Phosphatidylcholine-dependent protein kinase C activation

Effects of *cis*-fatty acid and diacylglycerol on synergism, autophosphorylation and Ca^{2+} -dependency

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A long-chain neutral phospholipid, dioleoylphosphatidylcholine, was found to support protein kinase C activation by *cis*-fatty acid and diacylglycerol (DAG). This effect of phosphatidylcholine (PC) is totally dependent on the presence of *cis*-fatty acid; PC greatly stimulates the *cis*-fatty acid-induced protein kinase C activity, but it does not activate protein kinase C at all, even in the presence of DAG, if *cis*-fatty acid is absent. DAG, however, plays a modulatory role in the presence of Ca^{2+} ; it further enhances the PC-potentiated *cis*-fatty acid activation of protein kinase C. Although the activities of all three protein kinase C subtypes tested (types I, II and III) are supported by this PC mechanism, type III is most sensitive to the DAG effect, and it is activated synergistically by *cis*-fatty acid and DAG. The potency of PC to support the synergistic activation of this subtype is equivalent to that of phosphatidylserine (PS). There are several differences, however, between PC- and PS-supported synergism observed in type III protein kinase C: (1) Ca^{2+} -sensitivity is different; PC requires higher concentrations of Ca^{2+} (10–20 μM - Ca^{2+}) than those required for PS (micromolar Ca^{2+}); (2) PC/*cis*-fatty acid/DAG-induced autophosphorylation of protein kinase C subtypes (types I, II and III) is very weak, whereas PS/*cis*-fatty acid/DAG strongly stimulate autophosphorylation of these subtypes under the conditions at which both PC and PS systems fully activate the protein kinase C in terms of histone phosphorylation. These observations suggest that a neutral phospholipid such as PC may also participate in the activation and differential regulation of protein kinase C.

INTRODUCTION

Phospholipid- and Ca^{2+} -dependent protein kinase (protein kinase C) has been widely demonstrated to play a pivotal role in transmembrane signal transduction in response to various hormones and neurotransmitters [1–3]. It has been shown that protein kinase C is activated by diacylglycerol (DAG) in the micromolar range of Ca^{2+} concentrations in the presence of acidic phospholipids such as phosphatidylserine (PS) [4]. In addition to the DAG-dependent activation mechanism, *cis*-unsaturated fatty acid has been shown to activate protein kinase C in the absence of phospholipids and DAG [5–11]. It has been further demonstrated that *cis*-fatty acid preferentially activates soluble protein kinase C, and it was suggested that the site of action of *cis*-fatty acid is not in the membrane, but rather in the cytosol [12].

Recent studies further revealed that *cis*-fatty acid and DAG activate protein kinase C co-operatively [13–17]. We have shown that the type III subtype of protein kinase C is particularly sensitive to this mode of activation, and it is synergistically activated by *cis*-fatty acid and DAG [17]. This mode is distinct from the DAG-independent *cis*-fatty acid activation in several aspects. First, the synergistic mode of activation is extremely sensitive to micromolar concentrations of Ca^{2+} . Secondly, the synergistic activation of type III protein kinase C does not require high concentrations of *cis*-fatty acid; micromolar concentrations of *cis*-fatty acid can strongly activate protein kinase C synergistically with DAG. Thirdly, the *cis*-fatty acid-induced synergism still requires PS, an acidic phospholipid, but it significantly decreases the PS requirement for the activation. These observations suggest that the generation of three second messengers, i.e. the increase in intracellular Ca^{2+} concentrations

and the formation of both *cis*-fatty acid and DAG in the cell, are necessary for the full activation of this protein kinase C subtype.

Although PS is most active in supporting protein kinase C activity in the presence of Ca^{2+} and DAG, other species of membrane phospholipids, such as phosphatidylethanolamine, sphingomyelin, polyphosphoinositides and lysophosphatidylcholine, can also modulate the protein kinase C activation, either positively or negatively [18–20]. Phosphatidylcholine (PC) has been shown to have an inhibitory effect on the PS/DAG-induced protein kinase C activity [18]. In the present study, we have characterized the synergistic action of *cis*-fatty acid and DAG on protein kinase C activity with respect to the requirement of phospholipids for the activation. Here we show that a long-chain PC is able to support the synergistic activation of protein kinase C through a mechanism which is distinct from the PS-dependent activation.

