資料２－１

高井参考人のご意見

1. 体外から摂取された sn-1,2-ジアシルグリセロールの細胞内への取り込みに関して、ジアシルグリセロールを構成する脂肪酸の種類による差異があるか否か(参考１，２，３)。
   (ア)長鎖脂肪酸の場合、細胞膜を透過して細胞内へ取り込まれるか否か。
   (イ)中鎖、短鎖脂肪酸の場合、細胞膜を透過して細胞内へ取り込まれるか否か。
   (ウ)細胞膜に物理的な傷害等があって、膜の透過性に変化が生じているような場合、長鎖脂肪酸により構成されるジアシルグリセロールが細胞内へ取り込まれるか否か。

細胞膜に加えた DAG が細胞内に取り込まれたかどうかは、DAG がホスファチジン酸（PA）に転換されたかどうかで測定しました。２つとも長い脂肪酸をもった DAG は細胞内に取り込まれにくいと考え、１つが長い脂肪酸、１つが短い脂肪酸をもった DAG、具体的には1-オレオイル、2-アセチル-グリセロール（OAG）を山之内製薬に合成していただき、これを細胞に作用させましたところ、PA に転移されました（参考論文５）。この OAG は in vitro で PKC を活性化することができます（参考論文３）。この実験は OAG が細胞レベルでも PKC を活性化することを示すために行ったものです。
したがいまして、ご質問の
(ア)につきましては、私は実験を行った記憶がありませんのでわかりません。
(イ)につきましては、私共の実験結果から少なくとも OAG は細胞内に取り込まれます。
(ウ)取り込まれやすくなるとは推定できますが、私はその根拠は知りません。

2. sn-1,2-ジアシルグリセロールを用いた皮膚塗布実験等、in vivo において、発がんプロモーション作用を確認した実験は行われているのか。
   (ア)　実験が行われていた場合、その実験条件は、どのようなものか。
   (イ)　実験が行われていた場合、その実験結果、データはどのようなものか。

(ア)、(イ)共にわかりません。

3. 細胞内において、sn-1,2-ジアシルグリセロールはどのように代謝されるのか。
   (ア)細胞内のジアシルグリセロールは速やかに分解されるか否か。
   (イ)ジアシルグリセロールが細胞内に蓄積するか否か。

(ア)、１.の(ア)でお答え致しましたように、細胞外から導入された OAG は少なくとも PA に代謝されます。
(イ)細胞外から導入された DAG は PKC を活性化しますので、少しの間は、DAG のままで存在していると考えられますが、その後は少なくとも PA に代謝されます。DAG がどのくらいの間 DAG として存在しているかは知りません。DAG と TPA との違いは、当時、TPA は代謝されにくいということでした。

4. すべての sn-1,2-ジアシルグリセロールが発がんプロモーション作用を有するのか否か。
   細胞膜の構成成分であるリン脂質が分解して生成されるジアシルグリセロールと同様の発がんプロモーション作用が細胞外から投与されたジアシルグリセロールにもあるのか否か。

DAG が PKC を活性化するためには少なくとも１つの脂肪酸が不飽和であることが必要であることを報告しております（参考論文１、２）。発がんプロテーター活性のないホルボールアステルは PKC を活性化しないことも報告しております（参考論文４）。従いまして、PKC

を活性化することができない DAG には発がんプロモーター活性があるとは考えにくいと思います。もちろん、PKC を活性化することができる DAG に発がんプロモーター作用があるかどうかは存じません。

5. 皮膚と舌とでは、sn-1,2-ジアシルグリセロールの細胞内取り込みに、どの程度の差があるのか。

DAG を投与するとき、DAG をどのような溶媒に溶かしているかによっても細胞内への取り込み方が違うと推定されます。また、皮膚の上皮と舌の上皮では組織学的な構造の違いもありますから、厳密にいえば、DAG の取り込みがこの両者の上皮で違っているとは思いますが、正確なことはわかりません。

6. 扁平上皮細胞に対して sn-1,2-ジアシルグリセロールはＴＰＡと同様の作用をするメディエーターであるが、腺細胞に対しても、ジアシルグリセロールは同様の働きをするのか。

扁平上皮細胞と腺細胞に OAG を作用させると、両細胞において PKC は活性化されると考えられますが、この OAG による PKC の活性化が両細胞の発がんプロモーションに同じように関係しているかはわかりません。

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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

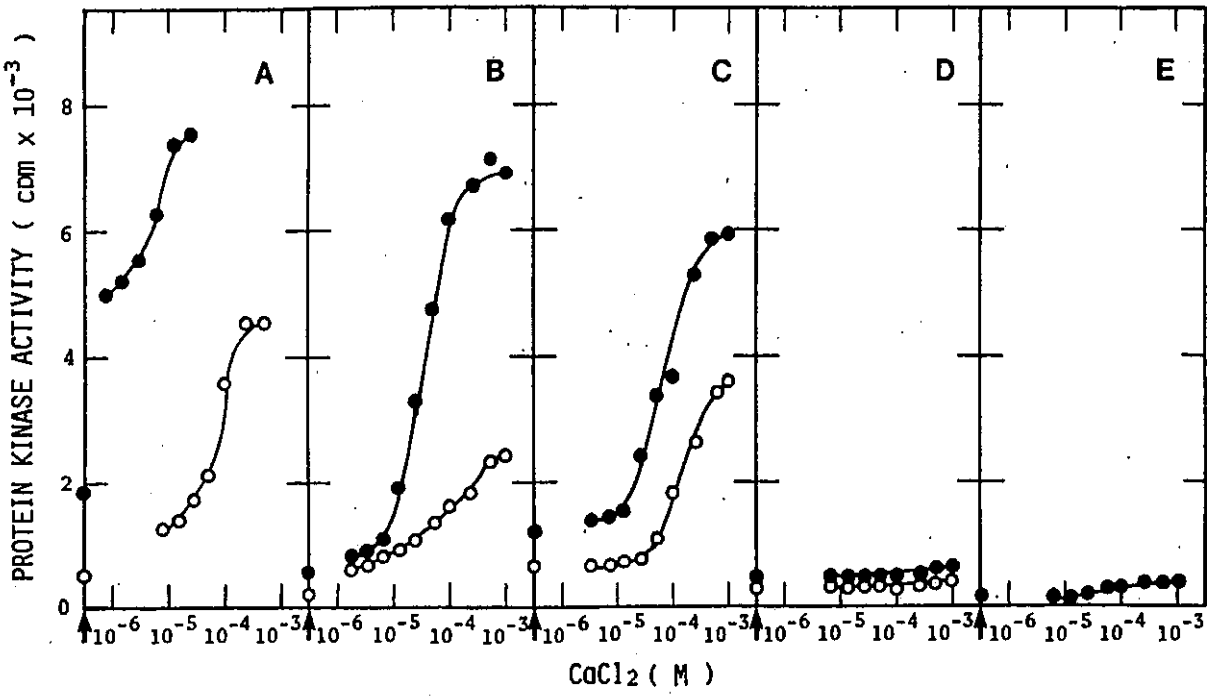

Fig. 1. Effect of diolein on reaction velocity of protein kinase C at various concentrations of $CaCl_2$. The complete reaction mixture (0.25 ml) contained 5 µmol of Tris/HCl at pH 7.5, 1.25 µmol of magnesium nitrate, 50 µg of H1 histone, 2.5 nmol of [γ-^{32}P]ATP (5 x 10^4 cpm/nmol), 0.4 µg of protein 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 experiments 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,

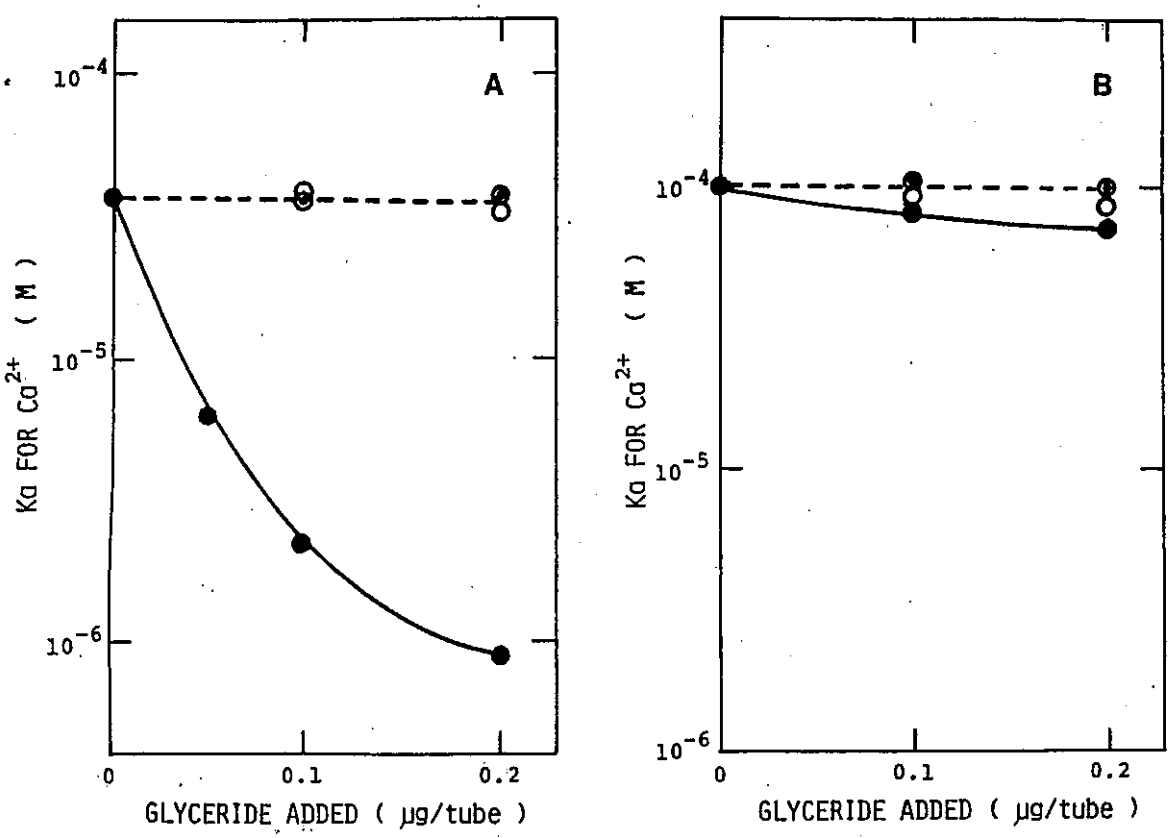

Fig. 2. Effects of mono-, di- and triolein on Ka value for Ca^{2+} of protein kinase C. The reaction mixture contained a fixed amount (2 µg each) of either PS or PI, and various amounts of glyceride as indicated. Other assay conditions were the same to those described in Fig. 1, and Ka value for Ca^{2+}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

(Received for publication, October 12, 1979, and in revised form, December 4, 1979)

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Ca^{2+}-activated, phospholipid-dependent protein kinase from various mammalian tissues (Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y (1979) *J. Biol. Chem.* 254, 3692-3695) was greatly stimulated by the addition of diacylglycerol at less than 5% (w/w) the concentration of phospholipid. This stimulation was due to an increase in the apparent affinity of enzyme for phospholipid and to a concomitant decrease in the K_a value for Ca^{2+} from about 1×10^{-4} M to the micromolar range. Diacylglycerol alone showed little or no effect on enzymatic activity over a wide range of Ca^{2+} concentrations. This effect was greatest for diacylglycerol which contained unsaturated fatty acid at least at position 2. The active diacylglycerols so far tested included diolein, dilinolein, diarachidonin, 1-stearoyl-2-oleoyl diglyceride, and 1-stearoyl-2-linoleoyl diglyceride. In contrast, diacylglycerols containing saturated fatty acids such as dipalmitin and distearin were far less effective. Triacyl- and monoacylglycerols were totally ineffective, irrespective of the fatty acyl moieties. Cholesterol and free fatty acids were also ineffective. Based on these observations, a possible coupling is proposed between the protein kinase activation and phosphatidylinositol turnover which can be provoked by various extracellular messengers.

