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BIOLOGICAL ACTION AND STRUCTURAL SPECIFICITY OF DIACYLGLYCEROL IN PLATELET ACTIVATION

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INDEXING WORDS

human platelet; 1,2-sn-dioctanoyl-glycerol; protein kinase C; diacylglycerol kinase; inositol phospholipid turnover

SYNOPSIS

Receptor-mediated hydrolysis of inositol phospholipids produces diacylglycerol for the activation of protein kinase C to transduce various extracellular signals. To explore further the mechanism of receptor-mediated degradation of inositol phospholipids, some synthetic, membrane-permeable diacylglycerols were used. In intact platelets, a synthetic membrane-permeable dioctanoyl-glycerol, having a 1,2-*sn*- but not 2,3-*sn*-configuration, activated protein kinase C directly. Serotonin release reaction from platelets was fully induced by this diacylglycerol in the presence of Ca^{2+}- inophore. 1,3-Dioctanoyl-glycerol was inactive for protein kinase C activation as well as for platelet release reaction. In both *in vitro* and *in vivo* systems, only 1,2-*sn*-dioctanoyl-glycerol, but not other isomers, was converted rapidly to the corresponding phosphatidic acid, 1,2-dioctanoyl-*sn*-3-phospho-

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Abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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ryl-glycerol. Therefore, the diacylglycerol which functions in stimulus-response coupling possesses the 1,2-*sn*-glycerol backbone, and other isomers are not involved in the signal transduction through the protein kinase C-dependent pathway.

INTRODUCTION

Receptor-mediated hydrolysis of inositol phospholipids is a common mechanism for transducing various extracellular signals into the cell. Under physiological conditions, protein kinase C is activated by diacylglycerol that is transiently produced from this receptor-mediated hydrolysis of inositol phospholipids.[34] However, the precise biochemical mechanism of this enzyme activation has not yet been fully clarified. In cells and tissues, diacylglycerol may be produced as an intermediate not only in phospholipid biosynthesis but in triacylglycerol metabolism. Therefore, there must be a highly specific lipid-protein interaction for this enzyme activation. An earlier report from this laboratory[17] has shown that a synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol, is permeable to the cell membrane to activate protein kinase C directly, and metabolized *in situ* to the corresponding phosphatidic acid. Lapetina *et al.*[26] and Davis *et al.*[11] have subsequently shown that 1,2-*sn*-dioctanoyl-glycerol and 1,2-*sn*-didecanoyl-glycerol are also permeable to the membrane and activate the enzyme. Rando and coworkers[8, 37] have reported that protein kinase C is activated *in vitro* by 1,2-*sn*-diolein but not by 2,3-*sn*-diolein nor by 1,3-diolein, suggesting that a special compartmentation or interaction of the diacylglycerol with membrane phospholipid bilayer for the activation of protein kinase C. The structures of 1-oleoyl-2-acetyl-*sn*-glycerol, 1,2-*sn*-dioctanoyl-glycerol, and their stereoisomers are shown in Fig. 1. Extending these observations, by using synthetic dioctanoyl-glycerol and its stereoisomers, I wish to describe here that this stereospecificity of diacylglycerol is also observed for its action on intact platelets, and that only diacylglycerol having a 1,2-*sn*-configuration may play roles in stimulus-response coupling.

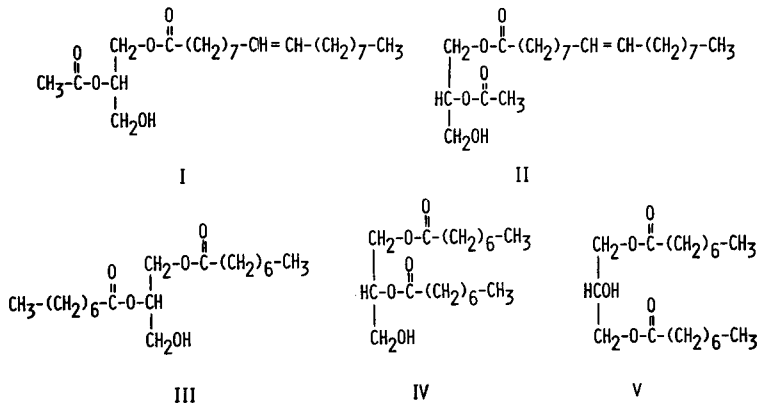

Fig. 1 Structures of membrane-permeable diacylglycerols and their stereoisomers. I, 1-oleoyl-2-acetyl-*sn*-glycerol; II, 2-acetyl-3-oleoyl-*sn*-glycerol; III, 1,2-*sn*-dioctanoyl-glycerol; IV, 2,3-*sn*-dioctanoyl-glycerol; V, 1,3-dioctanoyl-glycerol.

