Direct activation of purified protein kinase C by unsaturated fatty acids (oleate and arachidonate) in the absence of phospholipids and Ca^{2+}

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Unsaturated fatty acids (oleic acid and arachidonic acid) activate purified protein kinase C independently of phospholipid and Ca^{2+}. Oleic acid activation of protein kinase C is as effective as phosphatidylserine and Ca^{2+}. K_a values for oleic acid and arachidonic acid are 50 and 53 μM, respectively. In contrast to the *cis* fatty acids, a *trans* form (elaidic acid) or a saturated fatty acid (stearic acid) has little or no effect on protein kinase C activation. If *cis* fatty acid liberation is physiologically important, this suggests that another mechanism may exist for protein kinase C activation, in addition to phospholipase C/phosphatidylinositol turnover signaling, possibly via the liberation of *cis* fatty acids by the Ca^{2+}-dependent phospholipase A_2 system.

Protein kinase C *Unsaturated fatty acid* *Phospholipase A_2*

1. INTRODUCTION

Protein kinase C is a key enzyme in mediating transmembrane signaling. It was originally found as a proenzyme of a cyclic nucleotide-independent protein kinase [1,2] and later shown to be reversibly activated by phospholipid and Ca^{2+} [3]. Since diacylglycerol, a breakdown product of phosphatidylinositol by phospholipase C, increases protein kinase C activity at micromolar concentrations of Ca^{2+} [4], diacylglycerol is considered to be a second messenger for protein kinase C [5]. In concert with mobilization of Ca^{2+}, synthetic diacylglycerol or 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), which directly stimulates protein kinase C in the same manner as diacylglycerol [6], induces a physiological response in various systems [7,8]. Thus, protein kinase C has been claimed to have an absolute requirement for both phospholipid and Ca^{2+} and to be activated by diacylglycerol formation in a receptor-mediated fashion. Since fatty acids incorporated at acyl positions in diacylglycerol appear to be crucial for protein kinase C activation [4], their effect on activation is of interest. Although it was previously noted that free fatty acids have no stimulatory effect on protein kinase C [4], here, using a different method of lipid preparation, we demonstrate that *cis* unsaturated fatty acids can activate protein kinase C in the absence of phospholipid and Ca^{2+}.

2. EXPERIMENTAL

2.1. *Materials*

Oleic acid, arachidonic acid, elaidic acid, stearic acid, methyloleate, methylarachidonate, diolein phosphatidylserine, lysine-rich histone, ATP and DEAE-cellulose were obtained from Sigma. [γ-^{32}P]ATP (650 Ci/mmol) was purchased from ICN Radiochemicals. Chelex 100 and hydroxyapatite were from Bio-Rad. AcA34 was from LKB and phenyl-Sepharose CL-4B from Pharmacia Fine Chemicals. Phosphocellulose paper (Whatman P-81) was from Whatman. Other chemicals used were all reagent grade.

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2.2. *Preparation of purified protein kinase C*

Whole forebrain from 12 rats was used as a source of protein kinase C. The detailed purification procedure will be reported separately [9]. Briefly, homogenate was centrifuged at 100000 × *g* for 1 h. The supernatant, as a crude extract, was adjusted to 40 μM cAMP to dissociate the catalytic subunit of cAMP-dependent protein kinase before applying to a DEAE-cellulose column [10]. The active fraction was eluted with 0.0–0.3 M NaCl, concentrated and subjected to gel filtration (AcA34, 2.5 × 95 cm). The enzyme fraction was further purified using a hydroxyapatite column followed by a phenyl-Sepharose column. Protein kinase C was eluted with a 2.0–0.0 M NaCl gradient [11]. Protein kinase C was purified to 700–1000-fold from a crude extract and showed apparent homogeneity as judged by SDS gel electrophoresis. Isolated protein kinase C showed phospholipid/Ca^{2+} dependency (see fig.2), and addition of a small amount of diacylglycerol or TPA significantly reduced the requirement of Ca^{2+} as reported in [4].

2.3. *Assay for protein kinase C*

Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone (type III-S, Sigma) as a substrate. Phosphorylation reaction was initiated by adding 20 μl of 2 mM ATP (spec. act. 100–150 cpm/pmol) after 45 s incubation at 30°C. The reaction was terminated after 10 min with 50 μl of a EGTA + EDTA saturated solution. 200 μl of the reacted solution was spotted onto Whatman P-81 phosphocellulose paper and radioactivity was counted. The reaction was performed under the linear range with time and enzyme concentration.

