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ACTIVATION OF PROTEIN KINASE C BY *CIS*- AND *TRANS*-FATTY ACIDS AND ITS POTENTIATION BY DIACYLGLYCEROL

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Both *cis*- and *trans*-unsaturated but not saturated fatty acids activated protein kinase C purified to apparent homogeneity from rat brain. Fatty-acid-induced enzyme activation was not more than additive with that by phospholipids and was potentiated by diacylglycerol. Recently, we demonstrated that *cis*-and *trans*-unsaturated fatty acids induced platelet aggregation and phosphorylation of specific proteins. Both events were potentiated by a cell-permeable diacylglycerol [(1987) Biochem. Biophys. Res. Commun. 149, 762-768]. Thus, *trans*-unsaturated fatty acids may provide useful experimental tools for the study of protein kinase C activation *in vitro* and *in vivo*. Our results suggest that fatty acids and diacylglycerol may synergistically be involved in hormonal stimulation of protein kinase C, as certain hormonal stimuli cause release of diacylglycerol and fatty acids from phospholipids by parallel activation of phospholipases C and A_2.

Binding of various hormonal agonists to plasma membrane receptors leads to activation of phospholipase C, which catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol [1]. Diacylglycerol activates phospholipid/Ca^{2+}- dependent protein kinase C by increasing the apparent affinity of the enzyme for Ca^{2+} [2]. Therefore, diacylglycerol is considered to be the physiological activator of protein kinase C. In numerous cell types, phospholipase C-

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ABBREVIATIONS: AA, arachidonic acid; DO, 1,2-dioleoyl-*rac*-glycerol; ETYA, 5,8,11,14-ei-cosatetraynoic acid; PS, *L*-α-phosphatidyl-*L*-serine

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catalyzed polyphosphoinositide degradation is accompanied by phospholipase A_2-mediated release of AA from membrane phospholipids [1,3,4]. AA has been suggested to play a role as second messenger in the activation of protein kinase C [5,6]. *Cis*-unsaturated fatty acids, but not the *trans*-unsaturated elaidic acid or saturated fatty acids induce neuronal and platelet activation and phosphorylation of specific proteins [7,8]. These findings prompted us to investigate the stereospecificity of protein kinase C activation by fatty acids and the interaction of fatty acids with Ca^{2+}, phospholipids and diacylglycerol.

MATERIALS AND METHODS

Protein kinase C was purified from rat brain in a three-step procedure by novel combination of established chromatographic steps [9-11]. Briefly, rat brains were homogenized in a buffer containing 1 mM $CaCl_2$ [9]. Homogenates were centrifuged for 15 min at 40,000 x g. The pellet was rehomogenized in the same buffer supplemented with 5 mM EGTA and 2 mM EDTA instead of $CaCl_2$. After centrifugation for 15 min at 300,000 x g, protein kinase C was further purified by binding to and subsequent dissociation from erythrocyte inside-out vesicles and affinity chromatography using immobilized N-(2-aminoethyl) 5-isoquinolinesulfonamide (H-9) [10, 11]. By the procedure described above, the enzyme was purified to apparent homogeneity as judged by silver-stained SDS-polyacrylamide gels and revealed a molecular mass of 83 kDa.

Protein kinase C activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone (type III-S) (Sigma, Taufkirchen, F.R.G.). Reaction mixtures (50 µl) contained 0.75 ng of protein kinase C, 2 mM EGTA, $MgCl_2$ (5 mM free Mg^{2+}), either $CaCl_2$ (0.1 - 300 µM free Ca^{2+}) or 2 mM EGTA only without addition of $CaCl_2$, 20 µM Mg[γ-^{32}P] ATP (1 µCi/tube), histone (1 mg/ml), 1 mM dithiothreitol , 50 mM triethanolamine-HCl, pH 7.5, and lipids as indicated. Following incubation for 10 min at 37°C, aliquots (20 µl) were withdrawn, spotted onto 2 x 2 cm phosphocellulose strips (Whatman P-81) and immersed in 75 mM phosphoric acid (10 ml/sample) to terminate the reaction [12]. Phosphocellulose-bound radioactivity was determined by measuring Cerenkov radiation in a liquid scintillation spectrometer. Free Ca^{2+} and Mg^{2+} concentrations were calculated on the basis of metal chelator stability constants for EGTA and ATP tabulated in ref. 13. Data presented in the figures are the mean of assay duplicates or triplicates, which varied less than 5% from each other. Similar results were obtained in at least three independent experiments and using different enzyme preparations.

