipid, diacylglycerol and phorbol ester is needed.

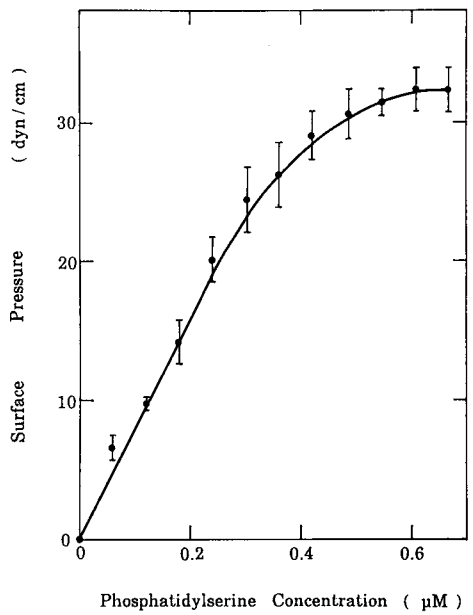

Fig. 1 Measurement of surface pressure of a film of phosphatidylserine. The surface pressure of the film of phosphatidylserine was measured as described under "EXPERIMENTAL PROCEDURES." Data were mean ± S.D. of 3 experiments.

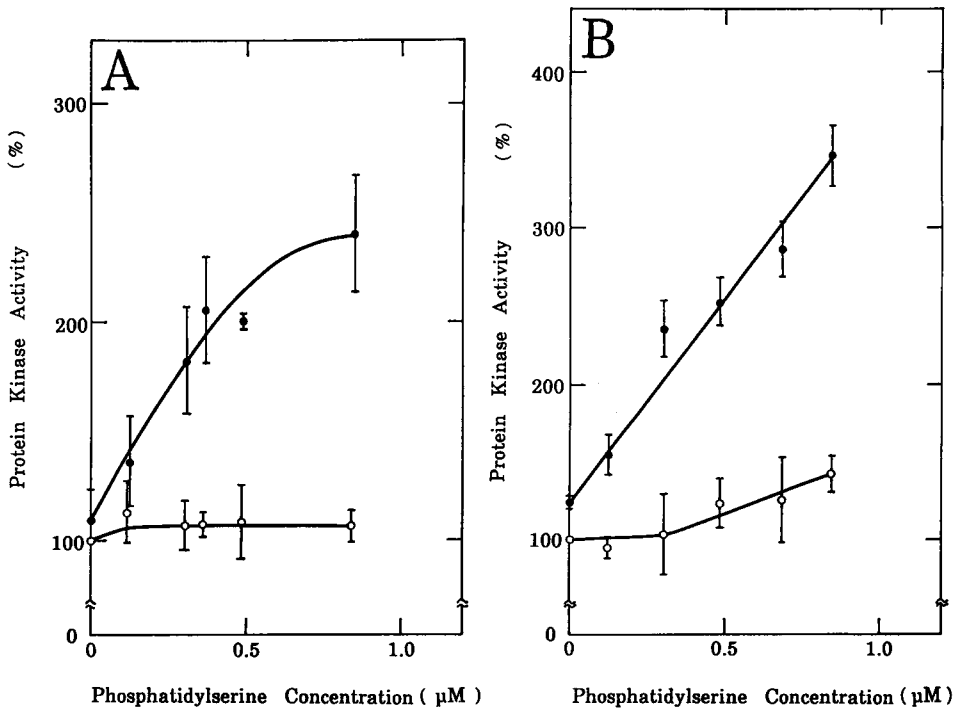

Fig. 2 Effect of phosphatidylserine on protein kinase C activity. Protein kinase C was assayed with various amount of phosphatidylserine in a monolayer or liposome system under the comparable conditions as described under "EXPERIMENTAL PROCEDURES." Free Ca^{2+} concentration was calculated to be 10 μM using Ca^{2+}-EGTA buffer. Basal activities measured in the absence of phosphatidylserine and TPA were 29.7 and 27.6 pmol/min/μg protein for the monolayer and liposome assay systems, respectively. Protein kinase C activity was expressed as percentages of the basal activity as 100%. Data were mean ± S.D. of 3 experiments. *A*, Effect of phosphatidylserine on protein kinase C activity in monolayer system. *B*, Effect of phosphatidylserine on protein kinase C activation in liposome system. (●——●), protein kinase C activity in the presence of TPA (0.162 μM) ; (○——○), protein kinase C activity in the absence of TPA.

