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Effect of *cis*-unsaturated fatty acids on aortic protein kinase C activity

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Long-chain *cis*-unsaturated fatty acids could substitute for phosphatidylserine and activate bovine aortic protein kinase C in assays with histone as substrate. The optimal concentration was 24–40 μM for oleic, linoleic and arachidonic acids. With arachidonic acid, the K_a for Ca^{2+} was 130 μM and kinase activity was maximal at 0.5 mM-Ca^{2+}. Diolein only slightly activated the oleic acid-stimulated enzyme at low physiological Ca^{2+} concentrations (0.1 and 10 μM). Oleic acid also stimulated kinase C activity, determined with a Triton X-100 mixed-micellar assay. Under these conditions, the fatty acid activation was absolutely dependent on the presence of diolein, but a Ca^{2+} concentration of 0.5 mM was still required for maximum kinase C activity. The effect of fatty acids on protein kinase C activity was also investigated with the platelet protein P47 as a substrate, since the properties of kinase C can be influenced by the choice of substrate. In contrast with the results with histone, fatty acids did not stimulate the phosphorylation of P47 by the aortic protein kinase C. Activation of protein kinase C by fatty acids may allow the selective phosphorylation of substrates, but the physiological significance of fatty acid activation is questionable because of the requirement for high concentrations of Ca^{2+}.

INTRODUCTION

Protein kinase C is a ubiquitous enzyme requiring both Ca^{2+} and phospholipid for activity (Nishizuka, 1986). At micromolar Ca^{2+} concentrations, the most effective phospholipid for activation is phosphatidylserine (PS) (Kaibuchi *et al.*, 1981). Diacylglycerol (DG) stimulates protein kinase C activity by markedly increasing the affinity of the enzyme for Ca^{2+}, so that enzyme activation can occur without an increase in intracellular Ca^{2+} (Nishizuka, 1986). DG can be produced by the hydrolysis of phosphoinositides, catalysed by a receptor-activated phospholipase C (Berridge, 1987), and by the degradation of other phospholipids such as phosphatidylcholine (Grillone *et al.*, 1988). Bazzi & Nelsestuen (1987*a*) have shown that the substrate can influence the co-factor requirements (PS, Ca^{2+}, DG) for kinase C activity. Therefore protein kinase C activity in the cell may be modulated by a number of different factors.

cis-Unsaturated fatty acids have been reported to stimulate protein kinase C activity by substituting for PS (McPhail *et al.*, 1984; Murakami & Routtenberg, 1985; Murakami *et al.*, 1986), raising the possibility that fatty acids could be physiological regulators of protein kinase C activity. McPhail *et al.* (1984) reported that fatty acid activation of protein kinase C required Ca^{2+}, whereas Murakami *et al.* (1986) observed activation in the presence of EGTA. Rat brain kinase C can be separated into three isoenzymes by chromatography on hydroxyapatite, and Sekiguchi *et al.* (1987) have shown that each of these isoenzymes responds differently to Ca^{2+} and activation by fatty acids. This suggests that it may be possible to activate one form of kinase C preferentially and hence to modulate a specific cellular event.

The effect of DG on the activation of protein kinase C by fatty acids has not been widely studied, and the available results are inconsistent. Diolein produced a 1.6-fold increase in arachidonic acid-stimulated protein kinase C activity in neutrophil extracts (McPhail *et al.*, 1984). For the rat brain enzyme, DG has been reported to have no effect on fatty acid activation (Sekiguchi *et al.*, 1987). In contrast, Verkest *et al.* (1988) have demonstrated an absolute dependence on diolein for the activation of rat brain protein kinase C by oleic acid.

We previously investigated the properties of a partially purified preparation of protein kinase C from bovine aortas with both histone and the platelet protein P47 as substrates (Dell *et al.*, 1988). Protein kinase C assayed with P47 was characterized by a greater sensitivity to PS and Ca^{2+} for activity as compared with assays with histone. Also, diolein increased kinase C activity measured with P47 at low concentrations of Ca^{2+} without producing a significant change in the K_a for Ca^{2+} (Dell *et al.*, 1988). The objective of the present work was to determine if fatty acids could activate aortic protein kinase C and, if so, if this activation was substrate-specific. We also wished to determine the effect of DG on fatty acid-stimulated protein kinase C activity.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (> 10 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Frozen bovine aortas were purchased from PelFreez Biologicals Inc. (Rogers, AR, U.S.A.). Outdated human platelets were donated by the Foothills Hospital Blood Bank (Calgary, Alberta, Canada). Histone III-S, elaidic acid,

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Abbreviations used: DG, diacylglycerol; PS, phosphatidylserine; P47, 47000 Da platelet protein.