3. RESULTS AND DISCUSSION

The direct activation of protein kinase C by oleic acid and arachidonic acid in the presence of 500 μM free Ca^{2+} is shown in fig.1. Protein kinase C was activated in the absence of phospholipid by the unsaturated fatty acids in a dose-dependent manner. The activity of protein kinase C stimulated with these unsaturated fatty acids is normalized relative to V_{max} for comparison with phosphatidylserine. Lineweaver-Burk plots reveal that K_a values for oleate and arachidonate are 50 and 53 μM, respectively. V_{max} for oleic acid is similar to that of phosphatidylserine (Sigma; isolated from bovine brain). The K_a value obtained for phosphatidylserine was 15.2 μg/ml. One cannot calculate the molar K_a value of phosphatidylserine because various fatty acids are incorporated into acyl chains in phospholipids. However, it is plausible to assume that the average M_r is about 800, since the predominant acyl moieties of phosphatidylserine used in these experiments are stearic acid (46%) and oleic acid (34%). The calculated molar K_a value is 19 μM. This value is comparable to those of unsaturated fatty acids. It has been reported that unsaturated fatty acids did not stimulate protein kinase C [4]. This may be attributed to the differences in lipid treatment prior to use. Fatty acids in this study were directly dispersed in buffer instead of first being dissolved in organic solvent (see fig.1).

There is a possibility that the stimulation by oleic acid of histone phosphorylation is due to the activation of a trace level of unidentified protein kinases rather than protein kinase C. If so, the effect of oleic acid should be additive with that of phosphatidylserine. This is not the case, since oleic acid (0, 40, 100, 200 and 400 μM) showed no further phosphorylation of histone under the conditions of full activation of protein kinase C in the presence of 100 μg/ml phosphatidylserine and 500 μM Ca^{2+}. Therefore oleic acid directly activates the phospholipid/Ca^{2+}-sensitive protein kinase C.

In contrast to unsaturated fatty acids, stearic acid failed to activate protein kinase C in the presence of 500 μM Ca^{2+} (see conditions listed in fig.1). It appears that fatty acids require at least one double bond for protein kinase C activation. We examined whether a *cis* double bond is important for protein kinase C activation using elaidic acid, the *trans* form stereoisomer of oleic acid. In the presence of 500 μM Ca^{2+}, elaidic acid was 80% less potent for protein kinase C activation than its *cis* isomer (not shown).

Since acidic phospholipids such as phosphatidylserine are responsible for protein kinase C activation, we also tested esterified neutral lipids. Both methyloleate and methylarachidonate failed to activate protein kinase C. This indicates that the negative charge of lipid is necessary for activation.

