Synergistic activation of type III protein kinase C by *cis*-fatty acid and diacylglycerol

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Micromolar concentrations of *cis*-fatty acid synergistically activate type III protein kinase C with diacylglycerol. This synergistic effect occurs at low concentrations of *cis*-fatty acid and diacylglycerol, and it is capable of inducing almost full activation of this protein kinase C subtype at a physiologically relevant Ca^{2+} concentration ($2\ \mu\text{M}$). The synergistic activation mode can be observed even in the absence of Ca^{2+} , but micromolar Ca^{2+} significantly enhances the type III protein kinase C activation. *cis*-Fatty acid also augments the diacylglycerol-induced activation of other subtypes (type I and II), although the effect is smaller than that observed in type III. Neither the diacylglycerol- nor the *cis*-fatty acid-dependent mode of activation can fully activate any of these subtypes at a physiological concentration of Ca^{2+} ($2\ \mu\text{M}$). Our results suggest that the generation of three second messengers, i.e. the increase in intracellular Ca^{2+} concentration and the generation of both *cis*-fatty acid and diacylglycerol in the cell, may be necessary signals for protein kinase C activation, particularly for type III protein kinase C.

INTRODUCTION

It is well established that protein kinase C is a Ca^{2+} - and phospholipid-dependent enzyme which is activated by diacylglycerol (DAG) resulting from receptor-mediated inositol phospholipid hydrolysis [1–3]. There is a mechanism, however, for the activation of protein kinase C that does not require any of these factors. *cis*-Fatty acids were shown to activate protein kinase C independently of DAG, Ca^{2+} and phospholipids [4–7]. In addition, a number of laboratories have shown that *cis*-fatty acids can potentiate DAG-induced protein kinase C activity additively or synergistically [8–12]. Since the individual subtypes of protein kinase C have different sensitivities for DAGs, *cis*-fatty acids or arachidonate metabolites [6,7,13–15], and since their cellular and subcellular localization is distinct [16–22], it is suggested that each protein kinase C subtype may be regulated differently by the series of phospholipid metabolites [23,24]. Seven protein kinase C subtypes (α , βI , βII , γ , δ , ϵ , ζ) have been identified in the brain [25]. Three distinct protein kinase C fractions, types I, II and III, have been separated by hydroxyapatite column chromatography [26–28]. Type I, II and III protein kinase C have been shown to correspond to γ , $\beta\text{I} + \beta\text{II}$, and α -protein kinase C, respectively [28,29]. To clarify the response of individual protein kinase C subtypes to the synergistic activation, we have purified these protein kinase C subtypes from rat brain and characterized them with respect to their sensitivity to *cis*-fatty acid and DAG. Here we report that *cis*-fatty acid and DAG synergistically activate type III protein kinase C at physiological concentrations of Ca^{2+} .

EXPERIMENTAL

Materials and chemicals

Histone (type III-S, Sigma), ATP, Triton X-100, leupeptin, phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT), 1,2-dioctanoyl-*sn*-glycerol (DiC_8), oleic acid, arachidonic acid and other fatty acids (sodium salts) were obtained from Sigma. Dioleoylphosphatidylserine (DOPS) was purchased from Avanti

Polar Lipids. [γ - ^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear. AcA 34 was from IBF Biotechnics. All other chemicals used were reagent grade.

Purification of protein kinase C subtypes

Whole brains from 25 rats were used as a source of protein kinase C. Protein kinase C was purified by a four-step liquid-chromatography procedure as described previously with modification [5]: (1) DEAE-cellulose ion exchange, (2) phenyl-Sepharose, (3) AcA 34 gel filtration and (4) phenyl-5PW h.p.l.c. column chromatography. Highly purified protein kinase C was further separated into three fractions (types I, II and III) by using a hydroxyapatite column [26,27] connected to a f.p.l.c. system (Pharmacia) [28]. Purification procedures described below were carried out at 4°C .

Homogenate. The 25 rats were killed by decapitation and brains were quickly removed. The fresh tissues were homogenized in 6 vol. of ice-cold buffer A (10 mM-EGTA, 2 mM-EDTA, 2 mM-DTT, 10 μg of leupeptin/ml, 1 mM-PMSF, 0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.5) with a Teflon/glass homogenizer (12 up-and-down strokes). The homogenate was centrifuged at 100 000 *g* for 60 min in a Beckman L5-50 ultracentrifuge (50.2 Ti rotor). The supernatant was collected as a crude extract.