EXPERIMENTAL

Materials and chemicals

Oleic acid (sodium salt), 1,2-dioctanoyl-*sn*-glycerol (DiC_8), lysine-rich histone (type III-S), ATP, Triton X-100, leupeptin, phenylmethanesulphonyl fluoride and dithiothreitol were obtained from Sigma. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) were purchased from Avanti Polar Lipids. 5(6)-Carboxyfluorescein (CF) was obtained from Kodak. [γ - ^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear. Chelex 100 and reagents for PAGE were from Bio-Rad. All other chemicals used were reagent grade.

Protein kinase C subtypes and assay

Whole brains from 25 rats were used as a source of protein

Abbreviations used: PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; DOPS, dioleoylphosphatidylserine; DAG, diacylglycerol; DiC_8 , 1,2-dioctanoyl-*sn*-glycerol; CF, 5(6)-carboxyfluorescein.

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kinase C. Protein kinase C was purified by a four-step liquid-chromatography procedure as described elsewhere [17]: (1) DEAE-cellulose ion exchange, (2) phenyl-Sepharose, (3) AcA 34 gel filtration and (4) phenyl-5PW h.p.l.c. column chromatography. This highly purified protein kinase C was further separated into three fractions (types I, II and III) by using a hydroxyapatite column [21,22] connected to a f.p.l.c. system (Pharmacia) [23]. Types I, II and III enzymes were eluted by a 20–250 mM-potassium phosphate gradient from the column. The apparent homogeneity of these purified protein kinase C subtypes was confirmed by silver staining. Protein kinase C activity was assayed by measuring the incorporation of [32 P]P_i from [γ - 32 P]ATP into lysine-rich histone (type III-S; Sigma) at 30 °C. The basic reaction mixture contained 100 μ l of diluted protein kinase C subtype (specific activity 2390, 1310 and 2370 nmol/min per mg of protein for types I, II and III respectively), 300 μ g of histone/ml, 5 mM-MgCl₂, 0.76 mM-EGTA, 58 μ M-[γ - 32 P]ATP (90–150 c.p.m./pmol) and 20 mM-Tris/HCl, pH 7.5, in a final volume of 260 μ l. Concentrations of DOPC, DOPS, DiC₈, oleic acid and free Ca²⁺ were adjusted and specified in each experiment. Free Ca²⁺ concentrations were calculated by taking account of Ca²⁺, Mg²⁺, ATP and EGTA as described previously [17], by using a computer program written by Dr. A. Gear (University of Virginia).

Lipid treatment

DOPC, DOPS and DiC₈ were first dissolved in a minimal amount of chloroform/methanol (9:1, v/v). Then a thin film layer was made under a stream of N₂ gas. After the organic solvent was evaporated, the lipids were dispersed into Chelex 100-treated 20 mM-Tris/HCl buffer (pH 7.5) by vigorous vortex-mixing and sonicated at 4 °C for 10 min. Oleic acid was dispersed into the buffer in the same manner without the treatment with organic solvent.

Autophosphorylation

Autophosphorylation of protein kinase C was performed as described by McFadden *et al.* [24], except that different protein kinase C-activating factors, such as PC and oleic acid, were used. The basic reaction mixture for autophosphorylation contained 20 mM-Mg²⁺, 100 μ M free Ca²⁺ and diluted protein kinase C subtype (9–14 ng) in 20 mM-Tris/HCl, pH 7.5 (final volume 60 μ l). Concentrations of DOPS, DOPC, DiC₈ and oleic acid were 70, 70, 10 and 100 μ M respectively. The reaction was initiated by addition of 25 μ M-[γ - 32 P]ATP (18 $\times$ 10³ c.p.m./pmol). The reaction was carried out at 30 °C for 3 min, and then terminated with 30 μ l of stopping buffer (125 mM-Tris/HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.04% Bromophenol Blue). The autophosphorylated samples were analysed by SDS/PAGE in a 10%-polyacrylamide gel. After the silver staining, the autophosphorylated bands were made visible by exposure of the dried gel to the X-ray film (Kodak X-Omat AR). The radioactivity of autophosphorylated protein kinase C was quantified with a Betascope analyser (Betagen).