MATERIALS AND METHODS

Washed human platelets were prepared by the method of Baenziger and Majerus.[4] Bovine thrombin was obtained from Mochida Pharmaceutical Company (Tokyo). A23187 was a product of Calbiochem. Carrier-free ^{32}Pi and [2-^{14}C]serotonin were obtained from New England Nuclear. [γ-^{32}P]ATP (3,000 Ci/mmole) was purchased from Amersham. Calf thymus H_1 histone and human erythrocyte phospholipid were prepared as described earlier.[22] Homogeneous protein kinase C was prepared from rat brain as described earlier.[21] Diacylglycerol kinase was partially purified from rat brain by DE-52, Sephadex G-150, and matrix greengel affinity chromatography, followed by hydroxyapatite column chromatography by the method of Kanoh *et al.*[19] 1,2-*sn*-Dioctanoyl-glycerol was prepared from 1,2-dioctanoyl-*sn*-3-phosphorylcholine by the use of a bacterial phospholipase C and purified by chromatography on a silicic acid column.[9, 10] This phospholipid was synthesized chemically from purified soy bean phosphatidylcholine by hydrolysis in 10% tetra-*n*-butyl ammonium hydroxide, followed by acylation with octanoyl chloride as described by Baer and Buchnea.[5] 2,3-*sn*-Dioctanoyl-glycerol was prepared chemically from D-mannitol as a starting material by the method of Virtanen *et al.*[40] 1,3-Dioctan-

oyl-glycerol was isolated from the mixture of 1,3-dioctanoylglycerol and 1,2-*rac*-dioctanoyl-glycerol by HPLC with a Toyo Soda Silica 60 column (0.46 x 25 cm) under the conditions described by Bocckino *et al.*[7] The mixture of dioctanoylglycerol isomers were prepared from glycerol and octanoic acid by the method of Rheineck *et al.*[38] The specific rotation values ($[\alpha]^{30}$) of 1,2-*sn*-, 2,3-*sn*-, and 1,3-dioctanoylglycerol were measured to be -3.2, +3.3, and 0, respectively. Diacylglycerols having 1,2-*sn*-, 2,3-*sn*, and 1,3-configurations were isomerized very rapid from one another,[28] and thus each dioctanoyl-glycerol was employed immediately after preparation. 1,2-Dioctanoyl-*sn*-3-phosphorylglycerol was prepared from 1,2-dioctanoyl-*sn*-3-phosphorylcholine by phospholipase D digestion.[20]

Assay for protein kinase C

Protein kinase C was assayed by measuring the incorporation of ^{32}Pi into H_1 histone from [γ-^{32}P]ATP under the conditions described earlier[22] with various synthetic diacylglycerols. The reaction mixture (0.25 ml) contained 5 µmol of Tris/HCl at pH 7.5, 1.25 µmol of magnesium acetate, 50 µg of H_1 histone, 2.5 nmol of [γ-^{32}P]ATP (5 to 15 x 10^4 cpm/nmol), 0.05 µg of protein kinase C, 7.5 nmol of $CaCl_2$, 4 µg of phospholipid, and diacylglycerols as indicated. Diacylglycerol and phospholipid were first mixed in a small volume of chloroform. After chloroform was removed under nitrogen stream, the residue was suspended in a small volume of 20 *mM* Tris/HCl at pH 7.5 by sonication for 3 min at 0C using a Kontes sonifier, Model K881440. The micelles thus prepared were added to the reaction mixture. The incubation was carried out for 5 min at 30C.