A recent report from this laboratory (1) has described a new species of cyclic nucleotide-independent multifunctional protein kinase, which is selectively activated by the simultaneous presence of Ca^{2+} and phospholipid. Membranes themselves from various sources such as brain synapses and hepatocytes are capable of supporting the enzymatic activity, and evidence has been presented that the enzyme may be activated by reversible association with membranes in the presence of Ca^{2+} (2). During the analysis of this mode of activation, we have noticed that the concentration of Ca^{2+} necessary for enzyme activation largely depends on the membranes employed and that, although the enzyme shows a marked preference for phosphatidylinositol and phosphatidylserine among various chromatographically pure phospholipid samples, an unfractionated total lipid fraction is most effective particularly at lower concentrations of Ca^{2+}. Subsequent analysis to clarify the reason for these observations has revealed that, in addition to phospholipid, a small quantity of diacylglycerol, particularly which contains unsaturated fatty acid, greatly enhances the reaction velocity with a concomitant decrease in the Ca^{2+} concentration giving rise to maximum enzyme activation. Since the first product of phosphatidylinositol breakdown is expected to be a diacylglycerol with an unsaturated fatty acid such as arachidonic or oleic acid (3-5), it is conceivable that the activation of newly found protein kinase may be directly related to phosphatidylinositol turnover which has been described first by Hokin and Hokin (6) and subsequently studied extensively by many investigators (for reviews see Refs. 7 to 9). The protein kinase to be discussed here will be referred to as protein kinase C.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Protein kinase C was prepared from rat cerebral cytosol as described previously (10). The enzyme preparation employed in this study was practically free of endogenous phosphate acceptors and interfering enzymes. Oleic acid, monoolein,[1] diolein, triolein, stearic acid, monostearin, distearin, tristearin, palmitic acid, monopalmitin, dipalmitin, tripalmitin, arachidonic acid, and cholesterol were purchased from Nakarai Chemicals, Kyoto. Linoleic acid, monolinolein, dilinolein, trilinolein, and triarachidonin were obtained from Gasukuro Kogyo, Tokyo. Lipase (*Rhizopus delemar*) was purchased from Seikagaku Kogyo, Tokyo. 1-Stearoyl-2-oleoyl diglyceride and 1-stearoyl-2-linoleoyl diglyceride were purchased from Serdary Research Laboratories. Monoarachidonin and diarachidonin were prepared from triarachidonin by treatment with lipase and were purified by thin layer chromatography. The solvent system used was ligroin/ethyl ether/acetic acid (70:30:1). With this solvent system 1,2- and 1,3-diacylglycerols could be separated from each other. Calf thymus H1 histone was prepared as described previously (12). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (13).

Extraction and Fractionation of Lipid—Total lipid was extracted from human erythrocyte ghosts with chloroform/methanol (2:1) as described by Folch *et al.* (14). Neutral lipid, glycolipid, and phospholipid were fractionated from the total lipid on a silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical Co.) column as described by Rouser *et al.* (15). The phospholipid fraction employed for the present studies was free of diacylglycerol as judged by thin layer chromatography.

Enzyme Assay—Protein kinase C was assayed by measuring the incorporation of $^{32}P_i$ into H1 histone from [γ-^{32}P]ATP. All reactions were carried out in plastic tubes. The standard 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 to 15 $\times 10^4$ cpm/nmol), 0.5 μg of protein kinase C, and various concentrations of

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[1] Unless otherwise specified, monoacylglycerol and diacylglycerol employed for the present study were mixtures of 1- and 2-monoacylglycerols, and 1,2- and 1,3-diacylglycerols, respectively. Diarachidonin enzymatically prepared as described in the text was mostly 1,2-diacylglycerol as judged by thin layer chromatography. Nevertheless, it has been reported that the isomers are convertible from each other under nonenzymatic conditions (11).

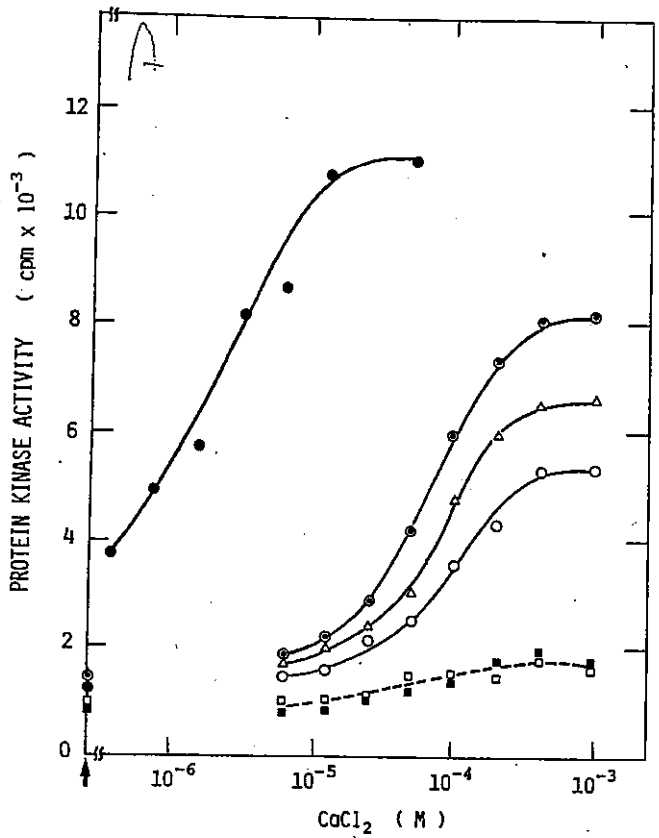

FIG. 1. **Response of protein kinase C to Ca^{2+} in the presence of various lipids.** Total lipid, neutral lipid, glycolipid, and phospholipid were separately suspended in 20 mM Tris/HCl at pH 7.5 by sonication with a Kontes sonifier K881440 for 5 min at 0°C. Protein kinase was assayed under the standard conditions except that lipids and $CaCl_2$ were added as indicated. Where indicated with an *arrow*, EGTA[2] (0.5 mM at final concentration) was added instead of $CaCl_2$. ●——●, with total lipid (5.8 μg; this total lipid was composed of phospholipid (4.1 μg), neutral lipid (1.4 μg), and glycolipid (0.3 μg)); ⊙——⊙, with phospholipid (16.4 μg); △——△, with phospholipid (8.2 μg); ○——○, with phospholipid (4.1 μg); ■- - -■, with neutral lipid (5.7 μg); □- - -□, with glycolipid (1.2 μg).

Ca^{2+} and lipid as indicated in each experiment. The incubation was carried out for 3 min at 30°C. The reaction was 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).

Removal of Ca^{2+} from Reagents—Ca^{2+}-free reagents were obtained by passing them through Chelex 100, a resin specific for chelating divalent cations, as described by Teo and Wang (16). Double-distilled water, Tris/HCl buffer at pH 7.5, and a solution of [γ-^{32}P]ATP were separately passed through a Chelex 100 column (3 × 0.5 cm). H1 histone was dissolved in Chelex 100-treated, double-distilled water, and was passed through a Sephadex G-25 column (20 × 0.5 cm) equilibrated with the same water. Plastic columns and connections were used throughout these procedures. Spectrograde magnesium nitrate containing less than 100 ppm of Ca^{2+} was purchased from Merck, E.G. and was dissolved in Ca^{2+}-free water. Protein kinase C and lipid preparations were diluted 100 to 1000 times with Ca^{2+}-free buffer and employed. These reagents were stored in plastic tubes.

Determinations—The radioactivity of ^{32}P-labeled samples was determined using a Nuclear Chicago Geiger Muller gas flow counter, model 4338 as described (17). Protein was determined by the method of Lowry *et al.* (18) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The response of protein kinase C to Ca^{2+} concentrations varied markedly with the membranes used. For instance, with rat brain synaptic and human erythrocyte membranes, the concentrations of Ca^{2+} needed for half-maximum activation (K_a) were 5×10^{-6} M and 2×10^{-5} M, respectively. A series of experiments to clarify the reason of this difference revealed that lipid composition of membranes significantly influenced the response of enzyme to Ca^{2+}. In fact, although the active lipid component was previously identified as phospholipid, particularly phosphatidylinositol and phosphatidylserine (1), unfractionated total lipid was more effective than any of the chromatographically pure samples of phospholipids, especially at lower concentrations of Ca^{2+}. In the experiment shown in Fig. 1, the reaction velocity in the presence of various lipids was measured and plotted against Ca^{2+} concentrations on a logarithmic scale. In the presence of phospholipid alone, relatively higher concentrations of Ca^{2+} were needed irrespective of the amount of phospholipid employed; the reaction velocity was accelerated by increasing amounts of phospholipid added. In the presence of a saturating amount of phospholipid (about 25 μg/tube), full enzymatic activity was observed even though the K_a value for Ca^{2+} remained high. Neither neutral lipid nor glycolipid alone showed a significant effect on enzyme activation over a wide range of Ca^{2+} concentrations. However, supplement of the phospholipid with neutral lipid markedly enhanced the reaction velocity and concomitantly decreased the Ca^{2+} concentration necessary for enzyme activation.[3] Kinetic analysis indicated that this enhancement of the reaction velocity by neutral lipid was due to an increase in apparent affinity of enzyme for both phospholipid and Ca^{2+}. Glycolipid could not substitute for neutral lipid for this enhancement.

Analysis of the neutral lipid fraction resulted in the identification of diacylglycerol containing unsaturated fatty acid as the active component which enhanced the phospholipid-dependent activation of protein kinase C. In the presence of phospholipid alone, higher concentrations of this cation were needed, and the reaction velocity was relatively slow (Fig. 2). If, however, a small quantity of diolein was added in addition to phospholipid, the reaction velocity was greatly enhanced. Concomitantly, the response of enzyme to Ca^{2+} shifted markedly and full enzymatic activity was obtained at much lower concentrations of this cation. Diolein alone showed practically no effect. Diolein at less than 5% (w/w) the concentration of phospholipid showed significant effect, decreasing the apparent K_a value for Ca^{2+} from 7×10^{-5} M to the micromolar range (Fig. 3*A*). At lower concentrations of Ca^{2+}, the reaction velocity was markedly enhanced by the addition of diolein (Fig. 3*B*). Diolein employed for the assay mentioned above was not phosphorylated or otherwise altered during the incubation. In addition, 1,2- and 1,3-dioleins were equally active, suggesting that diolein itself was the activating factor because these isomers of diacylglycerol should not be metabolized in the same manner (19). Neither triolein nor monoolein increased reaction velocity or decreased Ca^{2+} concentrations required for activation.

This unique effect of neutral lipid was greatest for diacylglycerol containing an unsaturated fatty acid. Essentially similar results were obtained with dilinolein, diarachidonin, 1-stearoyl-2-oleoyl diglyceride, and 1-stearoyl-2-linoleoyl diglyceride instead of diolein. Table I summarizes experiments which examined various synthetic neutral lipids. Diolein, dilinolein, diarachidonin, 1-stearoyl-2-oleoyl diglyceride, and 1-stearoyl-2-linoleoyl diglyceride were equally effective, whereas dipalmitin and distearin were far less effective in stimulating the Ca^{2+} and phospholipid-dependent activation of protein

[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid.

[3] Neutral lipid was inactive even when dispersed in Triton X-100 unless phospholipid was added. Thus, it was unlikely that the inability of neutral lipid to activate the enzyme was due to its insolubility.

