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UNSATURATED DIACYLGLYCEROL AS A POSSIBLE MESSENGER FOR THE ACTIVATION OF CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE SYSTEM*

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SUMMARY: A small quantity of unsaturated diacylglycerol (DG) sharply decreased the Ca^{2+} and phospholipid concentrations needed for full activation of a Ca^{2+}-activated, phospholipid-dependent multifunctional protein kinase described earlier (Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254. 3692-3695). In the presence of unsaturated DG and micromolar order of Ca^{2+}, phosphatidylserine (PS) was most relevant with the capacity to activate the enzyme, whereas phosphatidylethanolamine and phosphatidylinositol (PI) were far less effective. Phosphatidylcholine was practically inactive. It is possible, therefore, that unsaturated DG, which may be derived from PI turnover provoked by various extracellular stimulators, acts as a messenger for activating the enzyme, and that Ca^{2+} and various phospholipids such as PI and PS seem to play a role cooperatively in this unique receptor mechanism.

Hokin and Hokin (1) first presented evidence that PI[1/] turns over very rapidly in response to acetylcholine. Early work on such PI turnover was carried out with various types of secretory tissues such as pancreas (1,2), salivary gland (3) and salt-secreting gland (4). Subsequent studies developed by many investigators (for reviews see Refs. 5,6) have shown that the PI response can be provoked in a variety of tissues which are activated by various extracellular stimulators including α-adrenergic and muscarinic cholinergic neurotrans-

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1/ Abbreviations used are: PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; and PC, phosphatidylcholine.

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mitters as well as some peptide hormones. Nevertheless, all attempts to clarify the physiological significance of such PI turnover have been thus far uniformly unsuccessful. In preceding reports from this laboratory (7,8) a new species of multifunctional protein kinase has been identified in mammalian tissues which may be selectively activated by the simultaneous presence of Ca^{2+} and phospholipid. This communication will present evidence suggesting that PI turnover may be coupled with the activation of this protein kinase, and possible roles of various phospholipids in this receptor mechanism will be proposed. In order to relate to our previous papers (7,8) the Ca^{2+}-activated, phospholipid-dependent protein kinase will be referred to as protein kinase C.

EXPERIMENTAL PROCEDURES

Protein kinase C was purified partially from rat brain cytosol as described previously (9), and the preparation used was essentially free of endogenous phosphate acceptor proteins and interfering enzymes. The enzyme was assayed with H1 histone as phosphate acceptor in the presence of Ca^{2+}, phospholipid and neutral lipid. The detailed conditions are given in each experiment. PI (pig liver) was purchased from Serdary Research Laboratories, and was purified by thin layer chromatography on a Silica Gel H (E. Merck) plate as described previously (8). PS (bovine brain), PE and PC (human erythrocyte) were generous gifts of Dr. T. Fujii and Dr. A. Tamura, Kyoto College of Pharmacy. All samples employed were chromatographically pure. Mono-, di- and triacylglycerols employed were synthetic products which were obtained from commercial sources. Unless otherwise specified each sample of diacylglycerol was a mixture of 1,2- and 1,3-diacyl derivatives as judged by thin layer chromatography. 1-Stearoyl-2-oleoyl diglyceride and 1-stearoyl-2-linoleoyl diglyceride were products of Serdary Research Laboratories. Samples of monoacylglycerols were also mixtures of 1- and 2-acyl derivatives. H1 histone was prepared from calf thymus as described earlier (10). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (11). All materials and reagents employed for the present studies were taken up in water which was prepared by a double distillation apparatus followed by passing through a Chelex-100 column to remove Ca^{2+} as much as possible as specified by Teo and Wang (12). Protein was determined by the method of Lowry *et al.* (13) with bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

Protein kinase C normally present as an inactive form in the soluble fraction of mammalian tissues was activated by reversible association with membranes in the presence of Ca^{2+} (7). The active