DEAE-cellulose chromatography. The crude extract was adjusted to 40 μM -cAMP (cAMP treatment is used to decrease contamination of cAMP-dependent protein kinase [5]; 40 μM -cAMP dissociates the holoenzyme, and the catalytic subunit does not bind to the DEAE-cellulose column) and loaded on to a DEAE-cellulose column (2.5 cm $\times$ 12 cm) pre-equilibrated with buffer B (5 mM-EGTA, 2 mM-EDTA, 2 mM-DTT, 20 mM-Tris/HCl, pH 7.5). After loading, the column was washed with 4 bed vol. of the same buffer, followed with 2 bed vol. of buffer C (1 mM-EGTA, 1 mM-EDTA, 2 mM-DTT, 20 mM-Tris/HCl, pH 7.5). A linear gradient (600 ml) of 0–0.3 M-NaCl was applied to the column to elute protein kinase C at a flow rate of 60 ml/h.

Phenyl-Sepharose chromatography. The active protein kinase C fractions (150 ml) were pooled, adjusted to 1.5 M-NaCl, and applied to a phenyl-Sepharose CL-4B column (1 cm $\times$ 12 cm)

Abbreviations used: PS, phosphatidylserine; DOPS, dioleoylphosphatidylserine; DAG, diacylglycerol; DiC_8 , 1,2-dioctanoyl-*sn*-glycerol; DTT, dithiothreitol; cAMP, cyclic AMP.

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Fig. 1. SDS/PAGE of protein kinase C subtypes

Three protein kinase C subtypes (type I, II and III) were purified and resolved by a hydroxyapatite column connected to a f.p.l.c. system as described in the Experimental section. Samples (300–500 ng) of protein kinase C fractions (type I, lane 1; type II, lane 2; type III, lane 3) were analysed by SDS/PAGE (10% acrylamide gel). The gel was fixed and bands were made visible by silver staining [51]. Molecular-mass markers (indicated by stars; from top to bottom) were run in parallel: galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

pre-equilibrated with 1.5 M-NaCl in buffer C. The column was washed with 10 bed vol. of the same buffer. Protein kinase C was then eluted with a linear gradient of 2.0–0 M-NaCl in 120 ml of buffer C, followed by 100 ml of buffer C at a flow rate of 80 ml/h.

AcA 34 gel filtration. Fractions (50 ml) containing protein kinase C activity were collected and concentrated to 5 ml under N_2 with a YM 30 membrane ultrafiltration unit (Amicon). The ultrafiltered sample was applied to an AcA 34 column (2.5 cm $\times$ 95 cm), which was previously equilibrated with buffer C, and eluted with 500 ml of the same buffer. The flow rate was set at 25 ml/h.

Phenyl-5PW chromatography. The protein kinase C fractions (50 ml) from AcA 34 were pooled, adjusted to 1.0 M-NaCl, and then loaded on to a phenyl-5PW column (0.75 cm $\times$ 7.5 cm), which was connected to f.p.l.c. and pre-equilibrated with 1.0 M-NaCl in buffer C. After the column was washed with 30 ml of the same buffer, protein kinase C was eluted with a 20 ml linear gradient of 1.0–0 M-NaCl in buffer C, followed by 20 ml of buffer C.

Hydroxyapatite chromatography. The purified protein kinase C fractions (5 ml) from the phenyl-5PW column were then diluted with an equal volume of buffer D [20 mM-potassium phosphate, 10% (v/v) glycerol, 2 mM-DTT, pH 7.5], applied to a hydroxyapatite column (Kokken, type S: 0.78 cm $\times$ 10 cm), which was connected to the f.p.l.c. system and pre-equilibrated with buffer D. The column was washed with 30 ml of the same buffer. Three protein kinase C subtypes were separated by application of a 80 ml gradient of 20–250 mM-potassium phosphate at a flow rate of 30 ml/h. Types I, II and III were eluted from the column with the gradient at 64 mM-, 84 mM- and 140 mM-potassium phosphate respectively. The purification factor for three types of protein kinase C was about 600–900-fold with a

yield of 0.4–2%. The apparent homogeneity of these three fractions was confirmed by SDS/PAGE followed by silver staining (Fig. 1). These distinct fractions of protein kinase C subtypes (types I, II and III) were stored in the presence of 0.025% Triton X-100 and 10% glycerol at -80°C . These highly purified protein kinase C subtypes were used for biochemical studies.

Assay for protein kinase C

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 300 μg of histone/ml, 5 mM-MgCl₂, 0.77 mM-EGTA, 20 mM-Tris/HCl, pH 7.5, and 10–50 ng of purified protein kinase C in a final volume of 260 μl . Concentrations of DOPS, DiC₈, *cis*-fatty acids and free Ca²⁺ were adjusted and specified in each experiment. Free Ca²⁺ concentrations were calculated by taking account of multiple equilibria of Ca²⁺, Mg²⁺ and ATP in the presence of 770 μM -EGTA. Since the stability constant for Ca²⁺-EGTA has been shown to be smaller in the presence of buffer ions [30,31] than the old literature values ($\log K_{\text{Ca}^{2+}\text{-EGTA}} = 10.90\text{--}10.98$ [30,32]), we used the value of 9.07 estimated by Kim & Padilla [31]. Other stability constants were from the literature summarized in [33].