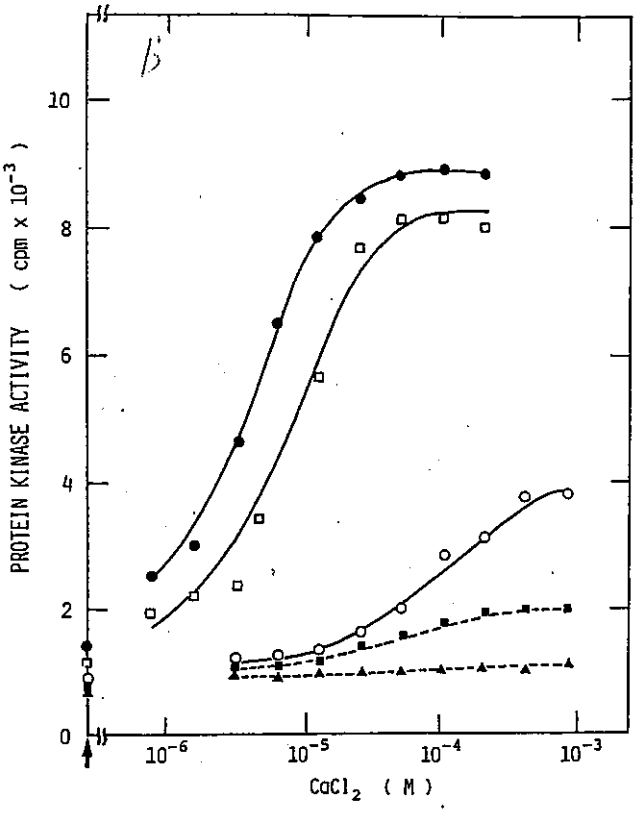

FIG. 2. **Effects of neutral lipid and diolein on reaction velocity of protein kinase C at various concentrations of Ca^{2+}.** Phospholipid was first mixed with neutral lipid or diolein in a small volume of chloroform. After the chloroform was removed *in vacuo*, each mixture was suspended in 20 mM Tris/HCl at pH 7.5 by sonication as described in Fig. 1. Protein kinase was assayed under the standard conditions except that lipids and $CaCl_2$ were added as indicated. Where indicated with an *arrow*, EGTA (0.5 mM at final concentration) was added instead of $CaCl_2$. O——O, with phospholipid (4.1 μg) alone; ■- - -■, with diolein (0.28 μg) alone; ▲- - -▲, with neutral lipid (1.4 μg) alone; ●——●, with phospholipid (4.1 μg) plus diolein (0.28 μg); □——□, with phospholipid (4.1 μg) plus neutral lipid (1.4 μg).

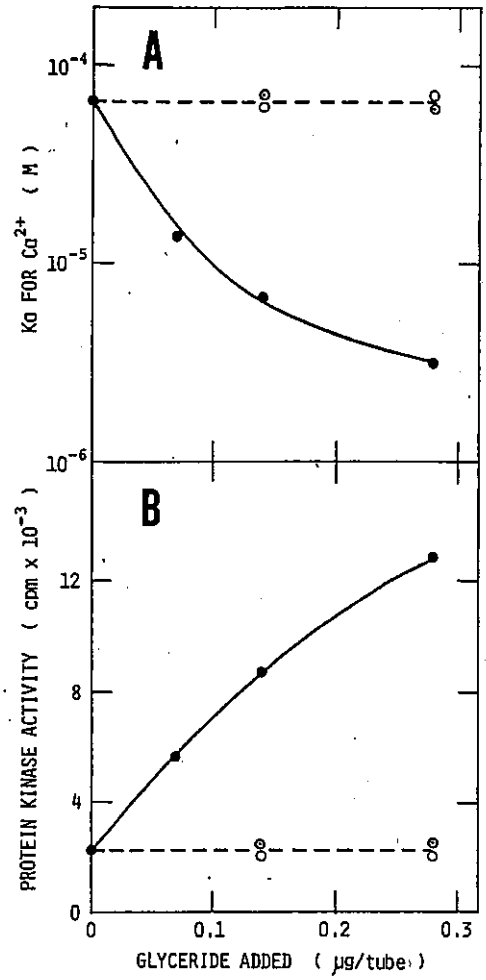

FIG. 3. **Effects of mono-, di-, and triolein on K_a value for Ca^{2+} and reaction velocity of protein kinase C.** The reaction mixture contained a fixed amount of phospholipid (4.1 μg) and varying amounts of glyceride as indicated. Other assay conditions were the same to those described in Fig. 2. *A*, K_a value for Ca^{2+} was plotted against concentration of glyceride added. *B*, reaction velocity at a fixed concentration of Ca^{2+} (6.4×10^{-6} M) was plotted against concentration of glyceride added. ●——●, with diolein; O- - -O, with monoolein; ⊙- - -⊙, with triolein.

kinase C.[4] Triacyl- and monoacylglycerols were ineffective irrespective of the fatty acyl moieties including palmitic, stearic, oleic, linoleic, and arachidonic acids. Cholesterol and free fatty acids were also ineffective under similar conditions.

Hokin and Hokin (6) presented the first evidence that phospholipid, particularly phosphatidylinositol, turns over very rapidly in response to various extracellular messengers. Early work on such phosphatidylinositol turnover was carried out with various acetylcholine-sensitive glands (20–23). Subsequent studies have shown that this phospholipid turnover can be stimulated in virtually any type of tissue stimulated by a variety of extracellular messengers including α-adrenergic and muscarinic cholinergic stimulators (for reviews see Refs. 7 to 9). It is well known that phosphatidylinositol in mammalian tissues is composed of mainly unsaturated fatty acids such as arachidonic and oleic acids, particularly at the position 2 (24). Since the first product of phosphatidylinositol turnover is expected to be such an active diacylglycerol (3–5), an enzyme which cleaves the phospholipid like phospholipase C appears to be of crucial importance for initiating both activation of protein kinase C and phosphatidylinositol turnover. Bell *et al.* (25) has recently proposed that arachidonic acid is released from thrombin-stimulated human platelets through

TABLE I

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

The reaction mixture contained phospholipid (4.1 μg), diglyceride indicated (0.2 μg), and varying concentrations of Ca^{2+}. Other conditions were the same to those described in Fig. 2. The protein kinase activity at 6.4×10^{-6} M $CaCl_2$ is given.

	K_a for Ca^{2+}	Protein kinase activity
	μM	cpm
None	70	980
Diolein	4	6500
Dilinolein	7	5510
Diarachidonin	4	6220
1-Stearoyl-2-oleoyl diglyceride	8	4610
1-Stearoyl-2-linoleoyl diglyceride	6	5670
Dipalmitin	30	1760
Distearin	70	950

[4] The differing activation by different diacylglycerols did not appear to be due to solubility problems rather than enzyme specificity. Dipalmitin and distearin were less effective over a wide range of concentrations even when added together with Triton X-100. The exact amount of diacylglycerols in the sonicated lipid bilayers is not known at this time.

two consecutive reactions catalyzed by phosphatidylinositol-specific phospholipase C and diglyceride lipase. Although the direct relationship between this proposal and the activation of protein kinase C described above has remained unexplored, it is reasonable to assume that the diacylglycerols produced by various extracellular messengers may also serve as substrates to produce unsaturated fatty acids which are in turn converted to prostaglandins. Unsaturated fatty acids, as well as prostaglandins, have been shown to stimulate guanylate cyclase, and the phosphatidylinositol turnover appears to parallel with the increase in cyclic GMP in many tissues (for reviews see Refs. 8 and 26).

It may also be emphasized that in the mechanism described in the present paper protein kinase C may be activated without a net increase in the intracellular Ca^{2+} concentrations, since the small quantity of diacylglycerol which results from the phosphatidylinositol hydrolysis would sharply increase affinity of the newly found protein kinase system for this divalent cation. It has been repeatedly documented that the response of cells to some hormones requires the entrance of Ca^{2+} into the cell (for reviews see Refs. 27 and 28). Nevertheless, the precise role of Ca^{2+}, as well as of various lipid, involved in such an unique mode of activation of protein kinase C remains to be explored. Although phosphatidylinositol and phosphatidylserine stimulated enzymatic activity more than other phospholipids, an unfractionated mixture of phospholipids was almost equally as effective as pure phosphatidylinositol and phosphatidylserine (1). Likewise, the effect of diacylglycerol was greatest when unfractionated phospholipid was employed. The exact structural features required for the specificity of the phospholipid are not yet known. Possibly, a lipid bilayer structure is important for rendering the enzyme more active.

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Specificity of the Fatty Acyl Moieties of Diacylglycerol for the Activation of Calcium-Activated, Phospholipid-Dependent Protein Kinase[1]

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The specificity of the fatty acyl moieties of diacylglycerol for the activation of Ca^{2+}-activated, phospholipid-dependent protein kinase was investigated. Diacylglycerol has been previously shown to activate this enzyme by increasing the affinity for Ca^{2+} and phospholipid, both of which are indispensable for the enzyme activation. Diacylglycerols containing at least one unsaturated fatty acid at either position 1 or 2 are fully active in this capacity, irrespective of the chain length of the other fatty acyl moiety in the range tested, C_2 to C_{18}. Diacylglycerols containing two saturated fatty acids such as dipalmitin and distearin are far less effective. Mono- and triacylglycerols and free fatty acids are totally inactive, indicating that the diacylglycerol structure is essential.

It has been well established that Ca^{2+} plays roles in various cellular activations by a wide variety of extracellular signals such as hormones and neurotransmitters (for reviews see Refs. *1* and *2*). In general, such extracellular signals that increase cytosolic Ca^{2+} concentrations stimulate phospha-

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tidylinositol turnover in their target cell membranes (for reviews see Refs. *3–5*). Preceding reports from this laboratory described a new species of multifunctional protein kinase which can be activated by the simultaneous presence of Ca^{2+}, phospholipid, and diacylglycerol (*6–10*). This diacylglycerol appears to be derived from phosphatidylinositol during its receptor-linked turnover. Kinetic analysis has revealed that diacylglycerol activates the enzyme by increasing its affinity for Ca^{2+} as well as for phospholipid (*8*). The present studies were undertaken to explore the structural specificity of fatty acyl moieties esterified to glycerol with various synthetic products. The protein kinase will be referred to hereafter as protein kinase C.

EXPERIMENTAL PROCEDURE

Materials and Chemicals—Protein kinase C was partially purified from rat brain as described previously (*11*). Phospholipid was extracted from bovine brain with chloroform–methanol (2 : 1) by the method of Folch *et al.* (*12*) and fractionated by silicic acid column chromatography as described by Rouser *et al.* (*13*). Diacylglycerols containing oleic acid at position 1 and various fatty acids at position 2 were synthesized by the method of Buchnea (*14*). An outline of this procedure is shown in Fig. 1. For this procedure, 1-monoolein was employed as a starting material. The monoacylglycerol was dissolved in a mixture of anhydrous pyridine and benzene (1 : 1). A solution of triphenylmethyl chloride in benzene was added under strictly anhydrous conditions. After the reaction mixture had been kept at 40°C for 24 h, the reaction product, 1-monoacyl-3-triphenylmethylglycerol (I), was isolated. This product was then acylated with the desired fatty acid chloride at the free 2-hydroxy group of the molecule. The resulting 1,2-diacylglycerol (III) was isolated and purified by chromatography on a silicic acid-boric acid column, which also removed the protective triphenylmethyl group. Diacylglycerols containing oleic acid at position 2 and various acyl moieties at position 1 were similarly synthesized. Other diacylglycerols, tri- and monoacylglycerols, and free fatty acids employed were obtained from commercial sources. All synthetic products were chromatographically pure. Calf thymus H1 histone was prepared as described earlier (*15*). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (*16*).