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stearic acid, oleoyl-CoA, ethyl oleate and fatty-acid-free bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PS (bovine brain), 1,2-diolein, oleic acid and arachidonic acid were purchased from Serdary Research Laboratories (London, Ontario, Canada). Linoleic acid was from Supelco Canada (Oakville, Ontario, Canada). DEAE-Sephacel and phenyl-Sepharose were purchased from Pharmacia (Mississauga, Ontario, Canada).

Purification of aortic protein kinase C and platelet protein P47

Protein kinase C was partially purified from bovine aortas by chromatography on DEAE-Sephacel and phenyl-Sepharose as previously described (Dell *et al.*, 1988). The platelet protein P47 was purified as outlined by Dell *et al.* (1988). Protein concentrations were determined by the Coomassie Blue spectrophotometric assay (Spector, 1978), by using dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.).

Protein kinase C assays

The activity of aortic protein kinase C was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to either histone III-S or P47 (Dell *et al.*, 1988). The reaction mixture contained enzyme (2–3 μg of the phenyl-Sepharose pool), 20 mM-Pipes/HCl (pH 6.5), 5 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 10 μM-[γ-^{32}P]ATP (100000 c.p.m./nmol), the appropriate substrate (0.2 mg of histone or P47/ml) and PS and diolein as described below. The total reaction volume was 250 μl. Sometimes Ca^{2+} concentrations were varied. Concentrations between 0.1 μM and 100 μM were determined with Ca^{2+}/EGTA buffers (Dell *et al.*, 1988), but for higher concentrations Ca^{2+} was added.

Two different assay methods were used. The first is termed the liposomal assay. Lipids in solvent were dried under N_2, sonicated into water and added to the reaction mixture to give final concentrations of 40 μg of PS/ml (51 μM) and, when present, 3.2 μg of diolein/ml (5 μM) (Dell *et al.*, 1988). Fatty acids were sonicated into 20 mM-Tris/HCl (pH 7.5) and added to the assay to give the indicated final concentration.

Protein kinase C activity was also determined with a mixed-micellar assay (Dell *et al.*, 1988) modified from the method described by Hannun *et al.* (1985). Lipids were dried under N_2 and resuspended by sonication into 0.3% (w/v) Triton X-100. The final reaction mixture contained 0.03% Triton X-100, PS (73 μg/ml; 20 mol% of Triton X-100) and diolein (57 μg/ml; 20 mol%) and the other assay components listed above. This procedure was also used when fatty acids (20–100 mol%) replaced PS in the assay mixture.

RESULTS

In the absence of diolein, the K_a for Ca^{2+} in assays of aortic protein kinase C with PS and histone was > 50 μM (Dell *et al.*, 1988). Consequently, kinase C activity in the presence of PS can be observed at Ca^{2+} concentrations of 10 μM and 500 μM, but not at 0.1 μM (Fig. 1, left panels). When PS was replaced by 40 μM-oleic acid, kinase C activity was not detectable at 0.1 μM- and 10 μM-Ca^{2+}, but oleic acid stimulated kinase C activity at 500 μM-Ca^{2+} to a rate that was approx. 60% of the activity determined in the presence of PS with histone as substrate (Fig. 1).

The fatty acid stimulation of protein kinase C activity had specific structural requirements, as shown in Fig. 2. *cis*-Unsaturated fatty acids (linoleic, oleic and arachidonic) activated protein kinase C. In contrast, neither a *trans*-unsaturated (elaidic acid) or saturated (stearic acid) fatty acid produced a significant activation of kinase C. Oleoyl-CoA, ethyl oleate, and oleic acid complexed to bovine serum albumin (4:1 molar ratio), also did not stimulate kinase C activity (results not shown).

The K_a values for linoleic, oleic and arachidonic acids were 21 μM, 14 μM and 9 μM respectively (Fig. 2). Maximal kinase C activity was measured with fatty acid concentrations of 24–40 μM. Increasing the fatty acid concentration further produced an inhibition of protein kinase C activity (Fig. 2). The addition of different concentrations (6–120 μM) of arachidonic acid to liposomal assays that contained a maximally activating concentration of PS (80 μg/ml) produced no significant change in kinase C activity (results not shown).