Lipid treatment

DOPS and DiC₈ were first dissolved in a minimal amount of chloroform/methanol (9:1, v/v). After the organic solvent was evaporated under N_2 , they were dispersed into Chelex 100-treated 20 mM-Tris/HCl buffer (pH 7.5) by sonication at 4°C . *cis*-Fatty acids were dispersed into the buffer directly without the treatment with organic solvent. Stearic acid and elaidic acid were dissolved in ethanol.

Protein determination

Protein concentration was determined by the Lowry method, or by a fluorimetric assay [34].

RESULTS

Synergistic activation of type III protein kinase C

Fig. 2 shows the activation of type III (α) protein kinase C at 2 μM -Ca²⁺ by DOPS, an effective cofactor for protein kinase C activation [5]. In the presence of DiC₈, a membrane-permeant DAG, DOPS activates this protein kinase C subtype in a dose-dependent manner, but slightly inhibits oleic acid-induced activation (Fig. 2a). A drastic increase in protein kinase C activity can be observed when the activation of type III protein kinase C is induced in the presence of both oleic acid and DAG: 8.3-fold and 1.7-fold increases over the activity obtained in the absence of oleic acid at 5 μM - and 74 μM -DOPS respectively. The degree of activation is more than the sum of the activities induced by DAG and by oleic acid alone in the entire range of DOPS concentrations tested, showing that the activation is synergistic. This synergism also leads to a great decrease in the requirement for phosphatidylserine (PS) for the protein kinase C activation. Less than 5 μM -DOPS is enough to induce a full activation of this protein kinase C subtype. The synergistic activation of protein kinase C is not limited to oleic acid. Eicosapentaenoic acid, an ω -3 fatty acid, also supports the synergy in a similar manner with oleic acid as shown in Fig. 2(b).

The dose-dependency of oleic acid in the activation mode is shown in Fig. 3. Activity of type III protein kinase C was measured in the presence or absence of 10 μM -DiC₈ at fixed concentrations of free Ca²⁺ (2 μM) and DOPS (10 μM). There was notable activation with as low as 6 μM -oleic acid in the presence of DAG and phospholipid. The K_a value (concentration required

Fig. 2. Effect of *cis*-fatty acid and DiC₈ on type III protein kinase C activation by DOPS at 2 μM free Ca²⁺

(a) DOPS-dependency of type III protein kinase C activity was examined at different concentrations of oleic acid and DiC₈: ■, 100 μM-oleic acid plus 10 μM-DiC₈; ▲, 25 μM-oleic acid plus 10 μM-DiC₈; △, 10 μM-DiC₈; □, 100 μM-oleic acid; ○, without oleic acid and DiC₈. We used DOPS, a synthetic PS, for the study because the potency of PS extracted from bovine brain often varied in different preparations. (b) Concentration of eicosapentaenoic acid (EPA) used was 100 μM, and other conditions were the same as those described for (a).

Fig. 3. Oleic acid-induced type III protein kinase C activation in the presence or absence of DAG

The requirement of DAG for the protein kinase C activation by oleic acid was examined in the absence (□) or presence (■) of 10 μM-DiC₈. Concentrations of free Ca²⁺ and DOPS were fixed at 2 μM and 10 μM respectively.

for half-maximal activation) is 20 μM, and the activation reaches a maximum at 100 μM-oleic acid. Comparison between protein kinase C activities observed in the presence and absence of DAG

Fig. 4. Dose-dependency of DiC₈ on type III protein kinase C activation at different concentrations of oleic acid

Activation of type III protein kinase C by DiC₈ was examined in the presence of different concentrations of oleic acid: ○, 0 μM; ▲, 25 μM; ■, 100 μM. Concentrations of DOPS and free Ca²⁺ were fixed at 12 μM and 2 μM respectively.

shows that oleic acid-induced activation in the presence of PS is DAG-dependent. The DAG-dependency of type III protein kinase C is most prominent when the oleic acid concentration is below 50 μM; in the presence of 10 μM-DiC₈, a 10–17-fold activity increase is observed compared with that in its absence at the same concentration of oleic acid.