Measurement of PC vesicle stability in the presence of *cis*-fatty acid

The effect of *cis*-fatty acid on PC vesicle stability was measured with CF as a fluorescent probe [25] by the following method. CF was purified on a hydrophobic LH-20 column (30 cm $\times$ 1 cm) [26]. To make CF-encapsulated PC vesicles, 8 mg of DOPC was sonicated in 1 ml of purified 30 mM-CF. CF-loaded PC vesicles were then separated from free CF on a Sephadex G-75 gel-filtration column (30 cm $\times$ 1 cm), which was pre-equilibrated with iso-osmotic buffer containing 34 mM-NaCl and 20 mM-

Tris/HCl, pH 7.4 (102 mosM); NaCl was included, to maintain the iso-osmotic condition inside and outside the PC vesicles. Fluorescence of CF excited at 470 nm was detected with a Perkin-Elmer LS-5 instrument at 525 nm in the iso-osmotic buffer. The concentration of PC was 150 μ M. Self-quenching of the fluorescence of encapsulated CF (30 mM) was first confirmed. A complete relief of quenching was obtained by adding Triton X-100 (final concn. 0.3%). At this concentration, the ratio of Triton X-100/DOPC (w/w), 16.7, is far greater than the reported values required for lamellar-vesicle/mixed-micelle transition (ratio 0.4–1.7) [27]. The effect of oleic acid on the fluorescence intensity increase owing to the relief of self-quenching was then measured and normalized with the fluorescence intensity in the presence of 0.3% Triton X-100. The PC concentration was quantified by measurement of P_i by using ammonium molybdate [28].

RESULTS

PC potentiation of synergistic activation of type III protein kinase C

The dose-dependency on PC of the activation of type III protein kinase C is shown in Fig. 1. In this study, we used defined species of phospholipids, DAG and *cis*-unsaturated fatty acid; DOPC, DOPS, DiC₈ and oleic acid were used as representatives of PC, PS, DAG and *cis*-fatty acid respectively. DOPC has no effect on the activation of protein kinase C, alone or together with DiC₈ (Fig. 1). This observation is consistent with the fact that PC fails to activate protein kinase C [29]. Oleic acid, a known protein kinase C activator, stimulates the activity of this subtype. DOPC was found to potentiate the protein kinase C activity significantly in the presence of oleic acid and DiC₈ in a dose-dependent manner (Fig. 1). The potentiating effect of PC absolutely requires oleic acid. These findings indicate that the PC effect is at least mediated by oleic acid-induced protein kinase C activation. To examine whether DAG is also required for the potentiation, we measured the effect of PC on *cis*-fatty acid-induced protein kinase C activity in the absence of DAG. As shown in Fig. 2, PC supports the oleic acid-induced protein kinase C activity and DAG is not required for the potentiation by PC. The PC potentiation of the protein kinase C activity requires Ca²⁺. In the presence of Ca²⁺, the oleic acid-induced

Fig. 1. Effect of oleic acid on type III protein kinase C activation in the presence of PC and DAG

Protein kinase C activity was determined at the indicated concentrations of DOPC (□), with addition of 10 μ M-DiC₈ (▲) or 10 μ M-DiC₈ and 100 μ M-oleic acid (●). Free Ca²⁺ concentration was fixed at 50 μ M.

Fig. 2. Effect of PC on oleic acid-induced type III protein kinase C activation

Protein kinase C activity was assayed at 100 μ M-oleic acid and various concentrations of DOPC in the presence of 0.5 mM- Ca^{2+} (■) or 0.75 mM-EGTA (□).