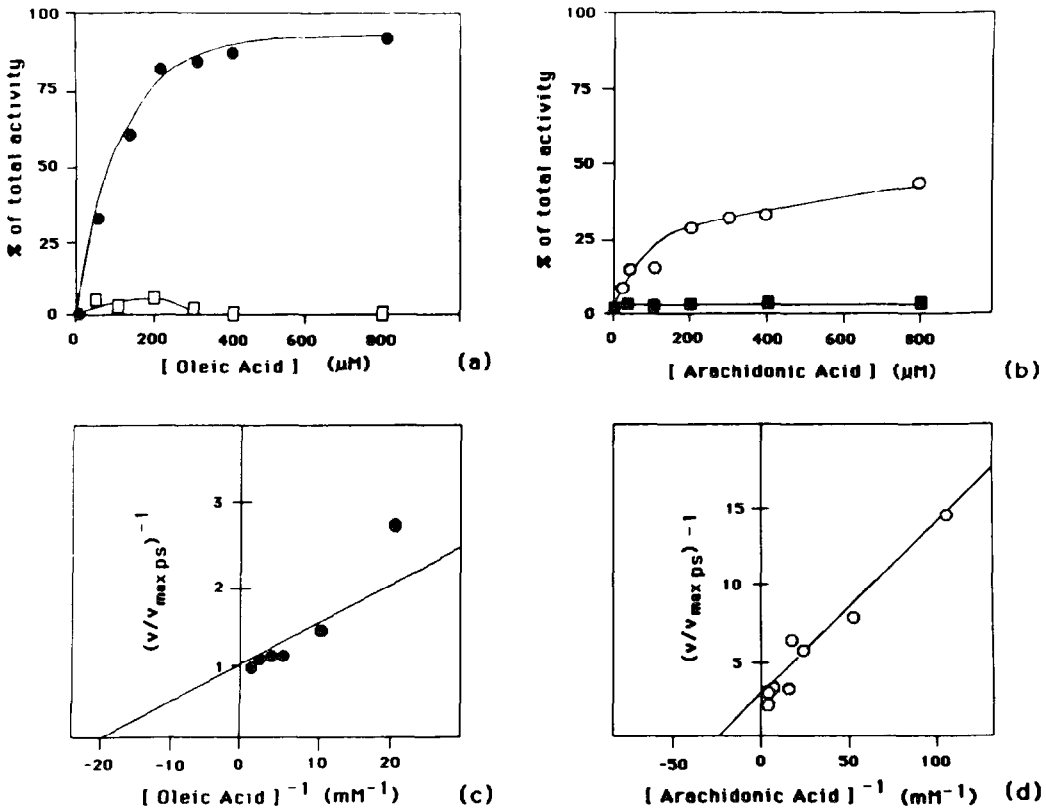

Fig.1. Protein kinase C activation by oleic acid and arachidonic acid in the presence of 500 μM free Ca^{2+}. The activities of protein kinase C observed are normalized to that by 30 μg/ml phosphatidylserine for comparison. (a) (●—●) Oleic acid; (□—□) methyloleate. (b) (○—○) arachidonic acid; (■—■) methylarachidonate. (c and d) Lineweaver-Burk plots of protein kinase C activation by oleic acid and arachidonic acid, respectively. The reaction mixture (240 μl) contained 300 μg/ml histone, 0.5 mM Ca^{2+}, 5 mM Mg^{2+} and various concentrations of fatty acids tested in 20 mM Tris-HCl, pH 7.5, along with purified protein kinase C (spec. act. 624 nmol/min per mg protein). To reduce the metal ion contamination as much as possible, deionized water and buffer used for protein kinase C assay were treated with chelex 100 and assay was performed in plastic test tubes. Unsaturated fatty acids were dispersed into 20 mM Tris-HCl by 1 min vigorous vortex-mixing, N_2 gas bubbling and 10 min sonication at 4°C.

It is not sufficient, however, as indicated by the failure of the saturated stearic acid to stimulate protein kinase C.

Fig.2 shows the Ca^{2+} dependency of the oleic acid effect on protein kinase C activation. Unlike the activation by phosphatidylserine, oleic acid activates protein kinase C directly without Ca^{2+}. A similar result was obtained for arachidonic acid. McPhail et al. [12] have shown the stimulatory effect of unsaturated fatty acids on protein kinase C from crude human neutrophils in the presence but not in the absence of Ca^{2+}. It was not clear, however, whether unsaturated fatty acid directly activates protein kinase C since the neutrophil extract may contain other protein kinases, phospholipids and ions. In our experiment, all the buffers were treated with chelex 100, and plastic test tubes were used to avoid Ca^{2+} contamination [13]. Ca^{2+} atomic absorption measurement of buffer system, fatty acids and phosphatidylserine used all showed the Ca^{2+} contamination to be below detection level (<0.05 mg/l, 1.2 μM). Since it is possible that oleic acid may bind to Ca^{2+} tightly, oleic acid was treated with 1 mM EGTA for 12 h before the assay. In this case, as well, protein kinase C was activated by oleic acid in the absence of Ca^{2+}.

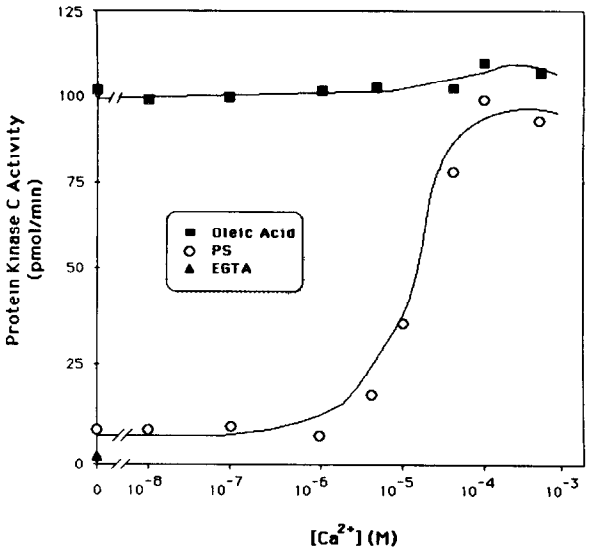

Fig.2. Ca^{2+} dependency of protein kinase C activation by oleic acid and phosphatidylserine. Protein kinase C assay was performed in the presence of oleic acid (400 μM, ■—■) or phosphatidylserine (30 μg/ml, ○—○), at various concentrations of free Ca^{2+}. (▲) Background activity without stimulators. Free Ca^{2+} concentration was controlled by Ca^{2+}-EGTA buffer. The other assay conditions were the same as in fig.1.