Fig. 4 shows the dose-dependency of DiC₈ for the activation of type III protein kinase C in the presence of different concentrations of oleic acid. Oleic acid greatly decreases the requirement for DiC₈ for the activation of this subtype; protein kinase C activity induced by 50 μM-DiC₈ alone is equivalent to that activated by 10 μM-DiC₈ + 25 μM-oleic acid or to that by 2 μM-DiC₈ + 100 μM-oleic acid. Conversely, an increase in DiC₈ concentration also decreases the requirement for *cis*-fatty acid for the activation; the activity attained in the presence of 100 μM-oleic acid + 10 μM-DiC₈ is equal to that activated by 25 μM-oleic acid + 50 μM-DiC₈. It is thus apparent that the concentration of *cis*-fatty acid required for the synergistic activation of type III protein kinase C is dependent on the DAG concentration.

Differential response of protein kinase C subtypes to the synergistic activation by *cis*-fatty acid and DAG

Three protein kinase C subtypes were compared with respect to their sensitivities to the synergistic activation mode. Arachidonic acid, an ω-6 *cis*-fatty acid and a precursor of the biologically important eicosanoids, also activates type III protein kinase C synergistically with DAG at a physiologically relevant concentration of Ca²⁺. As shown in Fig. 5(c), type III protein kinase C is strongly activated by arachidonic acid and DiC₈, whereas arachidonic acid or DiC₈ alone has little effect at 2 μM-Ca²⁺. At 50 μM, arachidonic acid induces almost full activation, with half-maximal activation at 17 μM in the presence of 10 μM-DiC₈ (Fig. 5f). Although the drastic synergism is not seen for type I and type II protein kinase C, arachidonic acid also augments the DAG-induced activation; the augmentation reaches its maximum at 25 μM-arachidonic acid (Figs. 5d and 5e). Among the three protein kinase C subtypes, type I protein kinase C is most sensitive to arachidonic acid, and 100 μM-arachidonic acid alone significantly activates this subtype (Figs. 5a–5c). This result is consistent with those observed in type I protein kinase C from hypothalamus [6] and cerebellum [7], which was shown to be highly sensitive to arachidonic acid in the absence of Ca²⁺.

Fig. 5. Synergism on the activation of protein kinase C subtypes by arachidonic acid and DAG, and its arachidonic acid dependency

(a)–(c) Synergistic activation of protein kinase C subtypes was studied in the presence of 100 μM -arachidonic acid and 10 μM -DiC₈ (+AA + DiC₈) and compared with the activity induced by 100 μM -arachidonic acid (+AA) or 10 μM -DiC₈ (+DiC₈) alone. Concentrations of DOPS and free Ca²⁺ were fixed at 10 μM and 2 μM respectively. (d)–(f) Dose-dependency of arachidonic acid activation of protein kinase C subtypes was examined in the presence of 10 μM -DOPS, 2 μM free Ca²⁺ and 10 μM -DiC₈. Protein kinase C activity was normalized to full activation obtained in the presence of 74 μM -DOPS (60 $\mu\text{g}/\text{ml}$), 10 μM -DiC₈, 100 μM -oleic acid and 0.5 mM free Ca²⁺.

Since arachidonic acid alone strongly activates protein kinase C, type I in particular, and since arachidonic acid at high concentrations (> 50 μM) rather suppresses the synergistic activation of type I and II protein kinase C (Figs. 5d and 5e), it is possible that the synergistic activation may be detected in these subtypes as well if low concentrations of the *cis*-fatty acid are used. As shown in Table 1, low concentrations (6–25 μM) of arachidonic acid activate type I protein kinase C synergistically with 10 μM -DiC₈, whereas only an additive effect is observed with type II. This suggests that the type I enzyme may also be sensitive to the synergistic activation mode. We further examined the synergy of the three subtypes of protein kinase C induced by various *cis*-fatty acids. As shown in Table 2, type III protein kinase C is most sensitive to this mode. This experiment also differentiates the response of type I and II protein kinase C to *cis*-fatty acid and DAG; type I protein kinase C is more responsive to the synergistic action than is type II, although the extent of synergy achieved is much less than for type III protein kinase C. There is a slight difference in the potency of *cis*-fatty acid for the

Table 1. Effect of low concentrations of arachidonic acid on the synergy in type I and II protein kinase C

Synergistic activation of type I and type II protein kinase C was examined in the presence of 6–25 μM -arachidonic acid (AA) and 10 μM -DiC₈ (+AA + DiC₈), and compared with the activity induced by arachidonic acid (+AA) or DiC₈ (+DiC₈) alone. Concentrations of DOPS and free Ca²⁺ were fixed at 10 μM and 2 μM respectively.