Fig. 3. Ca^{2+} -sensitivity of PC effect on type III protein kinase C

Protein kinase C activity was assayed in the presence of 74 μ M-DOPC (□), in combination with 10 μ M- DiC_8 (▲) or 100 μ M-oleic acid (■) or both (●), at various concentrations of Ca^{2+} .

activity is further stimulated by DOPC even in the absence of DAG. However, DOPC inhibits the oleic acid-induced activity if Ca^{2+} is absent. Although this inhibitory effect of PC is small, the effect is consistent. Interestingly, PS, a cofactor for protein kinase C activation, was also shown to inhibit the oleic acid-dependent type III protein kinase C activation in the absence of Ca^{2+} [17].

Ca^{2+} -dependency of PC potentiation

Since the potentiation effect of DOPC is dependent on the presence of Ca^{2+} , we examined the minimal requirement for Ca^{2+} for PC-supported activation of type III protein kinase C. Fig. 3 shows Ca^{2+} -dependency of the PC-supported protein kinase C activity in the absence or presence of *cis*-fatty acid and DAG. PC is totally ineffective for the activation in the absence of oleic acid. This is consistent with the indication in Figs. 1 and 2 that the PC effect is through *cis*-fatty acid-induced activation of protein kinase C, but not via a DAG-mediated mechanism. PC, together with oleic acid, stimulates the protein kinase C activity in a Ca^{2+} -

Fig. 4. Differential effect of PC/DAG on activation of protein kinase C subtypes

Protein kinase C activity was assayed in the presence of 74 μ M-DOPC, in combination with 10 μ M- DiC_8 (+ DiC_8) or 100 μ M-oleic acid (+OA) or both (+Both). Results were obtained in the presence of 50 μ M free Ca^{2+} (■) or 0.75 mM-EGTA (□).

dependent manner. The K_a value for Ca^{2+} , at which half-activation is achieved, is 40 μ M. No activation is observed in the absence of Ca^{2+} . This is presumably due to the PC inhibition of oleic acid-dependent protein kinase C activation, as shown in Fig. 2. Although DAG has no effect on protein kinase C activity in the presence of PC, it plays a modulatory role in the PC/*cis*-fatty acid-induced activation. DAG decreases the Ca^{2+} requirement for the activation (K_a for Ca^{2+} = 10 μ M).

Sensitivity of protein kinase C subtypes to the PC-dependent activation

Protein kinase C was recently shown to be activated additively or synergistically by *cis*-fatty acid and DAG in the presence of PS [12–17]. Our study further showed that, among the protein kinase C subtypes, type III is most sensitive to the synergistic activation mode [17]. We examined the sensitivity of three protein kinase C subtypes to the synergistic activation supported by PC. As shown in Fig. 4, in the presence of oleic acid, protein kinase C activity is supported by PC and Ca^{2+} , regardless of subtype. The synergistic effect of DAG on the PC/*cis*-fatty acid-mediated activation is different, depending on the protein kinase C subtypes; the DAG effect on the synergy is most prominent in type III protein kinase C, whereas the synergy is either small for type I or simply additive for type II. This order is similar to that observed in PS-supported synergism [17]. Thus the sensitivity of protein kinase C subtypes to the synergistic activation by *cis*-fatty acid and DAG appears to be independent of the type of phospholipids. There is a difference in Ca^{2+} requirement between the protein kinase C subtypes; unlike type III, activities of type I and type II can be supported by PC and *cis*-fatty acid even in the absence of Ca^{2+} .

Table 1. PC potentiation of type III protein kinase C activation

Protein kinase C activity was assayed in the presence of 70 μM -DOPC, in combination with 10 μM -DiC₈ or 100 μM -oleic acid (OA) or both. Ca²⁺ concentration was fixed at 50 μM unless indicated. The percentage of protein kinase C activity was normalized to that induced by 70 μM -DOPS, 10 μM -DiC₈ and 100 μM -OA. Data were represented as means $\pm$ S.E.M. of three separate experiments performed in duplicate.