The results shown in Fig. 1 indicated that activation of protein kinase C by oleic acid required a high concentration (500 μM) of Ca^{2+} in assays with histone as substrate. Fig. 3 shows the Ca^{2+}-dependency of enzyme activation by arachidonic acid. Activation occurred in a narrow range, with 0.5 mM-Ca^{2+} as the optimum and a K_a for Ca^{2+} at 130 μM. Concentrations of Ca^{2+} greater than 1 mM were inhibitory (Fig. 3). With oleic acid, the K_a for Ca^{2+} was 160 μM.

The K_a for Ca^{2+} of aortic protein kinase C was decreased from > 50 μM to 3 μM by diolein in assays with PS and histone as substrate (Dell *et al.*, 1988). In the presence of PS, addition of diolein (3.2 μg/ml) stimulated kinase C approx. 3.5- and 1.8-fold at 0.1 μM and 10 μM-Ca^{2+} respectively (Fig. 4). By comparison, diolein produced only a modest 1.5–2-fold increase in kinase C activity at 0.1 μM- and 10 μM-Ca^{2+} when oleic acid replaced PS in the assay (Fig. 4). Thus, at physiological concentrations of Ca^{2+} and in the presence of diolein, fatty acid-stimulated kinase C activity was only 20–25% of the corresponding activity measured with PS.

Hannun *et al.* (1985) have described a mixed-micellar assay system in which kinase C activity is completely dependent on the presence of DG. Using the modified micellar assay as described in the Materials and methods section, we previously showed an absolute dependence on diolein for aortic kinase C activity with Ca^{2+} and PS (Dell *et al.*, 1988). The activation of aortic protein kinase C by mixed micelles of Triton X-100 and oleic acid, with or without diolein, is shown in Fig. 5. Activity was almost completely dependent on the addition of diolein at all oleic acid concentrations used. In the presence of diolein and 0.5 mM-Ca^{2+}, the K_a for oleic acid was 42 mol% or 193 μM, with maximal activity at 60 mol% or 276 μM. The maximal activity measured with oleic acid was 39% of that measured with PS. The Ca^{2+}-dependency of enzyme activation with the micellar assay system was very similar to that seen with the liposomal assay. Even in the presence of 50 mol% diolein, the K_a for Ca^{2+} was 130 μM, with maximal activity between 0.5 mM- and 1 mM-Ca^{2+} with oleic acid (results not shown). This is in contrast with results seen with mixed micelles of PS, where diolein decreased the K_a for Ca^{2+} from > 100 to 2.5 μM (Dell *et al.*, 1988).