Activators	Protein kinase C activity (pmol/min)	
	Type I	Type II
[AA] = 6.3 μM		
+ AA	2.6	2.0
+ DiC ₈	28.6	14.7
+ AA + DiC ₈	39.6	18.1
[AA] = 12.5 μM		
+ AA	3.4	4.0
+ DiC ₈	28.6	14.7
+ AA + DiC ₈	55.3	19.7
[AA] = 25.0 μM		
+ AA	9.1	9.1
+ DiC ₈	28.6	14.7
+ AA + DiC ₈	56.5	27.3

Table 2. Effect of various *cis*-fatty acids on the synergistic activation of protein kinase C subtypes

To examine the extent of synergism, synergy index (S.I.) was defined as the ratio of protein kinase C activity in the presence of *cis*-fatty acid plus 10 μM -DiC₈ ($V_{\text{FA}+\text{DAG}}$) to the sum of the activities induced by either fatty acid (V_{FA}) or DAG (V_{DAG}) alone minus 1, i.e. $\text{S.I.} = V_{\text{FA}+\text{DAG}} / (V_{\text{FA}} + V_{\text{DAG}}) - 1$. Thus a S.I. value that is greater than 0 represents the synergistic effect. Both 25 μM - and 100 μM -*cis*-fatty acids were used to detect the synergistic effect. The data represent the averages of results obtained at each concentration and are expressed as means $\pm$ S.E.M. of three different sets of experiments performed in duplicate.

<i>cis</i> -Fatty acid	S.I.		
	Type I	Type II	Type III
Oleate	0.31 $\pm$ 0.17	0.04 $\pm$ 0.09	0.99 $\pm$ 0.26
Arachidonate	0.25 $\pm$ 0.13	0.08 $\pm$ 0.13	1.00 $\pm$ 0.34
Linoleate	0.19 $\pm$ 0.20	−0.10 $\pm$ 0.11	0.70 $\pm$ 0.26
Eicosapentaenoate	0.25 $\pm$ 0.15	0.02 $\pm$ 0.16	0.97 $\pm$ 0.37

synergy, but the order of sensitivity to the synergy, type III > type I > type II, was invariant for all *cis*-fatty acids tested in three separate experiments.

Ca²⁺-dependency of type III protein kinase C

We first characterized the Ca²⁺-dependency of the *cis*-fatty acid activation of the three subtypes in the absence of DAG. Because the Ca²⁺-independence of *cis*-fatty acid action on protein kinase C has been shown for the mixture of protein kinase C subtypes, it is possible that some of the subtypes may be Ca²⁺-dependent. Activation of the protein kinase C subtypes by *cis*-fatty acid (arachidonic acid and oleic acid) in the presence and absence of Ca²⁺ is shown in Fig. 6, which indicates that *cis*-fatty acids, arachidonic acid in particular, activate type I and II protein kinase C independently of Ca²⁺, especially at low concentrations. The type III enzyme, on the other hand, is Ca²⁺-dependent at these concentrations. The K_a values of various *cis*-

Fig. 6. PS/DAG-independent *cis*-fatty acid activation of protein kinase C subtypes in the presence and absence of Ca^{2+}

Dose-dependency of arachidonic acid (a-c) and oleic acid (d-f) on protein kinase C subtypes was examined in the presence of 0.5 mM free Ca^{2+} (●) or 0.8 mM-EGTA (○).

Table 3. K_a values of *cis*-fatty acids for protein kinase C subtypes in the presence or absence of Ca^{2+}

K_a values were determined from two to four separate experiments performed in duplicate.

<i>cis</i> -Fatty acid	K_a (μM)					
	0.5 mM- Ca^{2+}			0.75 mM-EGTA		
	Type I	Type II	Type III	Type I	Type II	Type III
Oleate ($\omega-9$)	17.9	19.0	40.8	31.5	25.3	106.9
Arachidonate ($\omega-6$)	24.3	12.1	15.0	20.6	14.5	84.0
Eicosapentaenoate ($\omega-3$)	20.2	12.4	20.7	19.5	9.8	95.0
Docosahexaenoate ($\omega-3$)	20.7	24.1	41.0	33.6	30.6	126.1

fatty acids in the presence and absence of Ca^{2+} are shown in Table 3. Comparison of K_a values in the presence of Ca^{2+} with those in its absence clearly shows that *cis*-fatty acid activation of type III protein kinase C requires Ca^{2+} , whereas type I and II enzymes are relatively Ca^{2+} -independent.