Protein Kinase Assay—Protein kinase C was assayed with a combination of unfractionated phospholipid and various synthetic diacylglycerols by measuring the incorporation of radioactive phosphate into H1 histone from [γ-^{32}P]ATP. All reactions were carried out in plastic tubes. The standard reaction mixture (0.25 ml) contained 5 μmol of Tris-HCl, pH 7.5, 1.25 μmol of magnesium nitrate, 50 μg of H1 histone, 2.5 nmol of [γ-^{32}P]-ATP (1×10^5 cpm/nmol), 0.5 μg of protein kinase C, 4 μg of unfractionated phospholipid, and various concentrations of Ca^{2+} and the diacylglycerol to be assayed. All reagents except $CaCl_2$ were taken up in water which was prepared with a double distillation apparatus followed by passing through a Chelex-100 column to remove as much Ca^{2+} as possible, as described previously (*9*). Phospholipid was first mixed with diacylglycerol in a small volume of chloroform. After the

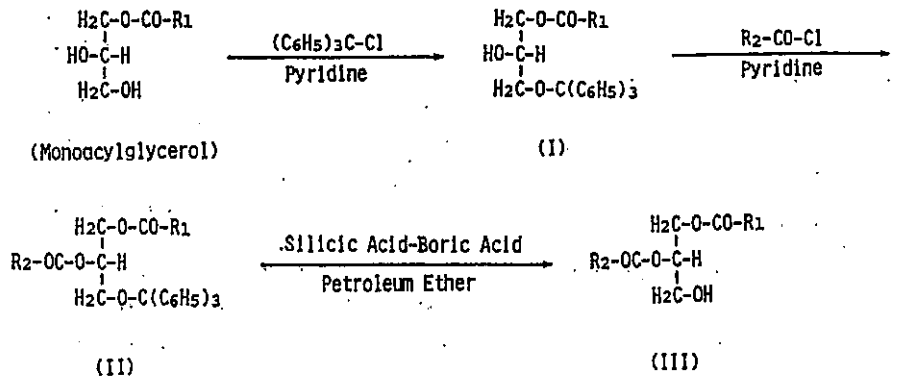

Fig. 1. An outline of diacylglycerol synthesis. R1-CO- and R2-CO- indicate oleoyl and saturated fatty acyl moieties, respectively.

chloroform was removed *in vacuo*, the mixture was suspended in 20 mM Tris-HCl at pH 7.5 by sonication as described previously (*8*), and then subjected to the assay. Incubation was carried out for 3 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable radioactive materials were collected on a Toyo-Roshi membrane filter.

Determinations—The radioactivity of ^{32}P-samples was determined with a Nuclear Chicago Geiger Muller gas flow counter, Model 4338. Protein was determined by the method of Lowry *et al.* (*17*) with bovine serum albumin as a reference protein.

RESULTS AND DISCUSSION

When protein kinase C was assayed in the presence of phospholipid alone, relatively higher concentrations of Ca^{2+} were needed and the reaction velocity was slow. If, however, small amount of diolein was added to the reaction mixture, the enzymatic activity was markedly enhanced with a concomitant decrease in the K_a value for Ca^{2+}, the concentration of Ca^{2+} needed for the half maximum activation of the enzyme. Kinetic analysis indicated that diolein greatly increased the apparent affinity of the enzyme for Ca^{2+} as well as for phospholipid, and thereby activated the enzyme, particularly at lower concentrations of this divalent cation. The experiment shown in Table I indicated that essentially similar effects were observed for unsaturated diacylglycerols including dilinolein and diarachidonin but not for saturated diacylglycerols such as dipalmitin and distearin as described previously (*8*, *9*). It was noted that neither mono- nor triacylglycerols were active in supporting enzymatic activity irrespective of the fatty acyl moieties. Further analysis shown in Fig. 2 with various synthetic diacylglycerols

TABLE I. Effects of various neutral lipids on the K_a values for Ca^{2+} and reaction velocities of protein kinase C. Protein kinase C was assayed with 0.8 μg/ml each of various neutral lipids and various concentrations of $CaCl_2$ under the standard conditions, and the K_a values for Ca^{2+} were obtained. Each sample of diacylglycerols was a mixture of 1,2- and 1,3-diacyl derivatives, and that of monoacylglycerols was a mixture of 1- and 2-monoacyl derivatives.

Neutral lipid	K_a for Ca^{2+}	Protein kinase activity at 4×10^{-6} M $CaCl_2$
	(μM)	(^{32}P incorporated, pmol/min)
None	90	5
Triolein	90	6
Diolein	8	29
Monoolein	90	5
Tripalmitin	90	7
Dipalmitin	50	10
Monopalmitin	90	6
Distearin	90	5
Dilinolein	7	33
Diarachidonin	4	31

TABLE II. Effects of various diacylglycerols on the K_a values for Ca^{2+} and reaction velocities of protein kinase C. The K_a values for Ca^{2+} and protein kinase activities at 4×10^{-6} M $CaCl_2$ were obtained from the data in Fig. 1. All protein kinase activities were assayed with 0.8 μg/ml of the diacylglycerol indicated.

Diacylglycerol R1-CO-[a]	R2-CO-[a]	K_a for Ca^{2+}	Protein kinase activity at 4×10^{-6} M $CaCl_2$
		(μM)	(^{32}P incorporated, pmol/min)
None		90	4
$C_{18:1}$	$C_{18:1}$	8	26
$C_{18:1}$	$C_{18:0}$	2	53
$C_{18:1}$	$C_{12:0}$	2	46
$C_{18:1}$	$C_{10:0}$	1	48
$C_{18:1}$	$C_{8:0}$	1	47
$C_{18:1}$	$C_{6:0}$	1	50
$C_{18:1}$	$C_{4:0}$	1	45
$C_{18:1}$	$C_{2:0}$	1	42
$C_{18:0}$	$C_{18:1}$	8	28
$C_{12:0}$	$C_{18:1}$	4	37
$C_{10:0}$	$C_{18:1}$	3	40
$C_{8:0}$	$C_{18:1}$	4	38
$C_{6:0}$	$C_{18:1}$	<1	58
$C_{4:0}$	$C_{18:1}$	1	47
$C_{2:0}$	$C_{18:1}$	1	44

[a] R1-CO- and R2-CO- indicate acyl moieties esterified at position 1 and 2 of glycerol, respectively.

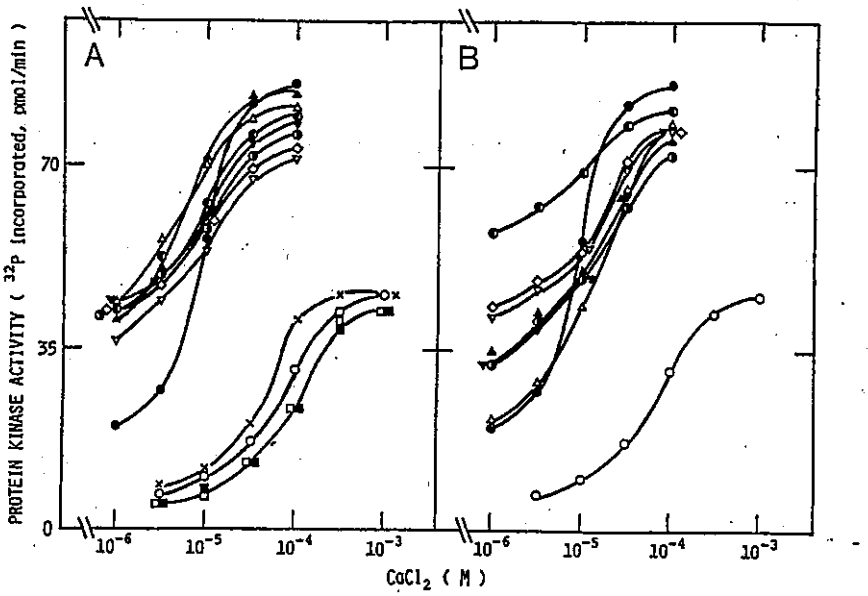

Fig. 2. Effects of various synthetic diacylglycerols on reaction velocities of protein kinase C at various concentrations of $CaCl_2$. Protein kinase was assayed with 0.8 μg/ml each of various diacylglycerols at various concentrations of $CaCl_2$ under the standard conditions. *A*: ○, phospholipid alone; ●, phospholipid plus DG ($C_{18:1}$ $C_{18:1}$); △, phospholipid plus DG ($C_{18:1}$ $C_{18:0}$); ▼, phospholipid plus DG ($C_{18:1}$ $C_{12:0}$); ▲, phospholipid plus DG ($C_{18:1}$ $C_{10:0}$); ◑, phospholipid plus DG ($C_{18:1}$ $C_{8:0}$); ◐, phospholipid plus DG ($C_{18:1}$ $C_{6:0}$); ◇, phospholipid plus DG ($C_{18:1}$ $C_{4:0}$); ▽, phospholipid plus DG ($C_{18:1}$ $C_{2:0}$); □, phospholipid plus DG ($C_{18:0}$ $C_{18:0}$); ■, phospholipid plus triolein; ×, phospholipid plus monoolein. *B*: ○, phospholipid alone; ●, phospholipid plus DG ($C_{18:1}$ $C_{18:1}$); △, phospholipid plus DG ($C_{18:0}$ $C_{18:1}$); ▼, phospholipid plus DG ($C_{12:0}$ $C_{18:1}$); ▲, phospholipid plus DG ($C_{10:0}$ $C_{18:1}$); ◑, phospholipid plus DG ($C_{8:0}$ $C_{18:1}$); ◐, phospholipid plus DG ($C_{6:0}$ $C_{18:1}$); ◇, phospholipid plus DG ($C_{4:0}$ $C_{18:1}$); ▽, phospholipid plus DG ($C_{2:0}$ $C_{18:1}$). DG indicates diacylglycerol.

showed that diacylglycerols containing one unsaturated fatty acid at either position 1 or 2 were all able to activate protein kinase C. Namely, diacylglycerols with oleic acid at one position and saturated fatty acids of various chain lengths from C_2 to C_{18} at the other position were similarly or even more effective than diolein. In the absence of phospholipid none of these diacylglycerols listed was active in supporting the activation of protein kinase C in a wide range of Ca^{2+} concentrations. The K_a values for Ca^{2+} and relative reaction velocities in the presence of various diacylglycerols are summarized in Table II. Free unsaturated fatty acids so far tested were all ineffective.

The results briefly presented above indicate that protein kinase C appears to distinguish specifically the diacylglycerol structure from other neutral lipids, and that only one unsaturated fatty acyl moiety esterified to glycerol seems to play a role of crucial importance in the enzyme activation. Apparently, unsaturated fatty acyl moieties esterified at position 1 and 2 are equally active, and the enzyme does not appear to recognize the structure of the second fatty acyl moiety. However, the unsaturated diacylglycerol does not serve simply as an allosteric activator of the enzyme, and kinetic analysis indicates that it sharply increases the affinity of the enzyme for Ca^{2+} as well as for phospholipid, both of which are indispensable for enzyme activation. Preliminary analysis has revealed that the enzyme is composed of at least two distinct domains; one is a hydrophobic phospholipid-binding domain and the other is a

hydrophilic catalytic domain (unpublished observation). It is likely, therefore, that the unsaturated fatty acyl moiety of diacylglycerol may be intercalated into the membrane phospholipid bilayer and seriously modulates it structure making the specific protein-lipid interaction more feasible. However, under physiological conditions, it is possible that this hydrophobic interaction may be modified more finely depending upon the structure of the unsaturated fatty acyl moiety esterified to glycerol. The detailed specificity of the unsaturated fatty acyl moiety and a more precise picture of this protein-lipid interaction remain for further investigations.