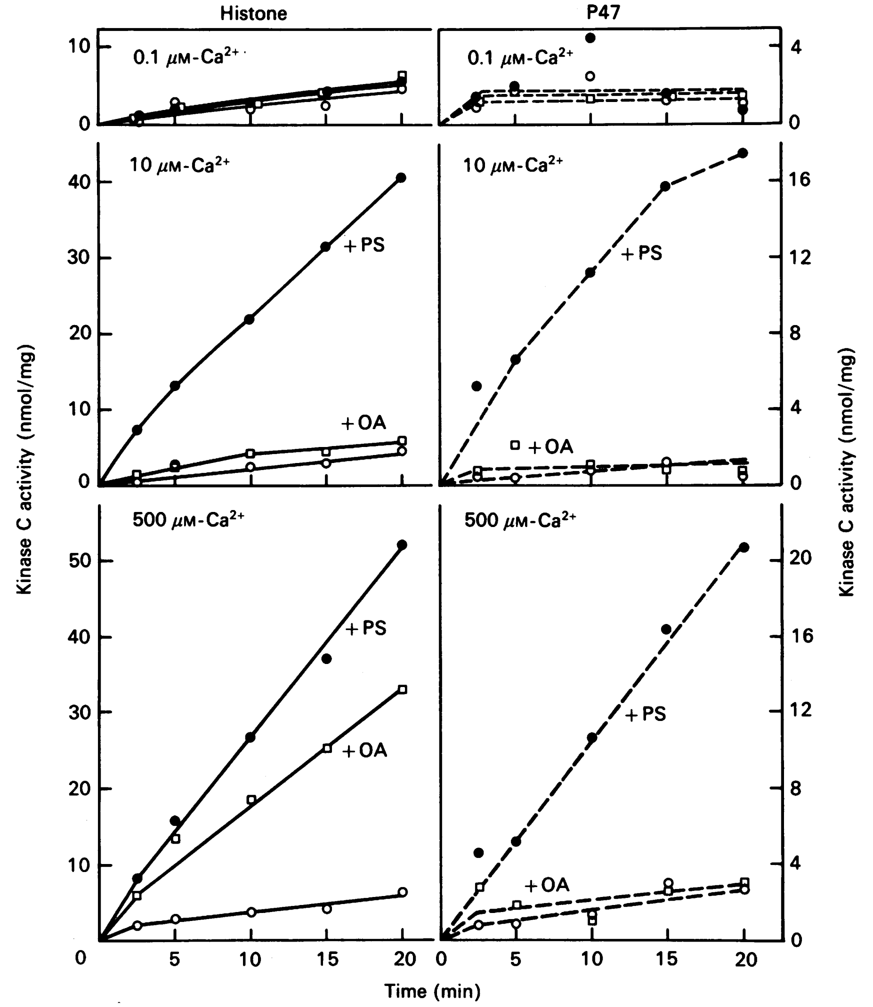

Fig. 1. Time course of protein kinase C activity

Kinase C activity was measured at the indicated times with histone (left panels and continuous lines) or P47 (right panels and broken lines) as substrate (liposomal assay). Assays were performed at the indicated concentrations of Ca^{2+} (0.1, 10, 500 μM) with no lipids (○), 40 μg of PS/ml (●) or 40 μM-oleic acid (OA, □).

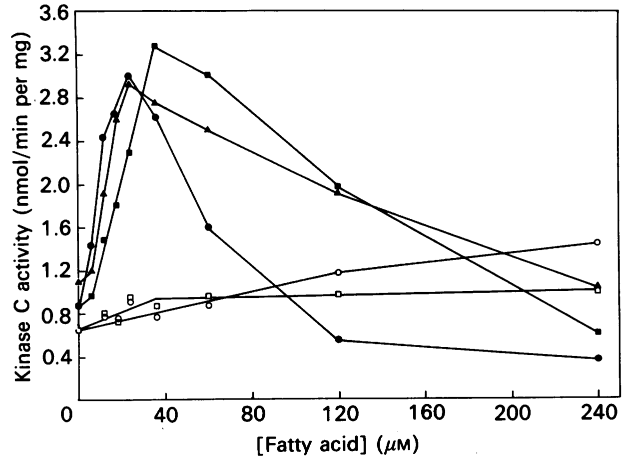

Fig. 2. Activation of protein kinase C by fatty acids

Protein kinase C was assayed with histone as substrate and in the presence of 500 μM-Ca^{2+} (liposomal assay) and with the indicated fatty acid concentrations: arachidonic acid (●), oleic acid (▲), linoleic acid (■), elaidic acid (○) and stearic acid (□).

Histone is most commonly used as a convenient substrate for protein kinase C; however, a number of other substrates *in vitro* and *in vivo* have been identified (Nishizuka, 1986). The platelet protein P47 is a substrate for kinase C *in vivo* (Kaibuchi *et al.*, 1983), and P47 can be phosphorylated *in vitro* by aortic protein kinase C (Dell *et al.*, 1988). The right-hand panels of Fig. 1 show the effects of PS and oleic acid on kinase C activity when P47 is the substrate. Protein kinase C activity could be measured at 10 μM- and 500 μM-Ca^{2+} when PS was present, but not at 0.1 μM-Ca^{2+}. These results are consistent with the reported K_a for Ca^{2+} of 5 μM when aortic protein kinase C activity is determined with P47 as substrate (Dell *et al.*, 1988). In contrast, oleic acid (40 μM) produced no increase in enzyme activity, even at 500 μM-Ca^{2+} (Fig. 1). The phosphorylation of P47 was not increased when the concentration of oleic acid was varied from 12 to 240 μM at Ca^{2+} concentrations of 0.1 and 500 μM (results not shown). The inability of arachidonic acid to stimulate protein kinase C activity measured with P47 at any Ca^{2+} concentration is also shown in Fig. 3. The addition of diolein to the reaction mixture had no effect on enzyme activity measured by P47 phosphorylation.