	PKC activity (pmol/min)	Activation (%)
PC	1.0 $\pm$ 0.4	1.2
PC + OA	34.8 $\pm$ 3.4	39.4
PC + DiC ₈	-0.04 $\pm$ 0.4	-0.1
PC + DiC ₈ + OA	60.0 $\pm$ 1.5	67.8
+ 100 μM -Ca ²⁺	84.5 $\pm$ 3.9	95.5
PS + DiC ₈ + OA	88.6 $\pm$ 7.1	100.1
+ 100 μM -Ca ²⁺	88.5 $\pm$ 3.3	100.0

Comparison of protein kinase C activities induced by PC and PS systems

Recent studies revealed that both DAG and *cis*-fatty acid are required for full activation of protein kinase C subtypes from rat brain [16,17]. To examine whether the synergistic activation of protein kinase C supported by PC is equivalent to that supported by PS, we compared the PC/DiC₈/oleic acid-dependent activity with that induced by PS/DiC₈/oleic acid. As shown in Table 1, when DAG and *cis*-fatty acid are present, DOPC and DOPS support the protein kinase C activities to almost the same extent at a high concentration (100 μM) of Ca²⁺. However, the activity supported by the PC-dependent mechanism is lower than that by the PS-dependent mechanism at a lower Ca²⁺ concentration. This difference in Ca²⁺ requirement is consistent with previous observations; PS-supported synergy does not require Ca²⁺ [16,17], whereas PC-supported synergism is Ca²⁺-dependent (Fig. 3).

Autophosphorylation of protein kinase C subtypes

Protein kinase C has been shown to be autophosphorylated when it is activated by PS, DAG and Ca²⁺ [30–33]. On the other hand, El Touny *et al.* [12] have shown that autophosphorylation of platelet protein kinase C is negligible when it is activated by oleic acid. These observations suggest that there is a distinct difference between active states of protein kinase C induced by PS/DAG/Ca²⁺ and *cis*-fatty acid. We have therefore examined the effect of PC on autophosphorylation of protein kinase C and compared with that induced by other activation mechanisms. Fig. 5 shows that the DOPC/DiC₈/oleic acid-induced autophosphorylation of protein kinase C (lane 4) is significantly less ($P < 0.01$ for type I and II, $P < 0.05$ for type III) compared with the PS-dependent autophosphorylation (lane 3), but it is not significantly different ($P > 0.1$) from that induced by oleic acid (lane 2). This phenomenon is observed in all three subtypes of protein kinase C tested under conditions at which both PC and PS systems fully activate the protein kinase C in terms of histone phosphorylation. It is also apparent that DOPS/DiC₈-induced autophosphorylation (lane 1) is slightly inhibited by oleic acid (lane 3) in all three subtypes.

Effect of *cis*-fatty acid on PC vesicle stability

Fatty acids are known to have detergent-like characteristics to some extent; they form micelles in aqueous solutions above a critical micelle concentration and exhibit colloidal behaviour. Since detergents solubilize phospholipid vesicles if the molar

Fig. 5. Autophosphorylation of protein kinase C subtypes induced by phospholipids and oleic acid

Autophosphorylation of protein kinase C subtypes was examined in the presence of DOPS and DiC₈ (lane 1), oleic acid alone (lane 2), DOPS, DiC₈ and oleic acid (lane 3), and in the presence of DOPC, DiC₈ and oleic acid (lane 4). The concentrations (μM) used were: DOPS, 70; DOPC, 70; DiC₈, 10; oleic acid, 100; free Ca²⁺, 100. (a) The autophosphorylated protein kinase C subtypes (arrow) were detected by autoradiography after SDS/PAGE (10% gel). (b) The radioactivity of each protein kinase C band on SDS/PAGE was counted with a Betascope analyser (Betagen). Autophosphorylation induced under the different conditions was normalized to that observed in the presence of PS and DiC₈. The data represent means $\pm$ S.E.M. of three separate experiments. Statistical significance of the increase in autophosphorylation relative to basal levels is indicated by asterisks; * $P < 0.1$, ** $P < 0.05$, *** $P < 0.01$. Symbols: ■, PS + DiC₈; □, basal; ▨, oleic acid only; ▩, PS + DiC₈ + oleic acid; ▤, PC + DiC₈ + oleic acid.