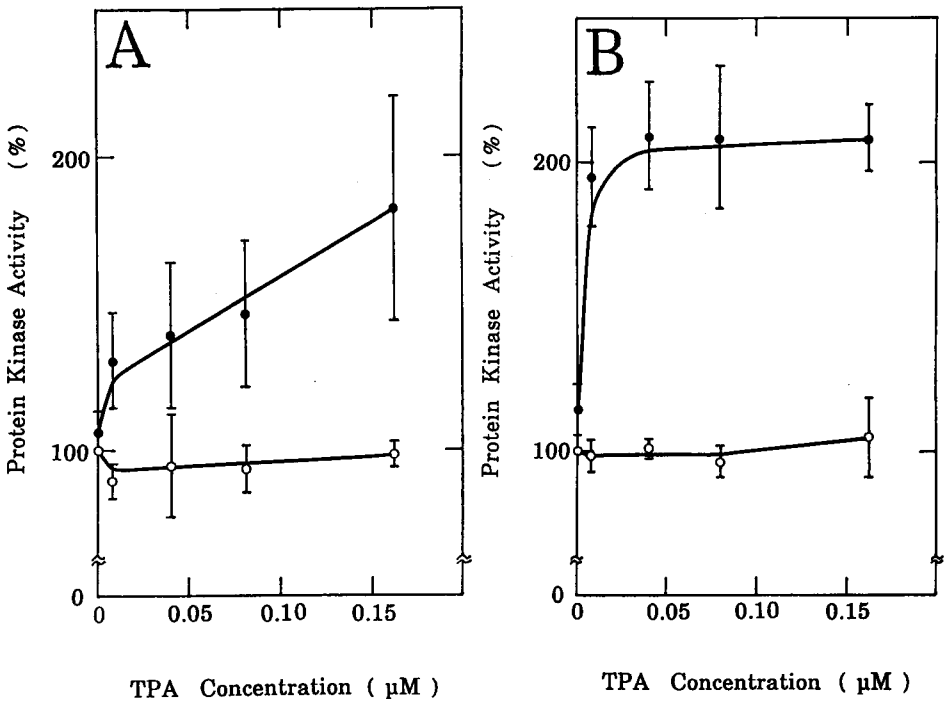

Fig. 3 Effect of TPA on protein kinase C activity. Protein kinase C was assayed with various amount of TPA in the presence of a monolayer or liposome of phosphatidylserine under the comparable conditions as described under "EXPERIMENTAL PROCEDURES." Free Ca^{2+} concentration was calculated to be 10 μ*M*. Basal activities measured in the absence of phosphatidylserine and TPA were 37.0 and 27.6 pmol/min/μg protein in the monolayer and liposome assays, respectively. Protein kinase C activity was expressed as percentages of the basal activity as 100%. Data were mean ± S.D. of 3 experiments. *A*, Effect of TPA on protein kinase C activity in monolayer system. *B*, Effect of TPA on protein kinase C activity in liposome system. (●——●), protein kinase C activity in the presence of phosphatidylserine (0.362 μ*M*); (○——○), protein kinase C activity in the absence of phosphatidylserine.

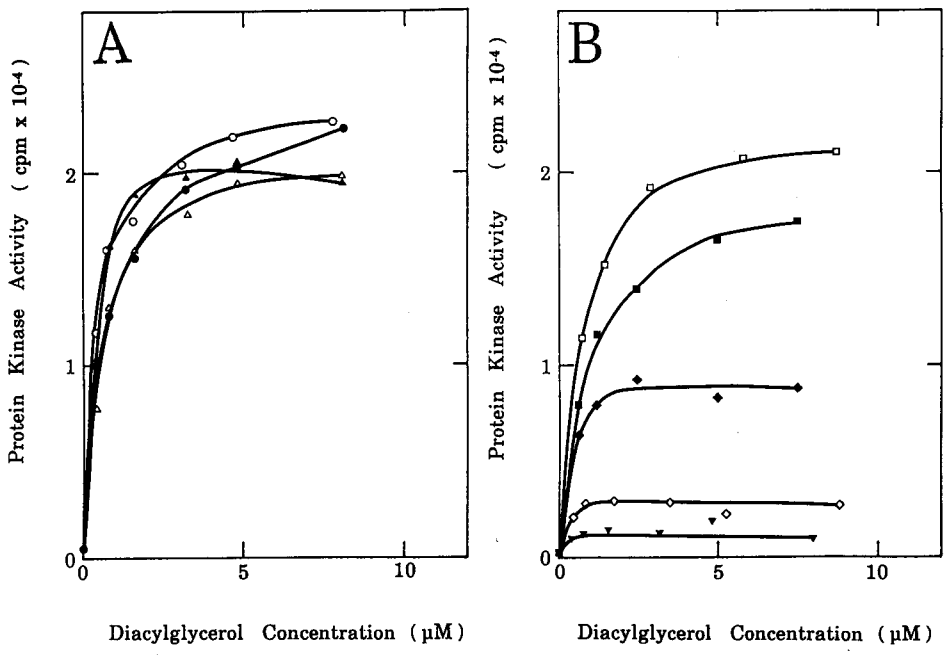

Fig. 4 Effect of 1,2-diacylglycerols on protein kinase C activity. Protein kinase C was assayed in the presence of 2 μg/ml phosphatidylserine, 0.816 *mM* $CaCl_2$, 1 *mM* EGTA, and various amount of diacylglycerols indicated as described under "EXPERIMENTAL PROCEDURES." Free Ca^{2+} concentration in the reaction mixture was calculated to be 1 μ*M*. *A*, Unsaturated diacylglycerols. (○——○), 1-stearoyl-2-arachidonylglycerol; (●——●), 1-palmitoyl-2-arachidonylglycerol; (△——△), 1-stearoyl-2-oleoylglycerol; (▲——▲), 1,2-dioleoylglycerol. *B*, Saturated diacylglycerols. (□——□), 1,2-dioctanoylglycerol; (■——■), 1,2-didecanoylglycerol; (◆——◆), 1,2-dimyristoylglycerol; (◇——◇), 1,2-dipalmitoylglycerol; (▼——▼), 1,2-distearoylglycerol.

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