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Direct Activation of Calcium-activated, Phospholipid-dependent Protein Kinase by Tumor-promoting Phorbol Esters*

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Tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) directly activate *in vitro* Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C), which normally requires unsaturated diacylglycerol. Kinetic analysis indicates that TPA can substitute for diacylglycerol and greatly increases the affinity of the enzyme for Ca^{2+} as well as for phospholipid. Under physiological conditions, the activation of this enzyme appears to be linked to the receptor-mediated phosphatidylinositol breakdown which may be provoked by a wide variety of extracellular messengers, eventually leading to the activation of specific cellular functions or proliferation. Using human platelets as a model system, TPA is shown to enhance the protein kinase C-specific phosphorylation associated with the release reaction in the total absence of phosphatidylinositol breakdown. Various phorbol derivatives which have been shown to be active in tumor promotion are also capable of activating this protein kinase in *in vitro* systems.

Although the cellular targets for the action of tumor-promoting phorbol esters have not been definitely identified, studies in cell culture systems strongly suggest that 12-*O*-tetradecanoylphorbol-13-acetate may act directly on cell surface membranes (for review, see Ref. 1). One of the earliest biological effects of phorbol esters is the induction of platelet aggregation associated with release reaction (2-5), and the structural requirements of tigliane-type diterpenes for tumor promotion appear to be similar to those for platelet activation (3, 4). A series of recent reports from this laboratory (6-8) has shown that a Ca^{2+}-activated, phospholipid-dependent protein kinase is activated by unsaturated diacylglycerol which may be transiently formed during the receptor-mediated turnover of phosphatidylinositol. It is suggestive that in human platelets the enzyme activated in this way plays roles in serotonin release, presumably through the phosphorylation of one protein having $M_r \sim 40{,}000$ (9, 10). This protein kinase is present in a wide variety of tissues, and shows apparently neither tissue nor species specificity (11, 12). The enzyme requires absolutely Ca^{2+} and phospholipid, particularly phosphatidylserine for its activation (7, 13). Kinetic analysis indicates that diacylglycerol sharply increases the affinity of enzyme for Ca^{2+} as well as for phospholipid, and thus initiates the selective activation of this protein kinase (7, 8). We wish to describe here that in human platelets tumor-promoting phorbol esters such as TPA[1] can substitute for unsaturated diacylglycerol and, thus activate the protein kinase directly without provoking phosphatidylinositol turnover. Ca^{2+}-activated, phospholipid-dependent protein kinase and cyclic AMP-dependent protein kinase will be referred to as protein kinase C and protein kinase A, respectively.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Protein kinase C and Ca^{2+}-dependent protease were prepared from soluble fraction of rat brain as described previously (14). The catalytic fragment (protein kinase M) of protein kinase C was prepared by limited proteolysis with Ca^{2+}-dependent protease under the conditions specified earlier (14). Rabbit muscle glycogen phosphorylase kinase was prepared by the method of Cohen (15). Rabbit muscle protein kinase A was prepared as described previously (16). These enzyme preparations were free of each other and of endogenous phosphate acceptor proteins. A mixture of phospholipids used for the present studies was extracted from bovine brain by the method of Folch *et al.* (17) and fractionated on a silicic acid column as described by Rouser *et al.* (18). Human platelet-rich plasma and washed platelets were prepared by the method of Baenziger and Majerus (19). TPA and other phorbol derivatives were obtained from P. Borchert, Eden Praire, MN. Diolein and dimethyl sulfoxide were purchased from Nakarai Chemicals. [^{3}H]Arachidonic acid (78.2 Ci/mmol) and [^{14}C]serotonin (58 mCi/mmol) were obtained from New England Nuclear and Amersham, respectively. Bovine thrombin was obtained from Mochida Pharmaceutical Co. [γ-^{32}P]ATP, calf thymus H1 histone, and other materials and chemicals were prepared as described earlier (6, 8).

Enzyme Assays—Protein kinase C was assayed by measuring the incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP. The standard 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 to 15 $\times$ 10^4 cpm/nmol), and 0.5 μg of protein kinase C. Phospholipid, diolein, phorbol esters, and Ca^{2+} were added as indicated in each experiment. All reagents were taken up in water which was prepared by a double distillation apparatus followed by passing through a Chelex 100 column to remove as much Ca^{2+} as possible as specified earlier (8). All reactions were carried out in plastic tubes. After incubation for 3 min at 30 °C, the reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials

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[1] The abbreviation used is: TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). The catalytic fragment of protein kinase C was assayed similarly except that Ca^{2+}, phospholipid, and diolein were omitted. Protein kinase A was assayed under similar conditions except that 250 pmol of cyclic AMP was added instead of Ca^{2+}, phospholipid, and diolein. Glycogen phosphorylase kinase was assayed by measuring the incorporation of ^{32}P into phosphorylase from [γ-^{32}P]ATP as specified earlier (20). Ca^{2+}-dependent protease was assayed with ^{125}I-labeled casein as a substrate (21).

Assay for Platelet Protein Phosphorylation—The washed platelets (4×10^9 cells) were labeled with 1 mCi of carrier-free $^{32}P_i$ in 2 ml of Buffer A (0.14 M NaCl, 15 mM Tris/HCl at pH 7.5, and 5.5 mM glucose) as described by Lyons *et al.* (22). The radioactive platelets (6×10^8/ml) were then stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of a half volume of a stop solution which contained 9% sodium dodecyl sulfate, 6% 2-mercaptoethanol, 15% glycerol, 0.186 M Tris/HCl at pH 6.7. The sample was boiled in a water bath for 3 min, and subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under the conditions described by Laemmli (23). The separating and stacking gels contained 11 and 3% acrylamide, respectively. The gel was stained with Coomassie brilliant blue. After destaining, the gel was dried on a Whatman No. 1 filter paper, and exposed to an x-ray film to prepare the autoradiograph. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph using a Shimadzu dual wavelength chromatogram scanner, Model CS-910.

Assays for Diacylglycerol Formation and ^{32}P Incorporation into Phospholipid—The platelet-rich plasma (36 ml) was incubated with 25 μCi of [^{3}H]arachidonic acid as described by Rittenhouse-Simmons (24), and platelets were isolated and washed as described (19). The radioactive platelets thus obtained were suspended in Buffer A (6×10^8 cells/ml) and stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of chloroform/methanol (1:2) and the radioactive lipid was extracted by the method of Bligh and Dyer (25). Diacylglycerol was separated from the other lipids by Silica Gel G plate thin layer chromatography with a solvent system of benzene/diethylether/ethanol/ammonia water (50:40:20:0.1). The area corresponding to diacylglycerol was scraped into a vial and the radioactivity was determined.

In another set of experiments to measure phosphatidylinositol turnover, the platelets which were separately labeled with $^{32}P_i$ were suspended in Buffer A (6×10^8 cells/ml), and stimulated by thrombin or TPA. At various periods of time, the incubation was terminated by the addition of chloroform/methanol (1:2). Phospholipids were then extracted and isolated by Silica Gel G plate thin layer chromatography with a solvent system of chloroform/methanol/acetic acid/H_2O (25:15:4:2). The areas corresponding to each phospholipid were scraped into a vial and the radioactivity was determined.

Assay for Serotonin Release—The platelet-rich plasma (20 ml) was incubated with 1 μCi of [^{14}C]serotonin as described by Haslam and Lynham (26), and platelets were isolated and washed as described (19). The radioactive platelets thus obtained were suspended in Buffer A (6×10^8 cells/ml) and stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of formaldehyde followed by centrifugation at 10,000 × *g* for 40 s by the method of Costa and Murphy (27). The radioactive serotonin released was determined.

Determinations—The radioactivity of ^{32}P-, ^3H-, and ^{14}C-labeled samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320. Protein was determined by the method of Lowry *et al.* (28) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Among various lipids tested so far, including monoacyl-, diacyl-, and triacylglycerols and free fatty acids, only unsaturated diacylglycerol was effective in the activation of protein kinase C (7, 8). However, it was found that, when TPA instead of unsaturated diacylglycerol was directly added to the reaction mixture, the enzymatic activity was greatly enhanced with the concomitant decrease in the Ca^{2+} concentration that was necessary for enzyme activation as shown in Fig. 1. Phospholipid was indispensable, and TPA alone was unable to activate the enzyme. Kinetic analysis indicated that TPA, like unsaturated diacylglycerol (7, 8), greatly increased the

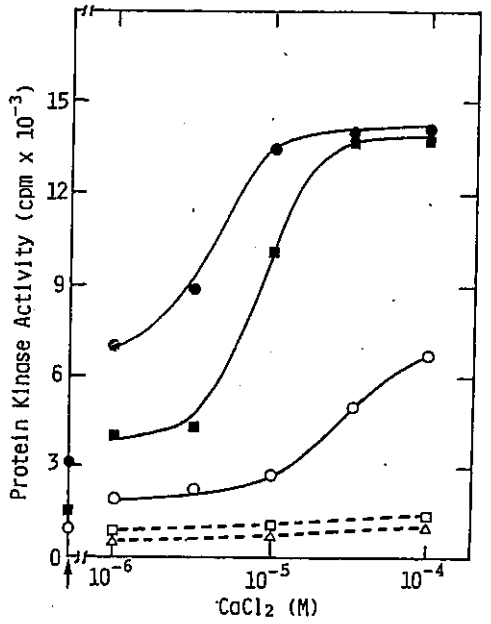

FIG. 1. **Activation of protein kinase C by TPA and unsaturated diacylglycerol at various concentrations of $CaCl_2$.** Protein kinase C was assayed under the standard conditions in the presence of $CaCl_2$ as indicated. TPA, diolein, and phospholipid were added as specified. Diolein, phospholipid, or both were dissolved 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 as described (7). TPA, which was dissolved in dimethyl sulfoxide, was directly mixed with phospholipid suspended in the buffer before being added to the reaction mixture. The final concentration of dimethyl sulfoxide in the reaction mixture was 0.01%. Where indicated with an *arrow*, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (0.5 mM at a final concentration) was added instead of $CaCl_2$. ○——○, in the presence of 20 μg/ml of phospholipid alone; □- - -□, in the presence of 10 ng/ml of TPA alone; △- - -△, in the presence of 0.8 μg/ml of diolein alone; ●——●, in the presence of 20 μg/ml of phospholipid plus 10 ng/ml of TPA; ■——■, in the presence of 20 μg/ml of phospholipid plus 0.8 μg/ml of diolein.

apparent affinity of enzyme for Ca^{2+} as well as for the phospholipid, and thus enhanced the enzyme activation. Namely, in the presence of phospholipid alone, relatively higher concentrations of Ca^{2+} were needed irrespective of the amount of phospholipid present, and the reaction velocity was accelerated by increasing amounts of phospholipid employed. When a saturating amount of phospholipid (about 100 μg/ml) was added, full enzymatic activity was obtained even though the K_a value for Ca^{2+}, the concentration needed for half-maximum activation, remained higher (about 7×10^{-5} M). If, however, a small amount of either TPA or diolein was supplemented to phospholipid, the K_a value for Ca^{2+} was dramatically decreased to be 10^{-6} M range. For instance, in the presence of TPA (10 ng/ml) or diolein (0.8 μg/ml) in addition to phospholipid (20 μg/ml), approximate K_a values of 2×10^{-6} and 8×10^{-6} M were obtained for this divalent cation, respectively; here diolein was not saturated. With saturating amount of TPA (more than 10 ng/ml) or diolein (more than 1.5 μg/ml), the same Ca^{2+} titration curves were obtained for these activators. In addition, TPA and diolein did not act as synergistic allies, and in the presence of a saturating amount of one of these two activators, no further enhancement of the reaction was observed by the addition of the other. However, at submaximal concentrations, the effects of TPA and diolein were apparently additive.