Assay for protein phosphorylation

Washed platelets (4 x 10^9 cells) were labeled with 1 mCi of carrier-free ^{32}Pi for 1 h at 37C as described by Lyons *et al.*[27] The radioactive platelets (1.2 x 10^8 cells) suspended in 0.2 ml of a buffer solution, consisting of 15 *mM* Tris/HCl at pH 7.5, 0.14 *M* NaCl, and 5.5 *mM* glucose (Buffer A), were stimulated for 2 min at 37C by dioctanoylglycerol. All incubations were made in siliconized test tubes. The synthetic diacylglycerol was suspended in 1% dimethylsulfoxide by sonication for 30 sec at 0C under nitrogen

using a Kontes sonifier, Model K881440. The final concentration of dimethylsulfoxide was 0.1%. In a parallel experiment the platelets were stimulated by thrombin (0.06 unit/1.2 x 10^8 cells) for 1 min. The incubation was terminated by the addition of 0.1 ml of 187.5 *mM* Tris/HCL at pH 6.7 containing 6% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.003% bromophenol blue. The mixture was heated for 90 sec in a boiling water bath, and then subjected directly to SDS-polyacrylamide slab gel electrophoresis as described by Laemmli.[23] The stacking and separation gels contained 3% and 12% acrylamide, respectively. After electrophoresis each slab gel was stained with Coomassie brilliant blue, dried on a Whatman 3MM filter paper, and exposed to a Fuji new AIF RX film to prepare an autoradiograph. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph at 430 nm using a Shimadzu dual wavelength chromatogram scanner, Model CS-910.

Assay for release of serotonin

The platelet-rich plasma (20 ml) was incubated with 1 μCi of [2-^{14}C]serotonin for 30 min at 37C as described by Haslam and Lynham,[14] and the platelets were isolated and washed as described earlier.[39] The radioactive platelets (1.2 x 10^8 cells) were suspended in 0.2 ml of Buffer A, and stimulated for 2 min at 37C by dioctanoylglycerol as indicated, A23187 (0.2 μ*M*), and $CaCl_2$ (1 *mM*). In a parallel experiment, platelets were stimulated by thrombin (0.06 unit/1.2 x 10^8 cells). The incubation was terminated by the addition of 0.02 ml of 6% formaldehyde containing 50 *mM* EDTA followed by immersion in an ice bath. After centrifugation for 40 sec at 10,000 x g, the radioactivity of serotonin recovered in the supernatant was determined.

Assay for diacylglycerol kinase

The standard reaction mixture of diacylglycerol kinase assay contained in a final volume of 0.25 ml, Tris-HCl (pH 7.4, 25 μmol), sodium deoxycholate (0.25 μmol), sodium fluoride (5 μmol), dithiothreitol (0.125 μmol), magnesium chloride (2.5 μmol), diacylglycerol (0.25 μmol), [γ-^{32}P]ATP (0.4 μmol) and enzyme source (100 μl). The complete mixture was incubated for 3 min at 30C. The reaction was terminated by addition of 0.1 ml of concentruted HCl, followed by 3 ml of water. The reaction products

were then extracted with 2.0 ml of 1-butanol. The radioactivity of the butanol extracts was, after being washed once with 2.0 ml of water saturated with butanol, measured in toluene/Triton X-100 scintillation fluid.

Assay for lipid metabolism

The washed platelets (4×10^9 cells) were labeled with 1 mCi of carrier-free ^{32}Pi as described above. The radioactive platelets (3×10^8 cells) were stimulated in 0.5 ml of Buffer A at 37C by thrombin for 2 min or dioctanoyl-glycerol for 5 min as indicated in each experiment. The reaction was terminated by the addition of 1.9 ml of chloroform/methanol/concentrated HCl (100:200:2;v/v/v). The radioactive phospholipids were directly extracted by adding 0.6 ml of chloroform and 0.6 ml of 0.1 *N* HCl. The organic phase was taken, and dried under nitrogen stream. The residue was dissolved in 0.1 ml of chloroform/methanol (1:1;v/v), and subjected to thin layer chromatography on a Silica Gel G60 plate (Merck) using chloroform/methanol/4 *N* ammonia water (45:35:10;v/v/v) as a solvent. The plate was exposed to a Fuji new AIF RX film. Phospholipids were identified by comparison with authentic markers.

Other determination

The radioactivity of ^{32}P-, and ^{14}C-samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 4640.