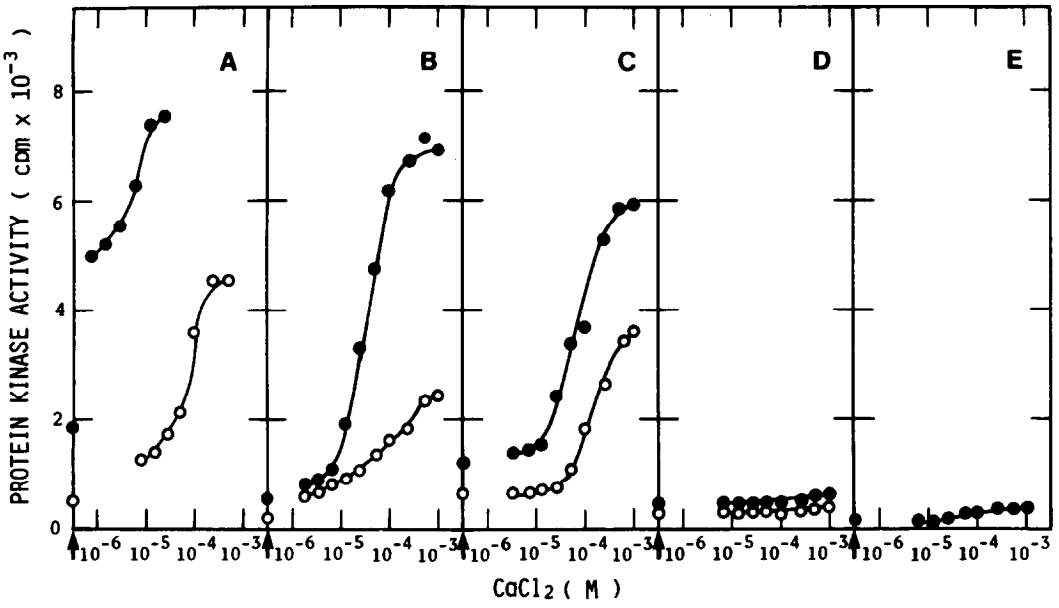

Fig. 1. Effect of diolein on reaction velocity of protein kinase C at various concentrations of $CaCl_2$. The complete reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium nitrate, 50 μg of H1 histone, 2.5 nmol of [γ-^{32}P]ATP (5 x 10^4 cpm/nmol), 0.4 μg of protein kinase C, 2 μg each of phospholipid indicated, and various concentrations of $CaCl_2$ as indicated. Where indicated diolein (0.2 μg/tube) was added. Each phospholipid was first mixed with diolein in a small volume of chloroform. After chloroform was removed in vacuo, the residue was suspended in 20 mM Tris/HCl at pH 7.5 by sonication with a Kontes sonifier K881440 for 5 min at 0°C, and employed for the assay. The incubation was carried out for 3 min at 30°C. The reactions were stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). The radioactivity was determined as described (14). Abscissa indicates the final concentration of $CaCl_2$ added. Where indicated with an arrow, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (0.5 mM at final concentration) was added instead of $CaCl_2$. A, with PS; B, with PE; C, with PI; D, with PC; and E, with diolein alone. (●——●), assayed in the presence of diolein; and (○——○), assayed in the absence of diolein.

factor in membranes was identified as phospholipid; particularly PI and PS were most effective to support enzymatic activity (8). Subsequent analysis on the mechanism of action of phospholipid revealed that coexistence of a very small quantity of diacylglycerol possessing unsaturated fatty acid particularly at the position 2 greatly enhanced the phospholipid-dependent activation of enzyme especially at lower concentrations of Ca^{2+}. A typical result of such experiment is shown in Fig. 1. In this figure the reaction velocities in the presence and absence of diolein were plotted against Ca^{2+} concentra-

tions in a logarithmic scale. The enhancement of reaction by diolein was most remarkable when PS was employed (Fig. 1A). Namely, supplement of a small quantity of diolein to PS greatly enhanced the reaction velocity with the concomitant decrease in Ca^{2+} concentrations giving rise to full activation of the enzyme. If, however, PE or PI was employed instead of PS, only reaction velocity was accelerated by the addition of diolein and relatively higher concentrations of Ca^{2+} were needed for activation of the enzyme (Fig. 1,B and C). PC was practically ineffective to support enzymatic activity irrespective of the presence and absence of diolein (Fig. 1D). Diolein alone showed a very little or no effect over a wide range of Ca^{2+} concentrations (Fig. 1E).

The enhancement of reaction by diolein in the presence of PS did not appear to be attributed simply to the increase in reaction velocity but was accompanied by the decrease in Ca^{2+} concentration which was needed for full activation of the enzyme as described above. Kinetic analysis indicated that the addition of diolein greatly increased an apparent affinity of the enzyme for PS as well as for Ca^{2+}. In the experiments shown in Fig. 2, Ka value for Ca^{2+}, the concentration needed for half maximum activation, was plotted against mono-, di- or triolein which was added together with either PS or PI. The results showed that, when PS was employed, Ka value for Ca^{2+} was decreased from about 5×10^{-5} M sharply to the micromolar order, and that diolein in an amount of less than 10% of that of PS showed remarkable effect (Fig. 2A). Again, diolein showed a very little effect when PS was replaced by PI (Fig. 2B). It may be noted that monoolein and triolein did not enhance reaction velocity nor decreased Ka value for Ca^{2+} under comparable conditions. Such a unique effect of neutral lipid was specific for diacylglycerol possessing unsaturated fatty acid, and essentially similar results were obtained for dilinolein,