All lipids were obtained from Sigma Chemie (Taufkirchen, F.R.G.) in the highest available degree of purity. [γ-^{32}P]ATP was prepared as described [14]. ETYA was a gift of Hoffmann-La Roche (Basel, Switzerland). All other chemicals and reagents were of analytical grade. Stock solutions of ETYA, arachidic, stearic and palmitic acid (15-20 mM) were prepared in dimethyl sulfoxide. Stock solutions of other fatty acids and methyl arachidonate (15 - 20 mM) were prepared in absolute ethanol under a nitrogen atmosphere, protected from light and stored at -20°C. Dilutions of fatty acids were made according to the method of Badwey et al. [15]. Ethanol and dimethyl sulfoxide up to their maximal concentrations (5%, v/v) in the assay mixtures containing 1 mM of fatty acids had no effect on protein kinase C activity. Stock solutions of PS and of DO (100

μg/ml) were prepared in 95% chloroform and 5% methanol (v/v) and stored at -20°C. Organic solvent was removed under a stream of nitrogen, and PS, DO or a mixture of both were dispersed in assay buffer by sonication.

RESULTS AND DISCUSSION

In the presence of 300 μM Ca^{2+}, *cis*-unsaturated fatty acids with 1-4 ethylenic bonds and chain lengths of between 14-20 carbon atoms activated protein kinase C (Fig. 1). The carboxyl group of fatty acids appeared to