ratio of detergent/phospholipid exceeds a value that is needed for lamellar/mixed-micelle transition [27], it is possible that *cis*-fatty acid may also disrupt the lamellar structure of PC vesicles and form *cis*-fatty acid/PC mixed micelles. To examine this possibility, we have tested whether oleic acid induces such micelle formation by monitoring the fluorescence intensity of CF encaps-

Fig. 6. Effect of oleic acid on PC-vesicle stability

The oleic acid effect on PC-vesicle stability was monitored by measuring the increase in the fluorescence intensity owing to the relief of the self-quenching of encapsulated CF (see the text). The percentage increase was calculated by the equation:

$$\% \text{ increase} = \frac{I_{\text{obs.}} - I_0}{I_{\text{max.}} - I_0} \times 100$$

where $I_{\text{max.}}$, $I_{\text{obs.}}$, and I_0 are the fluorescence intensity of CF in the presence of detergent (0.3% Triton X-100), oleic acid, and without detergent or oleic acid respectively. DOPC concentration was 150 μM .

sulated in DOPC vesicles. If micelle formation occurs, it can be observed as an increase in the fluorescence owing to the relief of self-quenched CF fluorescence through the leakage of CF encapsulated in PC vesicles. As shown in Fig. 6, oleic acid has little effect on the fluorescence intensity of CF up to 300 μM , which is twice as high as the DOPC concentration. This result clearly shows that oleic acid does not disrupt PC vesicles, indicating that it is vesicles, but not mixed micelles, that interact with protein kinase C and activate it in the presence of *cis*-fatty acid.

DISCUSSION

The potentiating effect of PC on protein kinase C activation was described in this study. A long-chain neutral phospholipid, DOPC, was found to be able to substitute for PS in the activation of protein kinase C by *cis*-fatty acid and DAG. Among the subtypes, type III protein kinase C is most sensitive to the PC potentiation, and it is synergistically activated by *cis*-fatty acid and DAG in the presence of PC. The PC activation mechanism is, however, distinct from that supported by PS. The PC potentiation absolutely requires *cis*-fatty acid and Ca^{2+} . Unlike the PS system, DAG is totally ineffective for protein kinase C activation in the absence of *cis*-fatty acid in the PC system. However, if *cis*-fatty acid is present, DAG further stimulates the PC-dependent protein kinase C activity and decreases the Ca^{2+} requirement for the activation (K_a for $\text{Ca}^{2+} = 10 \mu\text{M}$). Our study indicates that *cis*-fatty acid is essential, whereas DAG is modulatory, for the PC-dependent protein kinase C activation.

In contrast with the PC system, DAG is essential for protein kinase C activation in the PS system. DAG stimulates the PS/ Ca^{2+} -dependent protein kinase C activity and decreases the requirement for Ca^{2+} to the micromolar range [4]. *cis*-Fatty acid further enhances the PS/DAG-dependent activation [12,14–17]. This modulatory effect of *cis*-fatty acid, which is dependent on the presence of DAG, is two-fold: induction of a strong synergistic activation of protein kinase C, particularly type III, and nullifying the requirement of Ca^{2+} for the activation [16,17]. In short, DAG is the critical activation factor in the PS system, whereas *cis*-fatty acid acts as a modulator for the protein kinase C activity and its affinity to Ca^{2+} .

Another significant difference between PS- and PC-dependent mechanisms is autophosphorylation of protein kinase C. PS/DAG/ Ca^{2+} -dependent protein kinase C activation has been shown to result in autophosphorylation [30,33]. Autophosphoryl-

ation of protein kinase C was further shown kinetically to follow an intra-peptide mechanism, in which a single polypeptide phosphorylates itself [32]. Our study showed that PC/*cis*-fatty acid/DAG effect on protein kinase C autophosphorylation is much smaller than that induced by PS/*cis*-fatty acid/DAG under the conditions at which both the PS and PC systems activate protein kinase C equally. Such a difference in autophosphorylation induced by PS- and PC-dependent mechanisms indicates that protein kinase C undergoes quite different conformational changes with respect to active states. Recently, Flint *et al.* [34] have identified the major autophosphorylation sites of recombinant βII -protein kinase C. They found that these sites are distributed in both the regulatory domain (V1 region) and the catalytic domain (C4 region) as well as the V3 hinge region between the two domains. They suggested that protein kinase C, in the active state induced by PS/DAG/ Ca^{2+} , should be extremely flexible in order for these widely spread sites to be autophosphorylated by the intramolecular mechanism. Since the PC system is not as effective for autophosphorylation, regardless of protein kinase C subtypes, it is possible that the configuration of the active state of protein kinase C induced by PC/DAG/*cis*-fatty acid is more restricted.