The DAG-induced protein kinase C activation mode requires Ca^{2+} [1-3], whereas Ca^{2+} is not necessary for the *cis*-fatty acid activation of protein kinase C, although type III protein kinase C activation by *cis*-fatty acid is more sensitive to Ca^{2+} than is that of other subtypes. We examined whether the synergism induced by *cis*-fatty acid and DAG requires Ca^{2+} or not. As shown in Fig. 7, the synergistic activation does not require Ca^{2+} . Type III protein kinase C is activated even without Ca^{2+} when both DAG and *cis*-fatty acid are present. However, micromolar concentrations of Ca^{2+} significantly enhance the protein kinase C

activity induced by DAG and *cis*-fatty acid, with a K_a for Ca^{2+} of 2.5 μM . At higher concentrations of Ca^{2+} ($> 10 \mu\text{M}$), the protein kinase C activity is slightly inhibited.

DISCUSSION

In this study, we have characterized the synergistic activation of protein kinase C subtypes purified from rat brain. Our results show that *cis*-fatty acid and DAG synergistically activate type III protein kinase C. This mode of activation is distinct from the previously reported *cis*-fatty acid activation of protein kinase C in several aspects. First, unlike PS/ Ca^{2+} /DAG-independent *cis*-fatty acid activation, the synergistic activation mode is extremely sensitive to micromolar concentrations of Ca^{2+} . At 2 μM , Ca^{2+} significantly potentiates the activation of type III protein kinase

Fig. 7. Ca^{2+} -sensitivity of type III protein kinase C activation by DAG and oleic acid

Ca^{2+} -dependency of the synergistic activation was tested at different concentrations of DiC_8 and oleic acid: ■, 100 μM -oleic acid plus 10 μM - DiC_8 ; ▲, 25 μM -oleic acid plus 10 μM - DiC_8 ; △, 10 μM - DiC_8 ; □, 100 μM -oleic acid. DOPS concentration was fixed at 10 μM .

C by *cis*-fatty acid and DAG. This suggests that this mode is responsive to the increase in intracellular Ca^{2+} during excitation of the cells. Secondly, the synergistic activation of type III protein kinase C does not require high concentrations of *cis*-fatty acid. The synergistic effect is apparent with concentrations as low as 6 μM -oleic acid, with a K_a value of 20 μM (K_a for arachidonic acid is 17 μM), which is equivalent to that of DAG (10 μM). Thirdly, this activation is dependent on PS (Fig. 2) and DAG (Fig. 3). This suggests that the site of the synergistic activation is not in the cytosol, but is rather associated with the membrane phospholipids. It has been shown that the penetration of *cis*-fatty acid into the phospholipid layer is much greater than that of elaidic acid (*trans*-isomer of oleic acid) or of saturated fatty acid, which are inactive for protein kinase C activation [35].

The mechanism of the synergistic effect *in vitro* is unclear at present. However, it is apparent that *cis*-fatty acid does not competitively bind the same domain of protein kinase C as DAG for the activation, at least in type III enzyme; a synergistic increase in V_{max} would not be expected if *cis*-fatty acid and DAG activate protein kinase C by a competitive mechanism. This is consistent with the observations that DAG inhibits phorbol ester binding in a competitive manner [36,37] whereas *cis*-fatty acid inhibition does not appear to be competitive [37]. Ono *et al.* [38] have shown, using various deletions, and point mutations of protein kinase C, that cysteine-rich domain in conserved region, C1, is involved in phorbol ester binding and that another conserved region, C2, in the regulatory domain appears to regulate the Ca^{2+} -dependency of phorbol ester binding. The C2 region has also been suggested to be important for phospholipid binding to protein kinase C [39]. It was indicated that a monomeric form of *cis*-fatty acid interacts with protein kinase C [5], but the *cis*-fatty acid binding domain is not known. Studies using recombinant protein kinase C mutants engineered by molecular-biological techniques would provide more detailed topographical and mechanistic insights for the synergism of *cis*-fatty acid and DAG.

Using a protein kinase C mixture preparation from rat brain, Seifert *et al.* [9] have shown that various unsaturated fatty acids, including some *trans*-fatty acids, can potentiate DAG/phospholipid-induced protein kinase C activity. It has been reported that