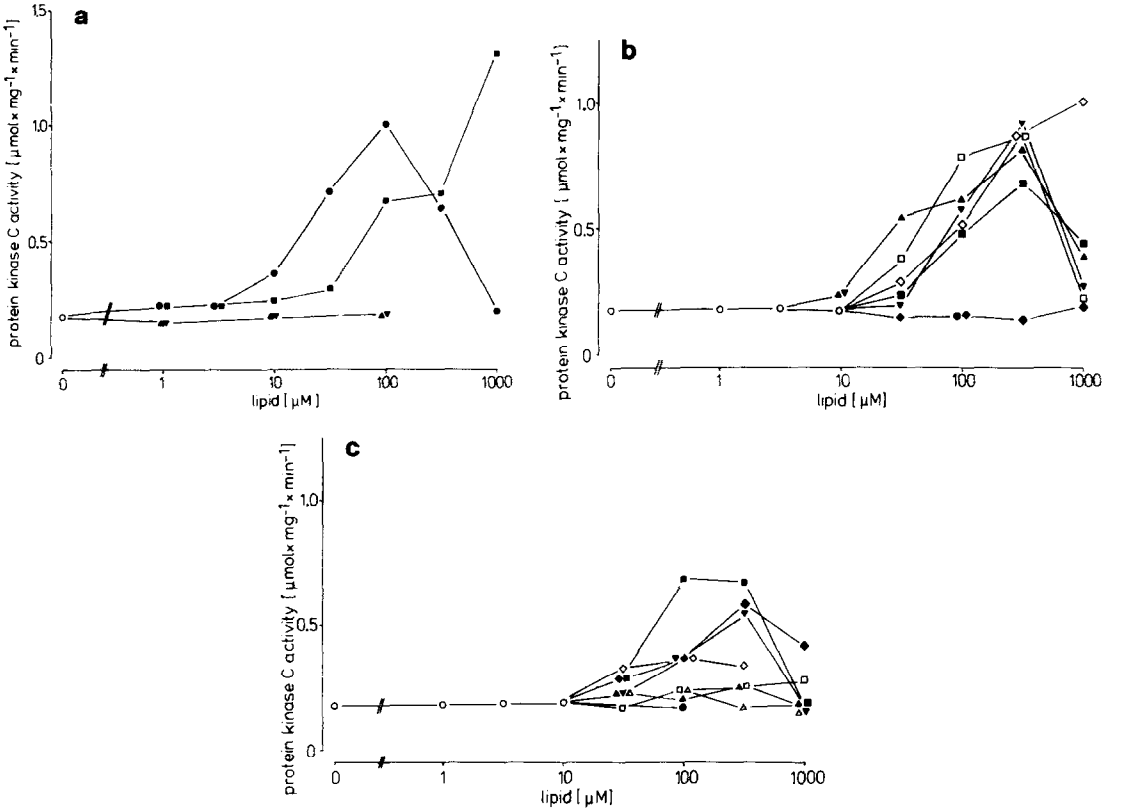

Fig. 1: Activation of protein kinase C by fatty acids, methyl arachidonate and sodium dodecyl sulfate. Protein kinase C activity was determined in the presence of 300 μM Ca^{2+} and lipids at the concentrations indicated:
a) Arachidic (n-eicosanoic) acid (▲), arachidonic (5,8,11,14-all-*cis*-eicosatetraenoic) acid (●), methyl arachidonate (▼), ETYA (■).
b) Stearic (n-octadecanoic) acid (●), oleic (*cis*-9-octadecenoic) acid (■), elaidic (*trans*-9-octadecenoic) acid (◆), *cis*-vaccenic (*cis*-11-octadecenoic) acid (▲), *trans*-vaccenic (*trans*-11-octadecenoic) acid (▼), linoleic (*cis*-9-*cis*-12-octadecadienoic) acid (□), linolelaidic (*trans*-9-*trans*-12-octadecadienoic) acid (◇).
c) Palmitic (n-hexadecanoic) acid (●), palmitoleic (*cis*-9-hexadecenoic) acid (■), palmitelaidic (*trans*-9-hexadecenoic) acid (◆), myristic (n-tetradecanoic) acid (▲), myristoleic (*cis*-9-tetradecenoic) acid (▼), lauric (n-dodecanoic) acid (□), sodium dodecyl sulfate (◇), capric (n-decanoic) acid (△).

be indispensible, as methyl arachidonate did not activate protein kinase C. Enzyme activation by *cis*-unsaturated fatty acids was maximal at concentrations between 30 and 300 μM. ETYA, the acetylenic analogue of AA, which is widely used as an inhibitor of lipoxygenases [16], also activated protein kinase C at concentrations above 10 μM. *Trans*-unsaturated fatty acids with 16 and 18 carbon atoms with the exception of elaidic acid activated protein kinase C to similar extents as did their *cis*-stereoisomers. Thus, the ability of fatty acids to activate protein kinase C is not restricted to *cis*-unsaturated fatty acids as was assumed by previous investigations using elaidic acid as the only *trans*-unsaturated fatty acid [6,17]. Saturated fatty acids with chain lengths of between 14 to 20 carbon atoms did not activate the enzyme. Lauric and capric acid as well as sodium dodecyl sulfate slightly activated protein kinase C. The maximum extent of enzyme activation obtained with optimally stimulatory concentrations of fatty acids increased with their carbon chain lengths, indicating that the ability of fatty acids to activate protein kinase C is related to their hydrophobicity [18]. The data also indicate that fatty acids require at least one double bond to be efficient activators of the enzyme and that the ability of fatty acids to activate the enzyme is probably not due to their detergent-like properties.

Trans-unsaturated fatty acids may provide useful experimental tools to study the regulation of the enzyme in intact cells, as they offer several advantages in comparison to *cis*-unsaturated fatty acids: *trans*-unsaturated fatty acids are not metabolized by cyclooxygenase and lipoxygenases and do not increase membrane fluidity [19,20]. In contrast to *cis*-unsaturated fatty acids, *trans*-unsaturated fatty acids do not stimulate other cellular signalling systems such as adenylate cyclase [21] and guanylate cyclase [22]. The utility of *trans*-unsaturated fatty acids as activators of protein kinase C in intact cellular systems was demonstrated in our recent study showing that linolelaidic acid induced platelet aggregation and protein phosphorylation [9].