It has been described previously (14) that protein kinase C may alternatively be activated through limited proteolysis by Ca^{2+}-dependent neutral protease. The enzyme activated in

this way (protein kinase M) was catalytically fully active in the absence of Ca^{2+}, phospholipid, and diacylglycerol and was not susceptible to TPA. The result seems to indicate that the tumor promoter does not interact with the catalytically active site of the enzyme. Rather, it is suggestive that TPA may associate with lipid lamellae or micelles and modify the phospholipid-enzyme interaction to express full catalytic activity at physiologically lower concentrations of Ca^{2+}. Ca^{2+}-dependent neutral protease was not affected by TPA as assayed with casein as a substrate. Experiments shown in Fig. 2 indicated that low concentrations of TPA in the order of nanograms/ml showed significant effects; this tumor promoter at an amount of roughly one- to five-thousandths of that of diacylglycerol fully activated the protein kinase *in vitro*. Dimethyl sulfoxide itself showed practically no effect at the concentrations employed in these experiments. In similar *in vitro* systems, neither protein kinase A nor calmodulin-dependent protein kinase such as glycogen phosphorylase kinase was affected by TPA.

The next set of experiments was conducted to examine whether in intact cells TPA activates directly protein kinase C and causes some cellular response in an analogous manner to receptor-linked natural extracellular messengers. For this purpose, human platelets were employed. Preceding reports from this laboratory (9, 10) have proposed that in thrombin-stimulated platelets protein kinase C is activated by diacylglycerol which is derived from the receptor-linked breakdown of phosphatidylinositol and that the enzyme thus activated is probably responsible for the release of serotonin. In the experiment given in Fig. 3, washed human platelets were preincubated with $^{32}P_i$, and then stimulated by either thrombin or TPA. Consistent with the recent observations made by Chiang *et al.* (5), when platelets were activated by TPA, some endogenous platelet proteins were rapidly phosphorylated; in the present experiment, most predominantly 40-kilodalton protein and to some extent another protein having $M_r \sim 20{,}000$ were labeled. It has been described earlier (9) that 40-kilodalton protein serves as a preferred substrate for protein kinase C *in vitro* and that the phosphorylation of this particular protein is most likely related to release reaction.[2] In fact, in all experiments thus far done with intact platelets, the diacylglycerol formation that was induced either by thrombin (Fig. 4*A*) or by exogenously added phospholipase C (9) was always associated with 40-kilodalton protein phosphorylation as well as with serotonin release. On the other hand, 20-kilodalton protein has been identified as myosin light chain, and another species of protein kinase, that is Ca^{2+}-calmodulin-regulated myosin light chain kinase, has been proposed to be responsible for the phosphorylation of this protein (30, 31). It is evident from the autoradiograph that 20-kilodalton protein was phosphorylated only slightly, when platelets were stimulated by TPA. It is likely that Ca^{2+} influx or movement may be limited at least in the early phase of the TPA-induced platelet activation. The rapid disappearance of diacylglycerol shown in Fig. 4*A* was probably due to the conversion to phosphatidic acid and also to further degradation to arachidonic acid and its metabolites.

In a marked contrast to thrombin, TPA induced serotonin release in parallel with 40-kilodalton protein phosphorylation, but did not produce diacylglycerol under similar conditions as shown in Fig. 4*B*. In the experiments shown in Fig. 4, *A* and *B*, however, the rates and extents of the release of serotonin were different, although the extents of 40-kilodalton protein phosphorylation in both systems were roughly the same. The reason for this difference is not known, but it is possible that Ca^{2+} plays some roles in the secretory process of serotonin. Another possibility which may not be ruled out is that diacylglycerol has additional roles during the platelet activation. For instance, diacylglycerol is known as a membrane fusigen (32, 33) and also serves as a precursor to ionophoric phosphatidic acid (for review, see Ref. 34) as well as to arachidonic

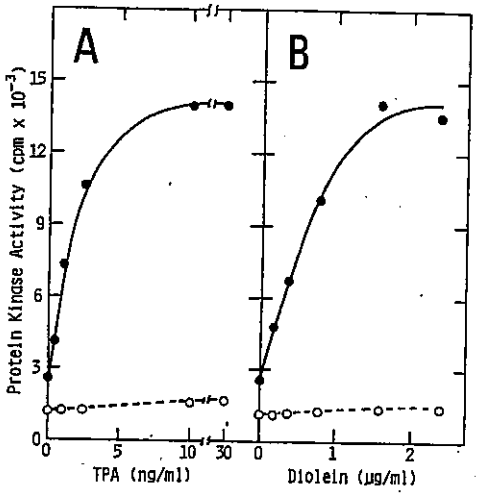

FIG. 2. **Dose-dependent activation of protein kinase C by TPA and diolein.** Protein kinase C was assayed under the standard conditions in the presence of 20 μg/ml of phospholipid, 1×10^{-5} M $CaCl_2$, and various amounts of either TPA or diolein as indicated. *A*, with TPA; *B*, with diolein. ●——●, in the presence of phospholipid plus $CaCl_2$; O- - -O, in the presence of $CaCl_2$ and without phospholipid. The enzymatic activity obtained in the presence of phospholipid and without $CaCl_2$ was nearly the same as that obtained in the presence of $CaCl_2$ and without phospholipid (data not shown).

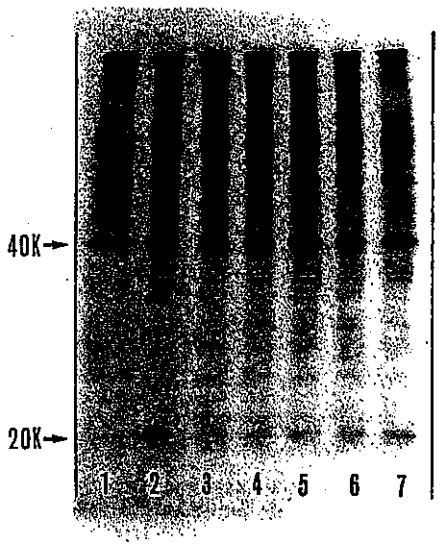

FIG. 3. **Autoradiograph of TPA-induced platelet protein phosphorylation.** The platelets, which were labeled with $^{32}P_i$, were stimulated at 37 °C by 0.25 unit/ml of thrombin or by 100 ng/ml TPA for various periods of time as indicated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were done as described under "Experimental Procedures." *Lane 1*, control; *Lane 2*, with thrombin for 60 s; *Lane 3*, with TPA for 10 s; *Lane 4*, with TPA for 30 s; *Lane 5*, with TPA for 60 s; *Lane 6*, with TPA for 90 s; *Lane 7*, with TPA for 120 s.

[2] This 40-kilodalton protein corresponds to the 47-kilodalton protein designated by other workers (for review, see Ref. 29). It is noted that the sites of phosphorylation in the purified *in vitro* 40-kilodalton protein-protein kinase C system are shown to be identical with those in the thrombin-stimulated platelet system *in vivo* as judged by fingerprint analysis (manuscript in preparation).

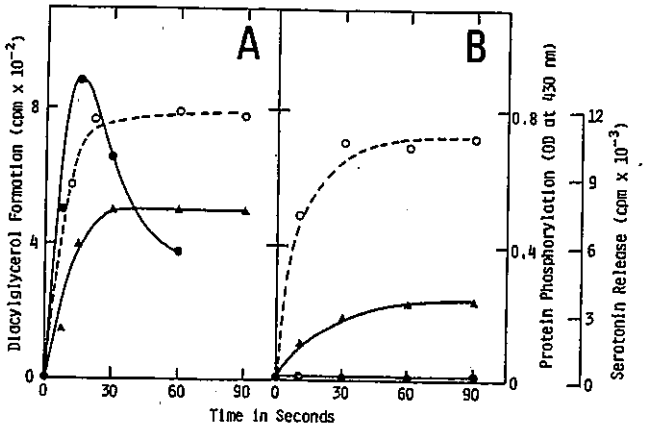

FIG. 4. **Time courses of diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release in platelets.** The platelets, which were labeled with either [^{3}H]arachidonic acid, $^{32}P_i$, or [^{14}C]serotonin, were stimulated at 37 °C by 0.25 unit/ml of thrombin or by 100 ng/ml of TPA for various periods of time as indicated. Diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release were assayed as described under "Experimental Procedures." *A*, with thrombin; *B*, with TPA. ●——●, diacylglycerol formation; O- - -O, 40-kilodalton protein phosphorylation; ▲——▲, serotonin release.

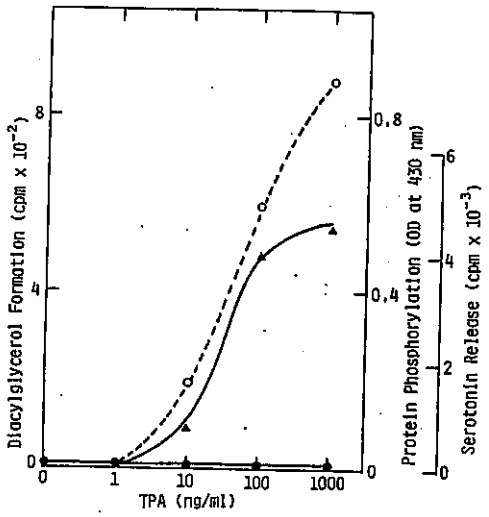

FIG. 5. **Effect of TPA concentration on diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release in platelets.** The platelets, which were labeled with either [^{3}H]arachidonic acid, $^{32}P_i$, or [^{14}C]serotonin, were stimulated for 1 min at 37 °C by various amounts of TPA as indicated. Diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release were assayed as described under "Experimental Procedures." The *symbols* are the same as those given in Fig. 4.

acid which is rapidly converted to thromboxane (35). Fig. 5 shows dose responses to TPA for such 40-kilodalton protein phosphorylation and release reaction. It is shown that the concentration of TPA necessary for platelet activation was roughly 10 times higher than that required for protein kinase C activation *in vitro*. Presumably, in intact platelets, more TPA is necessary to intercalate into the lipid bilayer structure leading to the protein kinase activation. Again, essentially no diacylglycerol was produced over a wide range of TPA concentrations. The results seem to indicate that this tumor promoter directly activates protein kinase C without provoking phosphatidylinositol breakdown. Further evidence supporting this assumption was provided by the fact that the incorporation of radioactive inorganic phosphate into phosphatidylinositol and phosphatidic acid (phosphatidylinositol turnover) was markedly accelerated by thrombin but not by TPA, although 40-kilodalton protein was actively phosphorylated and serotonin was released under the same conditions (data not shown). It has been described that TPA enhances the incorporation of radioactive choline into phosphatidylcholine in mouse epidermis (36) and bovine lymphocytes (37) several minutes or hours after stimulation, presumably as a result of increased membrane perturbation. However, in human platelets which were stimulated by TPA, the degradation of phosphatidylcholine as well as the incorporation of ^{32}P into this phospholipid was negligible at least at early phase of the platelet activation. The activation of platelets by TPA was usually completed within 1 min, and the appearance of lysophospholipids, if any, was negligible under the present conditions. Using human neutrophils (38), it has been recently reported that TPA shows no effect on arachidonate release at doses which maximally stimulate both degranulation and oxidative metabolism. Probably, the effect of TPA on phosphatidylcholine metabolism may largely depend on cell types employed and/or may be observed at a later phase of TPA actions. The detailed metabolic cascade of various phospholipids that might occur after the addition of TPA remains to be explored.

Table I shows relative activities of various phorbol derivatives to activate protein kinase C in *in vitro* systems. It was noted that phorbol derivatives showing tumor-promoting activity could activate protein kinase C as well. The structural requirements of phorbol-related diterpenes for tumor promotion on mouse skin (for review, see Ref. 39) appear to be roughly similar to those for protein kinase C activation.