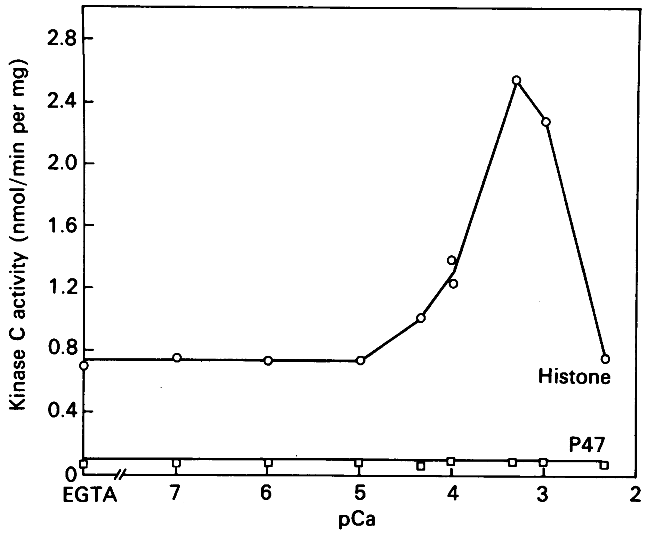

Fig. 3. Ca^{2+}-dependency of arachidonic acid-activated protein kinase C

Protein kinase C was assayed in the presence of 0.5 mM-EGTA or the indicated Ca^{2+} concentrations with 24 μM-arachidonic acid and either histone (○) or P47 (□) as substrate (liposomal assay). Results are from a representative experiment confirmed with at least two other enzyme preparations.

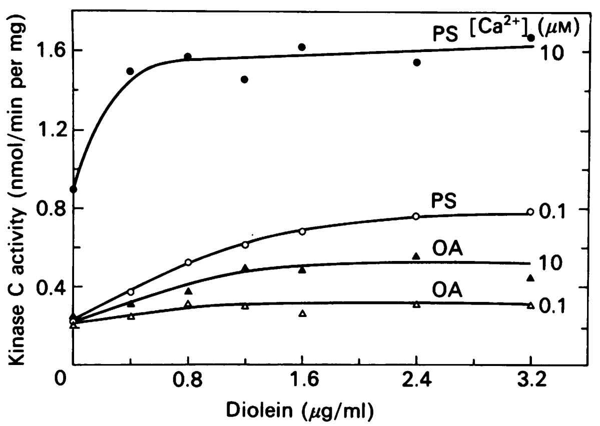

Fig. 4. Effect of diolein on protein kinase C activity

Protein kinase C activity was measured at the indicated diolein concentrations with histone as substrate (liposomal assay). Assays contained 0.1 μM- (○, △) or 10 μM-Ca^{2+} (●, ▲) as indicated and either 40 μM-oleic acid (OA; △, ▲; mean of two experiments) or PS (○, ●; mean of three experiments).

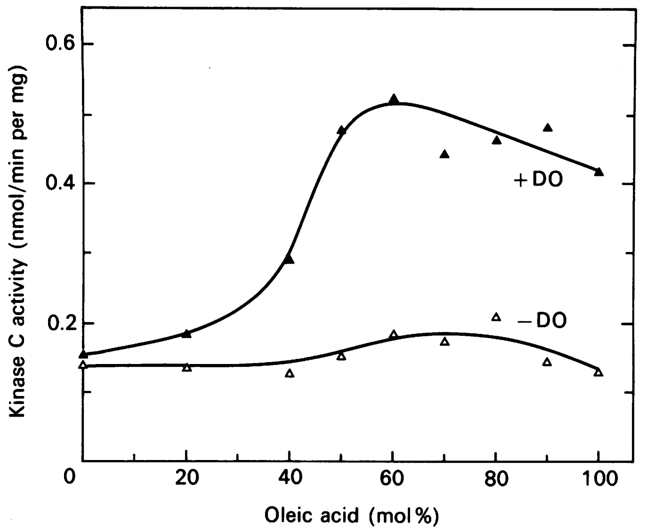

Fig. 5. Effect of oleic acid on protein kinase C activity measured with the mixed-micellar assay

Enzyme activity was measured with the micellar assay at 0.5 mM-Ca^{2+} in the absence (−DO, △) and presence (+DO, ▲) of diolein (20 mol%) and at the indicated oleic acid concentrations. Results were confirmed in at least two other experiments.

DISCUSSION

Aortic protein kinase C can be activated by *cis*-unsaturated fatty acids in the absence of PS, as reported in other investigations (Murakami & Routtenberg, 1985; Sekiguchi *et al.*, 1987). Ganong *et al.* (1986) have proposed a mechanism for kinase C activation in which Ca^{2+} forms a complex with four PS molecules via the carboxy groups of the serine head-group; possibly fatty acids form a similar complex with Ca^{2+} via their carboxy groups. With the liposomal assay, fatty acid concentrations of 24–40 μM gave optimal activity for the aortic protein kinase C. Other investigators have reported maximal activation at fatty acid concentrations ranging from 100 to 400 μM (Murakami & Routtenberg, 1985; Murakami *et al.*, 1986; McPhail *et al.*, 1984; Sekiguchi *et al.*, 1987). The lowest critical micellar concentration of oleic acid is 720 μM (Murakami *et al.*, 1986), and so the activation of aortic protein kinase C will almost certainly be the result of the monomeric form of the fatty acid.