trans-fatty acids containing two double bonds, such as linolelaic acid, activate protein kinase C in the presence of DAG [9]. We have found that, of the *trans*- and saturated fatty acids tested, linolelaic acid was also potent for the synergistic activation of type III protein kinase C. Elaidic acid also supported the synergistic activation, but to a much lesser extent, whereas saturated fatty acids such as stearic acid had no effect (results not shown). It has also been shown that protein kinase C purified from platelet cytosol, enriched in the α -isoform, is synergistically activated by oleic acid and DAG in the presence of high (300 μM) concentration of free Ca^{2+} [10]. Our study further showed that (1) physiological concentrations of Ca^{2+} (below 2 μM) are enough to support the synergistic activation of protein kinase C by *cis*-fatty acid and DAG and (2) type III protein kinase C is most sensitive to this mode of activation and is drastically activated by the synergism. *cis*-Fatty acids were also shown to augment the DAG-induced activation of other subtypes at 2 μM - Ca^{2+} , but the synergism is small (type I), or the effect is additive rather than synergistic (type II). Type I, II and III protein kinase C have been shown to correspond to γ , $\beta\text{I} + \beta\text{II}$ and α -protein kinase C respectively [28,29]. Another class of protein kinase C subtypes (δ , ϵ , ζ) has been cloned, but they have escaped biochemical detection, presumably owing to the different activation mechanism or substrate specificity [40]. However, we cannot rule out the possibility that our purified protein kinase C may contain these subtypes. We therefore used the chromatographic designation (type I, II and III) in this study.

The physiological significance of the synergism between unsaturated fatty acid and DAG has been shown, and their effects have been attributed to the activation of protein kinase C. In human lymphocytes, Szamel *et al.* [41] have shown that *cis*-fatty acids (linoleic acid and arachidonic acid) regulate interleukin 2 synthesis by inducing a prolonged protein kinase C translocation from the cytosol to the membrane in the presence of Ca^{2+} ionophore and DiC_8 , whereas these compounds alone have no such effect. They have also shown that DAG and *cis*-fatty acid induce protein kinase C translocation with a different temporal sequence; the former is transient, whereas the latter is long-lasting. They suggested that the incorporation of polyunsaturated fatty acids into plasma-membrane phospholipids is a signal for sustained protein kinase C activation. In human platelets, Seifert *et al.* [42] have demonstrated that DAG-induced activation of the platelets was enhanced by unsaturated fatty acids (arachidonic acid, linoleic acid and linolelaic acid) and that the effect was reversed by polymyxin B, a protein kinase C inhibitor. They have suggested that fatty acids and DAG may synergistically be involved in hormonal activation of protein kinase C. Since type III protein kinase C has been shown to be highly expressed in both human lymphoma [43] and platelets [44], it is possible that the activation of these cells by *cis*-fatty acid and DAG is mediated by the synergistic activation of type III protein kinase C.

Arachidonic acid release and inositol phospholipid hydrolysis are often stimulated concomitantly when cells are exposed to Ca^{2+} -mobilizing agents such as muscarinic, α_1 -adrenergic or peptidergic agonists in various tissues and cells (for review, see [45]). This concomitant generation of DAG and arachidonic acid (and possibly other *cis*-fatty acids) may be regulated by receptor-mediated activation of phospholipases. In thyroid cells, α_1 -adrenergic-receptor activation has been shown to stimulate both phospholipase A_2 (arachidonic acid release) and phospholipase C (DAG formation) through distinct G-proteins [46]. Alternatively, DAG and *cis*-fatty acid may act as regulators of phospholipase A_2 and C respectively. DAG has been shown to stimulate arachidonic acid metabolism in 3T3 fibroblasts [47]. It has been further demonstrated that DAG can directly activate isolated phospholipase A_2 from the same cell line [48]. Conversely,

arachidonic acid is capable of stimulating the inositol lipid breakdown and DAG formation in the 3T3 fibroblasts [49]. *cis*-Fatty acid has been shown to activate directly phosphatidylinositol-specific phospholipase C isolated from rat brain [50]. These cellular events may be responsible for the synergistic activation of type III protein kinase C described here. In conclusion, our results show that three second messengers, i.e. Ca^{2+} , *cis*-fatty acid and DAG, are necessary for the full activation of this protein kinase C subtype.