A number of kinetic studies using a variety of cell systems appear to suggest that the biochemical target of phorbol esters leading to activation of specific cellular functions or proliferation may be located on membranes. The pleiotropic actions as well as the structure-activity relations of these tumor promoters seem to be compatible with the supposition that there is a specific cell surface receptor that is widespread on various tissues and organs. The results presented above seem to indicate that one of the possible targets of TPA actions is protein kinase C, although it is not known at present whether the activation of this enzyme is directly related to the mechanism involved in the tumor promotion. Under normal conditions, the activation of this enzyme appears to be induced by a large number of hormones, neurotransmitters, and many other biologically active substances including epidermal growth factor and lymphocyte mitogens of plant origin, which are all able to provoke phosphatidylinositol turnover (34). However, this may not necessarily rule out a role of protein kinase C in the tumor promotion. It is possible that the tumor-

TABLE I

Effects of various phorbol derivatives on activation of protein kinase C in vitro

Protein kinase C was assayed under the standard conditions in the presence of 20 μg/ml of phospholipid, 1×10^{-5} M $CaCl_2$, and 10 ng/ml each of various phorbol derivatives.

Phorbol derivative	Protein kinase C activity
	%
TPA	100
Phorbol-12,13-didecanoate	81
Phorbol-12,13-dibutyrate	88
Phorbol-12,13-dibenzoate	100
Phorbol-12-tetradecanoate	0
Phorbol-13-acetate	0
4α-Phorbol-12,13-didecanoate	0
Phorbol	0

promoting phorbol ester once intercalated into membranes remains active for prolonged periods of time, since the diterpene is hardly metabolizable (1). In contrast, diacylglycerol, the natural activator of this enzyme, occurs transiently during the phosphatidylinositol turnover and disappears very quickly. Nevertheless, possible roles of this protein kinase in the regulation which may be essential to the activation of specific functions or proliferation of mammalian cells remain largely unexplored.

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Communication

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Synergistic Functions of Protein Phosphorylation and Calcium Mobilization in Platelet Activation*

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When human platelets were stimulated by synthetic diacylglycerol such as 1-oleoyl-2-acetyl-glycerol, which was a potent activator *in vitro* of Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) (Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y., and Fujikura, T. (1982) *J. Biochem. (Tokyo)* 91, 427–431), a protein having M_r ~ 40,000 (40-kilodalton protein) was rapidly phosphorylated, just as it was by natural extracellular messengers such as thrombin. Fingerprint analysis appeared to indicate that protein kinase C was indeed responsible for this 40-kilodalton protein phosphorylation in intact platelets. Under these conditions, neither inositol phospholipid breakdown nor endogenous diacylglycerol formation was observed, indicating that the synthetic diacylglycerol intercalated into the membrane and directly activated protein kinase C without interaction with cell surface receptors. During this process, the diacylglycerol was converted *in situ* to the corresponding phosphatidate, 1-oleoyl-2-acetyl-glyceryl-3-phosphoric acid. Experiments with the synthetic diacylglycerol and Ca^{2+} ionophore A23187 suggested that the protein phosphorylation catalyzed by protein kinase C was a prerequisite requirement for the release of serotonin, and that the receptor-linked protein phosphorylation and Ca^{2+} mobilization acted synergistically to elicit the full physiological cellular response.

A wide variety of neurotransmitters, peptide hormones, secretagogues, and many other biologically active substances have been repeatedly shown to provoke inositol phospholipid turnover in their target tissues (see for reviews, Refs. 1–3). In general, the stimulation of most of these receptors immediately mobilizes Ca^{2+}, and this divalent cation appears to play crucial roles in the cellular response to these extracellular messengers. A series of recent studies in our laboratories has suggested that diacylglycerol derived from this inositol phospholipid breakdown is directly involved in the transmembrane control of protein phosphorylation through activation of Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) (4–9). Nevertheless, neither the relationship between the phospholipid degradation and Ca^{2+} mobilization nor the biological role of this receptor-linked protein phosphorylation has yet been definitely established. This communication will describe that under appropriate conditions a synthetic diacylglycerol exogenously added to intact platelets may intercalate into membranes and directly activates protein kinase C without inducing the phospholipid degradation and Ca^{2+} mobilization. Thus, using diacylglycerol and Ca^{2+} ionophore, it is possible to show that either the protein phosphorylation or Ca^{2+} mobilization alone is a prerequisite but not a complete requirement, and both are synergistically effective for causing the full physiological cellular response such as release of serotonin.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Human platelet-rich plasma and washed platelets were prepared by the method of Baenziger and Majerus (10). OAG[1] and AOG were synthesized as described previously (11). 1-Oleoyl-2-acetyl-*sn*-glyceryl-3-phosphoric acid was synthesized from 1-oleoyl-2-acetyl-*sn*-glycerol by the method of Lammers and Van Boom (12). These synthetic products were chromatographically pure. Bovine thrombin was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). A23187 was a product of Calbiochem. [^{3}H]Arachidonic acid (78.1 Ci/mmol), carrier-free $H_3{}^{32}PO_4$, and [^{14}C]serotonin (58 mCi/mmol) were obtained from New England Nuclear, Japan Radioisotope Association, and Amersham, respectively. [γ-^{32}P]ATP and calf thymus H1 histone were prepared as described earlier (13). Other materials and chemicals were obtained from commercial sources.

Assay for Lipid Metabolism—The platelet-rich plasma (36 ml) was labeled with 25 μCi of [^{3}H]arachidonic acid under the conditions described by Rittenhouse-Simmons (14), and platelets were isolated and washed as described (7–9). The radioactive platelets were stimulated by either thrombin, synthetic diacylglycerol, or A23187 as indicated in each experiment. The incubation was terminated by the addition of chloroform/methanol (1:2), and the radioactive lipids were directly extracted by the method of Bligh and Dyer (15). Phospholipids, diacylglycerol, and arachidonic acid metabolites were separated by Silica Gel G plate thin layer chromatography. The solvent systems employed were methyl acetate, *n*-propanol, chloroform, methanol, 0.25% aqueous KCl (25:25:25:10:9) for phospholipids; benzene/diethylether/ethanol/ammonia water (50:40:2:0.1) for diacylglycerol; and the top phase of ethylacetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10) for HETE and HHT. The area corresponding to each lipid was scraped into a vial, and the radioactivity was determined.

In another set of experiments, the washed platelets (4×10^8 cells) were labeled with 1 mCi of carrier-free $^{32}P_i$ under the conditions described by Lyons *et al.* (16). The radioactive platelets were then stimulated by thrombin or synthetic diacylglycerol and the reaction was terminated by the addition of chloroform/methanol (1:2). The radioactive phospholipids were directly extracted, and separated by two-dimensional Silica Gel G plate thin layer chromatography using

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[1] The abbreviations used are: OAG, 1-oleoyl-2-acetyl-glycerol; AOG, 1-acetyl-2-oleoyl-glycerol; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; SDS, sodium dodecyl sulfate.

chloroform, methanol, 7 N ammonia water (12:7:1) as a 1st dimension solvent system, and chloroform/methanol/acetic acid/water (25:15:4:2) as a 2nd dimension solvent system. Under these conditions, 1-oleoyl-2-acetyl-glyceryl-3-phosphoric acid was well separated from other phospholipids. The plate was then exposed to a Kodak Royal X-Omat film to prepare an autoradiograph.

Assay for Protein Phosphorylation—The washed platelets were labeled with $^{32}P_i$ as described above, and stimulated by either thrombin, synthetic diacylglycerol, or A23187 under the conditions specified in each experiment. The radioactive platelets were then directly subjected to SDS-polyacrylamide gel electrophoresis, stained, dried on a Whatman No. 1 filter paper, and exposed to a Kodak Royal X-Omat film to prepare an autoradiograph. The relative intensity of each band was quantitated by densitometric tracing at 430 nm using a Shimadzu dual wavelength chromatogram scanner, Model CS-910.

Assay for Serotonin Release—The platelet-rich plasma (20 ml) was incubated with 1 μCi of [^{14}C]serotonin as described by Haslam and Lynham (17), and platelets were isolated and washed as described earlier (7–9). The radioactive platelets were stimulated by either thrombin, synthetic diacylglycerol, or A23187. The incubation was terminated by formaldehyde followed by centrifugation at 10,000 × *g* for 40 s, and the radioactive serotonin released was determined as described by Costa and Murphy (18).

Other Procedures—Fingerprint analysis of the ^{32}P-labeled platelet proteins was carried out under the conditions described previously (8, 9). Two-dimensional mapping of the tryptic phosphopeptides was made by the method of Beemon and Hunter (19). Protein kinase C was routinely assayed with calf thymus H1 histone as a phosphate acceptor (20). Lactic dehydrogenase was assayed by the method of Kornberg (21). The radioactivity of ^{32}P-, ^{3}H-, and ^{14}C-labeled samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320.

RESULTS AND DISCUSSION

Protein kinase C absolutely requires Ca^{2+} and phospholipid for enzymatic activity, and it was shown that unsaturated diacylglycerol increased its affinity for Ca^{2+} dramatically to the 10^{-7} M range, and thereby rendered this enzyme fully active at physiologically lower concentrations of Ca^{2+} (5, 6, 22). It was also described that various synthetic diacylglycerols such as OAG, AOG, and diolein were equally active in this capacity when tested in *in vitro* enzymatic reactions (11). In the experiment shown in Fig. 1*A*, OAG was suspended in a small amount of 1% dimethylsulfoxide solution, sonicated to prepare micelles, and then directly added to intact platelets. It was found that an endogenous protein having an approximate molecular mass of 40,000 daltons (40-kilodalton protein) was rapidly phosphorylated just as it was by natural extracellular messengers. As shown in Fig. 1*B*, when platelets were stimulated by thrombin, 40-kilodalton protein was phosphorylated, and this reaction was preceded by transient formation of endogenous diacylglycerol. Quantitative analysis of various phospholipids suggested that this diacylglycerol was derived most likely from the receptor-linked breakdown of phosphatidylinositol as previously described (7–9, 14).[2] The rapid disappearance of the diacylglycerol was presumably due to its conversion to phosphatidylinositol by way of phosphatidate and also due to its further degradation to arachidonic acid for thromboxane synthesis. In a marked contrast to thrombin, OAG did not produce endogenous diacylglycerol nor did it induce phosphatidylinositol breakdown. There was no sign for arachidonic acid release or thromboxane synthesis, and neither HETE nor HHT was detected under these conditions. Dimethylsulfoxide alone showed no effect on platelets at the concentration employed for the present studies. The radioactive 40-kilodalton protein was isolated from the platelets which were stimulated by OAG and also from those stimulated

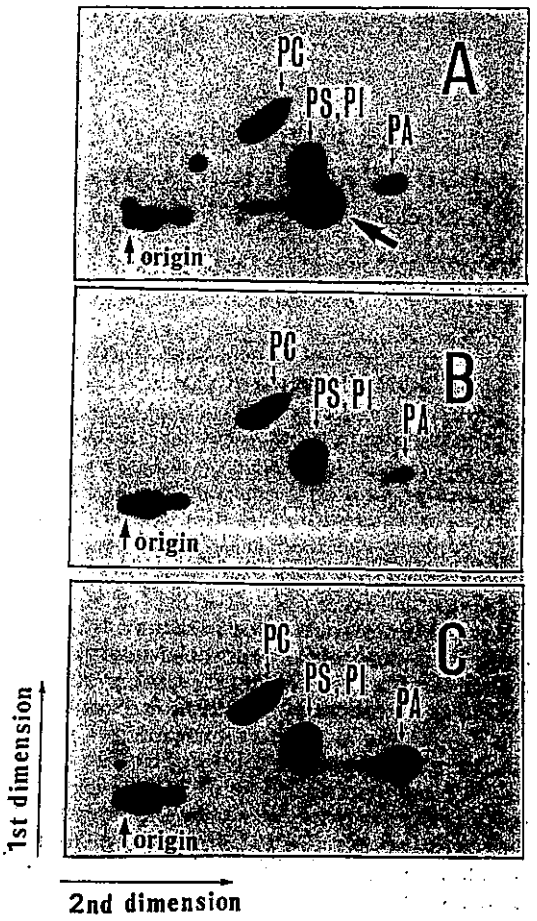

FIG. 2. **Autoradiograph of two-dimensional thin layer chromatograph of ^{32}P-labeled phospholipids of platelets.** The platelets prelabeled with $^{32}P_i$ were stimulated at 37 °C for 3 min by 50 μg/ml of OAG or 0.2 unit/ml of thrombin as indicated. The final concentration of dimethylsulfoxide was 0.1%. Thin layer chromatograph and autoradiograph were made as described under "Experimental Procedures." The spot shown by an *arrow* indicates 1-oleoyl-2-acetyl-glyceryl-3-phosphoric acid. *PC, PS, PI,* and *PA* indicate phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid, respectively. *A*, with OAG; *B*, control; *C*, with thrombin.