The *cis*-fatty acid effect on the autophosphorylation deserves some comment. *cis*-Fatty acid activation of protein kinase C in the absence of phospholipids does not significantly stimulate autophosphorylation of protein kinase C subtypes. This is in agreement with the observation made in platelet protein kinase C that the oleic acid-induced autophosphorylation is negligible [12]. We also showed that *cis*-fatty acid rather inhibits PS/DAG-induced autophosphorylation. These observations suggest that *cis*-fatty acid acts negatively, opposing the induction of the protein kinase C autophosphorylation. Although the significance of the inhibitory effect of oleic acid on autophosphorylation is not clear at present, there are some indications that it may be of some physiological relevance. Mochly-Rosen & Koshland [32] observed, using PS vesicles, that autophosphorylated protein kinase C by PS/DAG/ Ca^{2+} is no longer associated with PS multilamellar structures, suggesting that autophosphorylation may lead to the dissociation of protein kinase C from the membrane. Physiologically it has been shown that, whereas DAG induces a transient translocation of protein kinase C from the cytosol to the membrane fraction [35,36], the presence of *cis*-unsaturated fatty acids (linoleic acid, arachidonic acid) has a prolonged effect on the translocation [35]. It is possible that *cis*-fatty acid may exert its prolonged effect on the protein kinase C association with the membrane by negatively acting on the autophosphorylation. Further studies are required to clarify the relationship between the membrane association and autophosphorylation of protein kinase C activated by PC/*cis*-fatty acid system.

Short-chain PCs with acyl carbon-chain length of 4–8 atoms have been reported to activate protein kinase C [37,38]. In contrast with naturally occurring PCs, which have much longer acyl carbon chains, protein kinase C activation by these short-chain PCs does not require *cis*-fatty acid, but it is further stimulated in the presence of DAG [37]. In this respect, the short-chain-PC-dependent protein kinase C activation rather resembles PS-dependent activation. The concentrations of short-chain PCs required for the activation of protein kinase C strongly correlate with the critical micelle concentration of each PC [37]. It is further suggested that the activation by short-chain PCs is due to the interaction of micelle form of the short-chain PCs with protein kinase C [37]. DOPC, a long-chain and naturally occurring PC (acyl carbon chain length 18), is used in the present study. Since DOPC forms vesicles, not micelles or monomers, under our experimental conditions, as shown in Fig. 6, the

mechanism of short-chain-PC-dependent protein kinase C activation is quite different from that described here.

It has been demonstrated that long-chain PCs may play a role in signal transduction (for review, see [39]). In response to certain stimuli, such as muscarinic agonists, PC hydrolysis has been shown to serve as one of the important signalling pathways for DAG formation [40,41], which in turn could activate protein kinase C. The present study *in vitro* suggests that PC might be directly involved in the activation of protein kinase C, in addition to the formation of DAG via PC hydrolysis.

There are two different actions of *cis*-fatty acid on protein kinase C; protein kinase C can be activated by *cis*-fatty acid, either independently of DAG and PS [5–12] or in co-operation with DAG in the presence of PS [12,14–17]. Our previous study showed that type III protein kinase C is particularly sensitive to the latter mode of activation, and it is synergistically activated by *cis*-fatty acid and DAG in the presence of PS [17]. Our present study further revealed that the synergism can be supported by PC, with a similar $V_{\max}$ obtained with the PS-dependent mechanism. However, the PC and PS effects were shown to be different with respect to synergism, autophosphorylation and Ca^{2+} -dependency. These unexpected observations suggest that PC may play a role in the differential regulation of protein kinase C.

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