With respect to the physiological relevance of protein kinase C activation by fatty acids, we further studied the interaction of fatty acids (AA) with Ca^{2+}, phospholipids (PS), and diacylglycerol (DO) (Figs 2 and 3). At Ca^{2+} concentrations of up to 10 μM, AA only weakly activated protein kinase C; Ca^{2+} at higher concentrations enhanced the effects of AA. Similar to AA, PS required Ca^{2+} above 10 μM to activate protein kinase C. At Ca^{2+} concentrations above 30 μM, AA and PS activated protein kinase C not more than additively. In contrast to DO, AA did not increase the apparent affinity of the enzyme for Ca^{2+}. DO synergistically enhanced the stimulatory effect of AA in the absence or presence of Ca^{2+} and PS. The quantitative extent of synergistic interaction between DO and AA depended on the concentration of either lipid (Fig. 3). In the presence of 1 μM Ca^{2+}, DO at concentrations up to 0.3 μg/ml did not enhance the stimulatory effects of AA. By increasing the DO concentration from 1 to 10

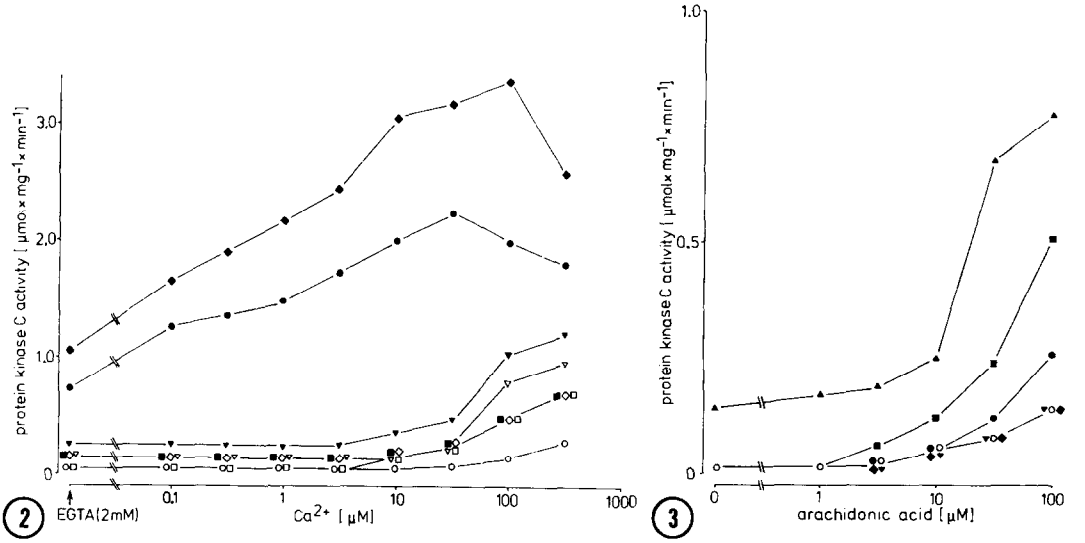

Fig. 2: Ca^{2+}-dependency of protein kinase C activation by arachidonic acid with diacylglycerol and phospholipids. Protein kinase C activity was measured in the presence of Ca^{2+} at the concentrations indicated and lipids at the following concentrations: Control (○), 30 μM AA (◇), 10 μg/ml PS (□), 2 μg/ml DO (■),10 μg/ml PS + 2 μg/ml DO (●), 30 μM AA + 10 μg/ml PS (▽), 30 μM AA + 2 μg/ml DO (▼), 30 μM AA + 10 μg/ml PS + 2 μg/ml DO (◆).

Fig. 3: Synergistic activation of protein kinase C by arachidonic acid and diacylglycerol. Protein kinase C activity was determined in the presence of 1 μM Ca^{2+}, AA at the concentrations indicated on the abscissa with varying concentrations of DO: Control (○), 0.1 μg/ml DO (▼), 0.3 μg/ml DO (◆), 1 μg/ml DO (●), 3 μg/ml DO (■), 10 μg/ml DO (▲).