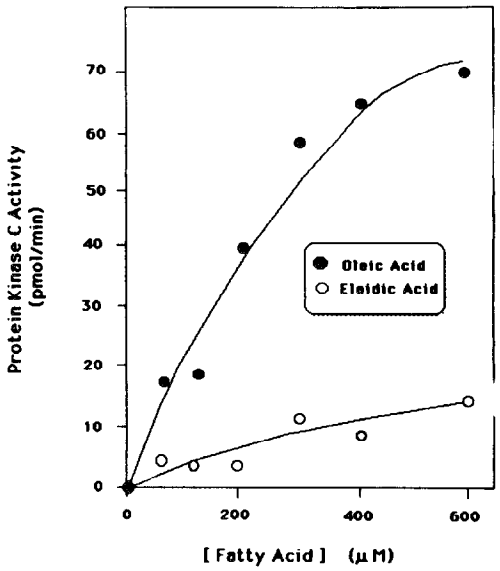

Fig.3. *cis* and *trans* fatty acid effects on protein kinase C activation in the absence of Ca^{2+} at various concentrations of (●—●) oleic acid and (○—○) elaidic acid. Protein kinase C activity was assayed under the same conditions as in fig.1 except using 0.75 mM EGTA instead of 0.5 mM Ca^{2+}. Total Ca^{2+} level was below 0.05 mg/l by atomic absorption.

To determine whether oleic acid activation of protein kinase C was due to limited proteolysis by any remaining Ca^{2+}-dependent protease, i.e. calpain, protein kinase C activation was monitored in the presence of 20 μg/ml leupeptin. The result showed that addition of leupeptin did not block the oleate-induced protein kinase C activation. This rules out the involvement of limited proteolysis. One cannot, however, rule out the possibility that a trace amount of Ca^{2+} may still play a role in protein kinase C activation as the protein kinase C concentration in the assay is less than 1 nM. It is nevertheless clear that unsaturated fatty acid is able to activate protein kinase C in the absence of added phospholipid at or below the physiological concentration of Ca^{2+}.

In the absence of Ca^{2+}, we compared *cis* and *trans* fatty acid effects on protein kinase C activation. Oleic acid showed much higher potency as an activator of protein kinase C than elaidic acid, as shown in fig.3. Fatty acid and lysophosphoglyceride, hydrolyzed products by phospholipase A_2, are known as fusogens [14,15]. Oleic acid shows high fusogenic activity in erythrocyte membrane [14], and has been proposed as 'a natural fusogen' [16]. On the other hand, elaidic acid, a *trans* form fatty acid, is far less effective than oleic acid as a fusogen [14]. Seelig and Seelig [17] have demonstrated using magnetic resonance that the presence of a *cis* double bond at the acyl moiety in membrane phospholipids causes a more disordered conformation of hydrocarbon chains and increases membrane fluidity. These observations along with the present data indicate that the fluidized domain in the membrane, created by the liberation of unsaturated fatty acid by phospholipase A_2, may provide an active site at which protein kinase C can interact with endogenous substrates [18] and can be activated at physiological levels of Ca^{2+}.

Our results demonstrate that protein kinase C can be activated independently of phospholipid and Ca^{2+} by *cis* unsaturated fatty acids. We do not know the physiological importance of this type of activation at the moment, however, the present data suggest that there may be a protein kinase C activation mechanism in addition to the receptor-mediated phospholipase C/diacylglycerol formation sequence. Since unsaturated fatty acids are normally present at very small concentrations and are liberated from membrane phospholipids by

phospholipase A_2, their liberation could serve as a signal for protein kinase C activation. Indeed, a striking rise in total fatty acids has been demonstrated in the brain after electroconvulsive shock [19]. Since phospholipase A_2 requires Ca^{2+} for its activation, the transient elevation of cytosolic Ca^{2+} concentration would stimulate phospholipase A_2-induced unsaturated fatty acid liberation which, in turn, would activate protein kinase C even after the cytosolic concentration of Ca^{2+} returns to resting level.

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