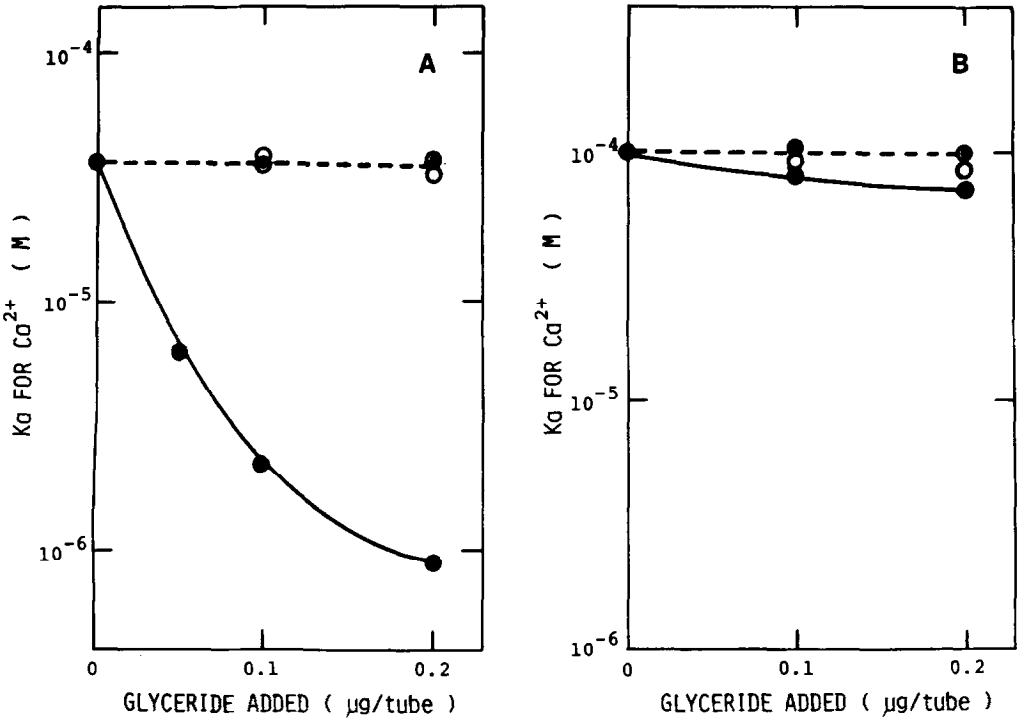

Fig. 2. Effects of mono-, di- and triolein on Ka value for Ca^{2+} of protein kinase C. The reaction mixture contained a fixed amount (2 μg each) of either PS or PI, and various amounts of glyceride as indicated. Other assay conditions were the same to those described in Fig. 1, and Ka value for Ca^{2+} was estimated. A, with PS; and B, with PI. (●—●), assayed in the presence of diolein; (⊙--⊙), assayed in the presence of monoolein; and (○--○), assayed in the presence of triolein.

diarachidonin, 1-stearoyl-2-oleoyl diglyceride and also for 1-stearoyl-2-linoleoyl diglyceride as shown in Table I. Both dipalmitin and distearin were less effective. Monoacylglycerol and triacylglycerol tested thus far were totally ineffective irrespective of the fatty acyl moieties. Neither cholesterol nor glycolipid could substitute for unsaturated diacylglycerols mentioned above.

It appears to be established that PI turnover which is provoked by various extracellular stimulators is initiated by hydrolysis of the phosphodiester linkage in a manner of phospholipase C (15-20). Thus, the primary product of this reaction is expected to be diacylglycerol which is very effective to potentiate the Ca^{2+} and phospholipid-dependent activation of protein kinase C, since PI of most

Table I

Effects of various diacylglycerols on Ka value for Ca^{2+} and reaction velocity of protein kinase C

Diacylglycerol added	Ka for Ca^{2+}	Protein kinase activity
	(μM)	(cpm)
None	50	970
Diolein	2	6,830
Dilinolein	3	5,040
Diarachidonin	6	4,270
1-Stearoyl-2-oleoyl diglyceride	4	4,890
1-Stearoyl-2-linoleoyl diglyceride	5	6,630
Dipalmitin	20	2,340
Distearin	50	950

The reaction mixture contained PS (2 μg) and diacylglycerol indicated (0.1 μg each). Other conditions were the same to those described in Fig. 1, and Ka value for Ca^{2+} was estimated. The protein kinase activity at 6.4×10^{-6} M $CaCl_2$ is given.

mammalian origins is well known to be composed of unsaturated fatty acid such as arachidonic or oleic acid particularly at the position 2 (21). Therefore, PI turnover may be directly related to the activation of this unique protein kinase in such a way that signals of extracellular stimulators induce the activation of a phospholipase C-type enzyme which is presumably specific for PI. This activation of phospholipase C may initiate PI turnover on one hand and, on the other hand, the resulting unsaturated diacylglycerol may serve as a messenger which in turn activates protein kinase C in the presence of Ca^{2+} and phospholipid. At lower concentrations of Ca^{2+} the highest enzymatic activity was obtained with the combination of PS and unsaturated diacylglycerol as described above. Presumably, some lipid bilayer structure is necessary for rendering the enzyme more active, and better physiological picture will be clarified by further investigations. Nevertheless, Ca^{2+} and various phospholipids such as PI and PS seem to play a role cooperatively in this unique receptor mechanism.

It may also be emphasized that in this mechanism protein kinase C can be activated without net increase in Ca^{2+} concentrations within the cell, since the unsaturated diacylglycerol markedly increases the affinity of this protein kinase system for this divalent cation.

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