μg/ml, the minimum AA concentration required to elicit a synergistic interaction was reduced from 100 to < 10 μM. With Ca^{2+} at such low concentrations, protein kinase C activation by AA (100 μM) and DO (10 μg/ml) was five times higher than with either lipid alone.

A previous investigation failed to reveal a synergism between fatty acids and diacylglycerol on protein kinase C [17]. Our results suggest that fatty acids and diacylglycerol may synergistically be involved in hormonal stimulation of protein kinase C as certain hormonal stimuli cause the release of diacylglycerol and fatty acids from phospholipids by parallel activation of phospholipase C and phospholipase A_2, respectively [1,3,4]. This hypothesis is supported by our finding that fatty acid-induced platelet activation was potentiated by a cell-permeable diacylglycerol [8]. In the latter study we observed distinct differences in the kinetics of platelet aggregation and of protein phosphorylation between fatty acids and diacylglycerol. In addition, we found that platelet aggregation induced by diacylglycerol, fatty acids or by fatty acids plus diacylglycerol was differentially inhibited by the inhibitors of protein kinase C, polymyxin B, H-7 and staurosporine [23]. From these data it may be concluded that fatty acids activate protein kinase C by a mechanism different from that of diacylglycerol.

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REFERENCES

1. Abdel-Latif, A.A. (1986) Pharmacol. Rev. 38, 227-272.
2. Nishizuka, Y. (1984) Nature 308, 693-698.
3. Ohta, H., Okajima, F. and Ui, M. (1985) J. Biol. Chem. 260, 15771-15780.
4. Burch, R.M., Luini, A. and Axelrod, J. (1986) Proc. Natl. Acad. Sci. USA 83, 7201-7205.
5. McPhail, L.C., Clayton, C.C. and Snyderman, R. (1984) Science 224, 622-625.
6. Murakami, K. and Routtenberg, A. (1985) FEBS Lett. 192, 189-193

7. Linden, D.J., Murakami, K. and Routtenberg, A. (1986) Brain Res. 379, 358-363.
8. Seifert, R., Schächtele, C. and Schultz, G. (1987) Biochem. Biophys. Res. Commun.149, 762-768.
9. Sahyoun, N., Le Vine III, H., McConnell, R., Bronson, D. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. USA 80, 6760-6764.
10. Wolf, M., Cuatrecasas, P. and Sahyoun, N. (1985) J. Biol. Chem. 260, 15718-15722.
11. Inagaki, M., Watanabe, M. and Hidaka, H. (1985) J. Biol. Chem. 260, 2922-2925.
12. Roskoski, Jr., R. (1983) Methods Enzymol. 99, 3-15.
13. Holloway, J.H. and Reilly, C.N. (1960) Anal. Chem. 32, 249.
14. Johnson, R.A. and Walseth, T.F. (1979) Adv. Cycl. Nucl. Res. 10, 135-167.
15. Badwey, J.A., Curnutte, J.T. and Karnovsky, M.L. (1981) J. Biol. Chem. 256, 12640-12643.
16. Aharony, D., Smith, J.B., and Silver, M.J. (1984) In The Leukotrienes (Chakrin, L.W., and Bailey, D.M., eds.), pp. 116-123, Academic Press, New York.
17. Sekiguchi, K., Tsukada, M., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1987) Biochem. Biophys. Res. Commun. 145, 797-802.
18. Smith, R. and Tanford, C. (1973) Proc. Natl. Acad. Sci. USA 70, 289-293.
19. Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. and Lefkowith, J.B. (1986) Ann. Rev. Biochem. 55, 69-102.
20. Karnowski, M.J., Kleinfeld, A.M., Hoover, R.L. and Klausner, R.D. (1982) J. Cell. Biol. 94, 1-6.
21. Orly, J. and Schramm, M. (1975) Proc. Natl. Acad. Sci. USA 72, 3433-3437.
22. Glass, D.B., Frey, W. II, Carr, D.W. and Goldberg, N.D. (1977) J. Biol. Chem. 252, 1279-1285.
23. Schächtele, C., Seifert, R. and Osswald, H. (1988) Biochem. Biophys. Res. Commun. 151, 542-547.