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REFERENCES

- Nishizuka, Y. (1984) *Nature* (London) **308**, 693–698
- Nishizuka, Y. (1984) *Science* **225**, 1365–1370
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Murakami, K. & Routtenberg, A. (1985) *FEBS Lett.* **192**, 189–193
- Murakami, K., Chan, S. Y. & Routtenberg, A. (1986) *J. Biol. Chem.* **261**, 15424–15429
- Naor, Z., Shearman, M. S., Kishimoto, A. & Nishizuka, Y. (1988) *Mol. Endocrinol.* **2**, 1043–1048
- Shearman, M. S., Naor, Z., Sekiguchi, K., Kishimoto, A. & Nishizuka, Y. (1989) *FEBS Lett.* **243**, 177–182
- Verkest, V., McArthur, M. & Hamilton, S. (1988) *Biochem. Biophys. Res. Commun.* **152**, 825–829
- Seifert, R., Schächtele, C., Rosenthal, W. & Schulz, G. (1988) *Biochem. Biophys. Res. Commun.* **154**, 20–26
- El Touny, S., Khan, W. & Hunn, Y. (1990) *J. Biol. Chem.* **265**, 16437–16443
- Shearman, M. S., Shinomura, T. & Nishizuka, Y. (1991) *FEBS Lett.* **279**, 261–264
- Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5149–5153
- Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U. & Nishizuka, Y. (1987) *Biochem. Biophys. Res. Commun.* **145**, 797–802
- Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988) *J. Biochem. (Tokyo)* **103**, 759–765
- Wooten, M. W. & Wrenn, R. W. (1988) *Biochem. Biophys. Res. Commun.* **153**, 67–73
- Brandt, S. J., Nidel, J. E., Bell, R. M. & Young, W. S., III. (1987) *Cell* **49**, 57–63
- Mochy-Rosen, D., Basbaum, A. I. & Koshland, D. E., Jr. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4660–4664
- Kose, A., Saito, N., Ito, H., Kikkawa, U., Nishizuka, Y. & Tanaka, C. (1988) *J. Neurosci.* **8**, 4262–4268
- Stichell, C. C. & Singer, W. (1988) *Exp. Brain Res.* **72**, 443–449
- Hosoda, K., Saito, N., Kose, A., Ito, A., Tsujino, T., Ogita, K., Kikkawa, U., Ono, Y., Igarashi, K., Nishizuka, Y. & Tanaka, C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1393–1397
- Saito, N., Kose, A., Ito, A., Hosoda, K., Mori, M., Hirata, M., Ogita, K., Kikkawa, U., Ono, Y., Igarashi, K., Nishizuka, Y. & Tanaka, C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3409–3413
- Ito, A., Saito, N., Hirata, M., Kose, A., Tsujino, T., Yoshihara, C., Ogita, K., Kishimoto, A., Nishizuka, Y. & Tanaka, C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3195–3199
- Nishizuka, Y. (1988) *Nature* (London) **334**, 661–665
- Nishizuka, Y. (1989) *J. Am. Med. Assoc.* **262**, 1826–1833
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1988) *J. Biol. Chem.* **263**, 6927–6932
- Huang, K.-P., Nakabayashi, H. & Huang, F. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8535–8539
- Jaken, S. & Kiley, S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4418–4422
- Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K. & Nishizuka, Y. (1987) *FEBS Lett.* **217**, 227–231
- Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. & Nishizuka, Y. (1987) *Science* **236**, 1116–1120
- Bartfei, T. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 219–242
- Kim, Y. S. & Padilla, G. M. (1978) *Anal. Biochem.* **89**, 521–528
- Boyd, S., Bryson, A., Nancollas, G. H. & Torrance, K. (1965) *J. Chem. Soc.* 7353–7358
- Scharff, O. (1979) *Anal. Chim. Acta* **109**, 291–305
- Böhlen, P., Stein, S., Dairman, W. & Indenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220
- MacDonald, R. C. & MacDonald, R. I. (1988) *J. Biol. Chem.* **263**, 10052–10055
- Sharkey, N. A., Leach, K. L. & Blumberg, P. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 607–610
- Sharkey, N. A. & Blumberg, P. M. (1985) *Biochem. Biophys. Res. Commun.* **133**, 1051–1056
- Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4868–4871
- Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R. & Südhof, T. C. (1990) *Nature* (London) **345**, 260–263
- Huang, K.-P. (1989) *Trends Neurosci.* **12**, 425–431
- Szamel, M., Rehmann, B., Krebs, B., Kurre, R. & Resch, K. (1989) *J. Immunol.* **143**, 2806–2813
- Seifert, R., Schächtele, C. & Schulz, G. (1987) *Biochem. Biophys. Res. Commun.* **149**, 762–768
- Sawamura, S., Ase, K., Berry, N., Kikkawa, U., McCaffrey, P. G., Minowada, J. & Nishizuka, Y. (1989) *FEBS Lett.* **247**, 353–357
- Tsukuda, M., Asaoka, Y., Sekiguchi, K., Kikkawa, U. & Nishizuka, Y. (1988) *Biochem. Biophys. Res. Commun.* **155**, 1387–1395
- Abdel-Latif, A. A. (1986) *Pharmacol. Rev.* **38**, 227–272
- Burch, R. M., Luini, A. & Axelrod, J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7201–7205
- Burch, R. M., Ma, A. L. & Axelrod, J. (1988) *J. Biol. Chem.* **263**, 4764–4767
- Burch, R. M. (1988) *FEBS Lett.* **234**, 283–286
- Takuwa, N., Takuwa, Y. & Rasmussen, H. (1988) *J. Biol. Chem.* **263**, 9738–9745
- Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. (1979) *Biochem. J.* **178**, 497–500
- Merrill, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437–1438

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