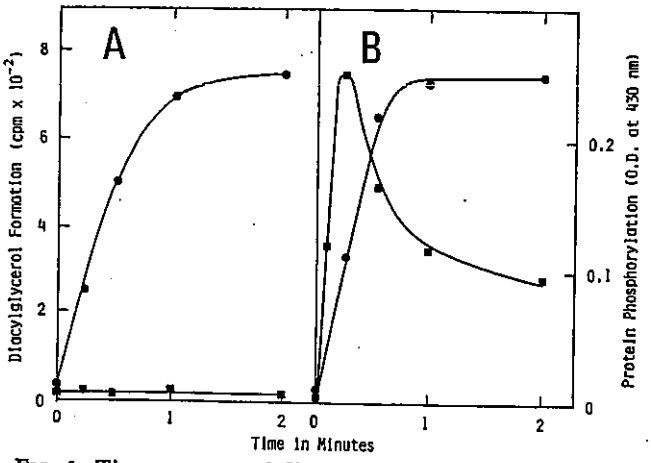

FIG. 1. **Time courses of diacylglycerol formation and 40-kilodalton protein phosphorylation in platelets.** The platelets, which were labeled with [^{3}H]arachidonic acid or $^{32}P_i$, were stimulated at 37 °C by 50 μg/ml of OAG or 0.2 unit/ml of thrombin for various periods of time indicated. The final concentration of dimethylsulfoxide was 0.1%. Diacylglycerol formation and 40-kilodalton protein phosphorylation were assayed as described under "Experimental Procedures." *A*, with OAG; *B*, with thrombin. ■——■, diacylglycerol formation; ●——●, 40-kilodalton protein phosphorylation.

[2] Polyphosphoinositides were not quantitated in the present studies.

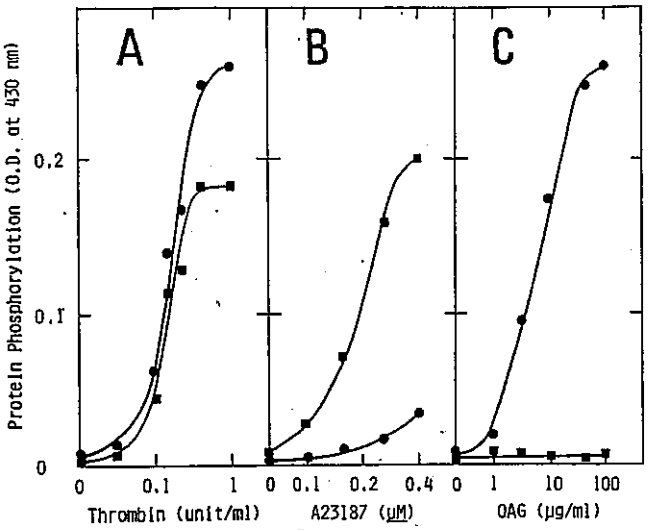

FIG. 3. **Effects of thrombin, A23187, and OAG concentrations on phosphorylation of 40-kilodalton and 20-kilodalton proteins.** The platelets prelabeled with $^{32}P_i$ were stimulated at 37 °C for 1 min by various amounts of either thrombin, A23187, or OAG. The final concentration of dimethylsulfoxide was fixed at 0.1%. The phosphorylation of 40-kilodalton and 20-kilodalton proteins was assayed as described under "Experimental Procedures." *A*, with thrombin; *B*, with A23187; *C*, OAG. ●——●, 40-kilodalton protein phosphorylation; ■- - -■, 20-kilodalton protein phosphorylation.

by thrombin using SDS-polyacrylamide gel electrophoresis, and both preparations were subjected to fingerprint analysis. The mapping patterns of the tryptic phosphopeptides thus obtained were identical with the pattern obtained from the 40-kilodalton protein preparation which was phosphorylated *in vitro* by a homogeneous preparation of protein kinase C in the presence of Ca^{2+}, phospholipid, and diacylglycerol.

The results presented above seem to indicate that OAG may intercalate into the phospholipid bilayer and directly activates protein kinase C without interaction with any of cell surface receptors. The observed effect of OAG did not appear to be simply due to the damage of platelet membranes, since cytoplasmic enzymes such as lactic dehydrogenase did not leak into the medium. It was noted that the exogenously added diacylglycerol was rapidly converted *in situ* to the corresponding phosphatidate, that is 1-oleoyl-2-acetyl-glyceryl-3-phosphoric acid, presumably by the action of diacylglycerol kinase. The large spot shown by an *arrow* in Fig. 2*A* was identified as such by comparison with an authentic sample of the synthetic product. This unique compound was not found in the resting platelets nor in the platelets stimulated by thrombin (Fig. 2, *B* and *C*). The rapid phosphorylation of the 40-kilodalton protein in platelets was also observed with AOG instead of OAG. Diacylglycerols possessing two long fatty acyl moieties such as diolein were practically ineffective to induce 40-kilodalton protein phosphorylation *in vivo*, presumably due to their inability to intercalate into the membrane, although this diacylglycerol was highly active to support enzymatic reaction *in vitro* as described earlier (5, 6, 11).

The next series of experiments was designed to examine if the protein phosphorylation catalyzed by protein kinase C or Ca^{2+} mobilization or both were essential for eliciting physiological response such as release of serotonin. It was repeatedly shown that, when platelets were activated by natural messengers such as thrombin, collagen, and platelet-activating factor, an additional protein having a molecular mass of 20,000 dalton (20-kilodalton protein) was also phosphorylated (7–9, 16, 17). This protein was identified as myosin light chain, and a specific calmodulin-dependent protein kinase was proposed to be responsible for its phosphorylation (23). In fact, as shown in Fig. 3*A*, thrombin induced the phosphorylation of both 40-kilodalton and 20-kilodalton proteins in a parallel manner, indicating that the natural messenger provoked inositol phospholipid breakdown as well as Ca^{2+} mobilization. If, however, platelets were stimulated by low concentrations of A23187, only 20-kilodalton protein was significantly phosphorylated as shown in Fig. 3*B*. This ionophore at lower concentrations did not induce 40-kilodalton protein phosphorylation nor produced endogenous diacylglycerol. On the other hand, OAG induced the phosphorylation of 40-kilodalton protein to the extent that was induced by thrombin, whereas 20-kilodalton protein was not phosphorylated under the conditions given in Fig. 3*C*. These results seem to provide the rationale that, under the given conditions, the activation of protein kinase C and mobilization of Ca^{2+} may be independently induced by the exogenous addition of the synthetic diacylglycerol and Ca^{2+} ionophore, respectively. In the experiments shown in Fig. 4, human platelets were stimulated by OAG in the presence or absence of a low concentration of A23187. It was found that the 40-kilodalton protein phosphorylation reaction proceeded by the addition of OAG irrespective of the presence and absence of the Ca^{2+} ionophore (Fig. 4*A*). In a marked contrast to this protein phosphorylation, serotonin was not sufficiently released by the addition of OAG alone, and the full physiological response was observed when both OAG and A23187 were added (Fig. 4*B*). This ionophore *per se* at the concentration employed (0.4 μM) did not induce endogenous diacylglycerol formation and 40-kilodalton protein phosphorylation nor caused serotonin release. At higher concentrations (more than 0.5 μM), A23187 alone caused the phosphorylation of 40-kilodalton protein in addition to 20-kilodalton protein, and released serotonin concomitantly. This is presumably due to nonspecific degradation of phospholipids and/or to activation of protein kinase C by a large increase in the Ca^{2+} concentration (5, 6, 22). Likewise, OAG alone at higher concentrations (more than 50 μg/ml) caused the release of a significant quantity of serotonin. The exact reason for this enhancement is not known, but it is possible

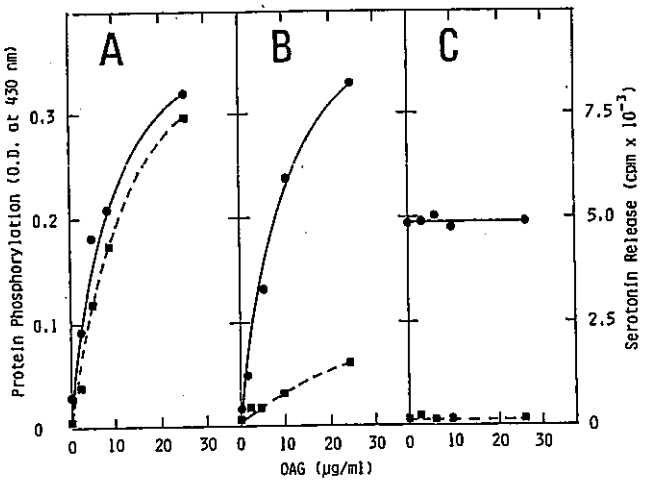

FIG. 4. **Synergistic effects of synthetic diacylglycerol and Ca^{2+} ionophore on platelet activation.** The platelets, which were labeled with $^{32}P_i$ or [^{14}C]serotonin, were preincubated with various concentrations of OAG as indicated for 1 min at 37 °C, and then stimulated by 2 mM Ca^{2+} plus 0.4 μM A23187 at 37 °C for 30 s. The final concentration of dimethylsulfoxide was 0.1%. The phosphorylation of 40-kilodalton and 20-kilodalton proteins and serotonin release were determined as described under "Experimental Procedures." *A*, 40-kilodalton protein phosphorylation; *B*, serotonin release; *C*, 20-kilodalton protein phosphorylation. ●——●, with OAG and A23187; ■- - -■, with OAG alone.

that the diacylglycerol may act as a membrane fusigen in this exocytotic process (24). Under the given conditions, the phosphorylation of 20-kilodalton protein induced by Ca^{2+} ionophore was not significantly affected by the addition of OAG (Fig. 4*C*).

The present studies seem to provide additional evidence that the receptor-mediated breakdown of inositol phospholipid is directly coupled to the activation of protein kinase C, which is responsible for the phosphorylation of 40-kilodalton protein. It is evident that the phosphorylation of this protein, if not a sole target of protein kinase C, is a prerequisite for the full physiological response that is release of serotonin, although the precise role of 40-kilodalton protein in platelet activation remains unknown. It is also conceivable that thrombin, like various other extracellular messengers which provoke inositol phospholipid turnover, immediately mobilizes Ca^{2+} as judged by the phosphorylation of 20-kilodalton protein. Obviously, Ca^{2+} may play diverse roles in the activation of cellular functions, but the present experiments with synthetic diacylglycerol and Ca^{2+} ionophore strongly suggest that the receptor-linked protein phosphorylation and Ca^{2+} mobilization act synergistically to elicit the full physiological cellular response. Nevertheless, the evidence described above is insufficient to discuss the relationship between the phospholipid degradation and Ca^{2+} mobilization. The event occurring immediately after stimulation of the receptors also remains unknown.

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