Activation of aortic protein kinase C by fatty acids required high concentrations of Ca^{2+} (K_a of 130–160 μM). McPhail *et al.* (1984) and Wooten & Wrenn (1988) also found that Ca^{2+} was needed for fatty acid activation of protein kinase C. In contrast, brain kinase C could be fully activated in the presence of EGTA by high (400 μM) concentrations of fatty acids (Murakami *et al.*, 1986; Murakami & Routtenberg, 1985). However, at fatty acid concentrations of 25–100 μM, protein kinase C activity was increased by Ca^{2+} concentrations > 10 μM (Murakami *et al.*, 1986). Protein kinase C isoenzymes II and III from rat brain (Sekiguchi *et al.*, 1987) were activated by fatty acids in the absence of Ca^{2+}, but kinase C activity measured with 400 μM fatty acid was markedly stimulated by increasing Ca^{2+}. Ca^{2+} increased type I protein kinase C activity at low concentrations of fatty acids, but inhibited activity measured in the presence of 400 μM-arachidonic acid (Sekiguchi *et al.*, 1987). Results such as these suggest that the Ca^{2+} and fatty acid requirements of the enzyme may vary with the source and isoenzyme of kinase C studied. Addition of diolein to the liposomal assay produced only a modest activation of fatty acid-activated kinase C at low physiological concentrations of Ca^{2+} (0.1 and 10 μM) as compared with assays with PS. Although fatty acid activation of kinase C assayed with mixed micelles was almost completely dependent on DG, this activation also required high concentrations of Ca^{2+} (K_a 130 μM).

From previous studies (Dell *et al.*, 1988), aortic protein kinase C phosphorylated P47 in assays with PS and Ca^{2+} (K_a 5 μM). However, fatty acids were unable to stimulate the phosphorylation of P47 under any experimental condition. Bazzi & Nelsestuen (1987*b*) have proposed that the interaction of substrate with phospholipid is essential for phosphorylation by kinase C. Since lysine-rich histone is a basic protein and P47 is slightly acidic (Imaoka *et al.*, 1983), it is quite feasible that they may react differently with fatty acids. This may explain the difference in phosphorylation of these two proteins, and suggests that varying the lipid environment of protein kinase C may potentially allow for selective substrate phosphorylation.

Fatty acids had no effect on aortic protein kinase C activity measured in the presence of PS; similar results have been reported by Murakami & Routtenberg (1985). Consequently, fatty acids would likely have no effect on membrane-associated protein kinase C activity. However, certain isoenzymes of protein kinase C may be localized in the cell cytosol (Knopf *et al.*, 1986). Thus it is possible that fatty acids may specifically activate cytosolic forms of kinase C and hence stimulate phosphorylation of particular cell proteins.

Although fatty acids will activate aortic protein kinase C under certain experimental conditions, the physiological significance of this activation is questionable. First, although intracellular fatty acids may be present at concentrations of 10–50 μM (Hunneman & Schweickhardt, 1982; Van der Vusse *et al.*, 1982), most intracellular fatty acids are protein-bound, and oleic acid bound to bovine serum albumin did not activate aortic protein kinase C. Second, optimal enzyme activation required 0.5 mM-Ca^{2+}, which is well above Ca^{2+} concentrations normally found in a cell. Even in the presence of diolein, significant activation did not occur at physiological Ca^{2+} concentrations. It is possible that enzyme activation might occur in some pathological situations where intracellular Ca^{2+} may be abnormally high.

In summary, the activation of bovine aortic kinase C by *cis*-unsaturated fatty acids was observed in both liposomal and mixed-micellar assays when histone was the substrate. Fatty acid activation was completely dependent on DG in the micellar assay. Maximum activity occurred at 0.5 mM-Ca^{2+}, with very little activity at physiological Ca^{2+} concentrations, even in the presence of diolein. Fatty acid activation was substrate-specific, since it could be observed with histone but not with platelet protein P47 as substrate. Further experiments are needed to determine if this means of activating kinase C has any physiological significance.

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