RESULTS

Protein phosphorylation

Dioctanoylglycerol which was capable of activating protein kinase C showed stereospecificity *in vitro*. Fig. 2A shows effects of increasing amount of the three isomers on the enzymatic activity. Only 1,2-*sn*-dioctanoyl-glycerol was highly acitve, whereas 2,3-*sn*-dioctanoyl-glycerol and 1,3-dioctanoyl-glycerol showed little activity. The slight activity observed for 2,3-*sn*-dioctanoyl-glycerol may be ascribed to a small amount of 1,2-*sn*-dioctanoyl-glycerol that could be produced from 2,3-*sn*-dioctanoyl-glycerol by rapid racemization during the experiments. On the

other hand, the slight activity of 1,3-dioctanoyl-glycerol does not appear to be due to racemization since its spontaneous acyl migration to form the 1,2-*sn*- or 2,3-*sn*-isomer was not detected under the experimental conditions. A slight activation of the enzyme by 1,3-dioctanoyl-glycerol was due to either its intrinsic activity of non-specific hydrophobic interaction with the enzyme that was often observed for various fatty acids such as arachidonate and oleate.[29, 33] These isomers of dioctanoylglycerol did not interact with one another for the activation of protein kinase C. Fig. 3 shows the initial reaction velocity in the

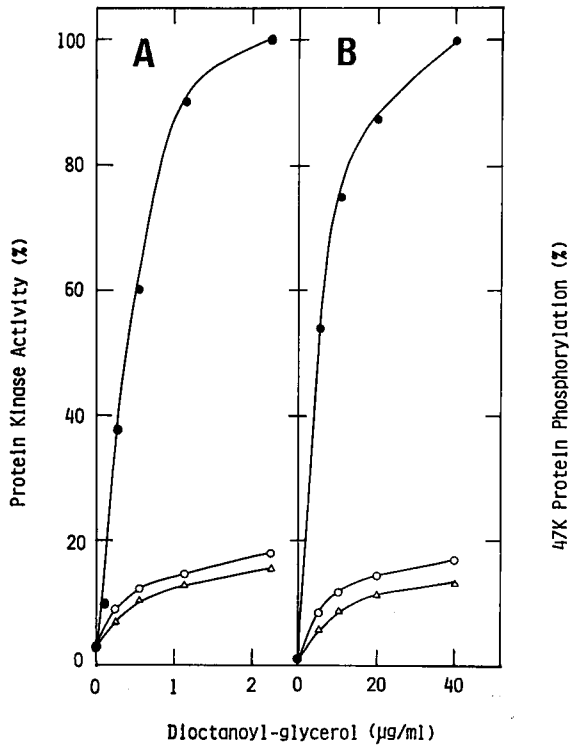

Fig. 2. Effect of three isomers of dioctanoyl-glycerol on protein kinase C. *A*; activation *in vitro*. Protein kinase C was assayed in the presence of each dioctanoyl-glycerol indicated. *B*; activation *in vivo*. Phosphorylation of 47K protein was assayed in the presence of each dioctanoyl-glycerol indicated. Other details were described under "MATERIALS AND METHODS." With 1,2-*sn*-dioctanoyl-glycerol, (●); with 2,3-*sn*-dioctanoyl-glycerol, (○); with 1,3-dioctanoyl-glycerol, (△).

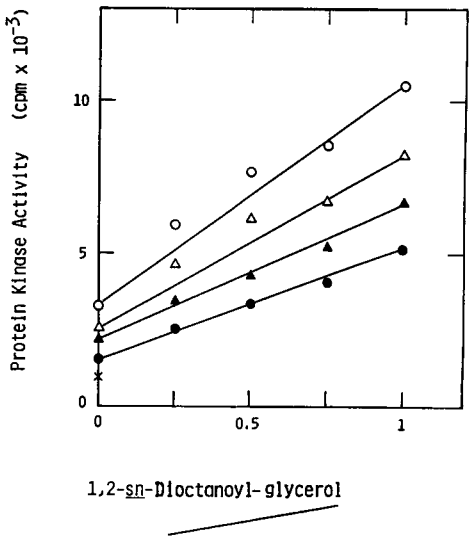

Fig. 3 The initial reaction velocities of protein kinase C in the presence of various ratios of 1,2-*sn*-dioctanoyl-glycerol and 2,3-*sn*-dioctanoyl-glycerol. Protein kinase C was assayed in the presence of various amounts of dioctanoyl-glycerol with ratios of 1,2-*sn*-dioctanoyl-glycerol over 1,2-*sn*-dioctanoyl-glycerol and 2,3-*sn*-dioctanoyl-glycerol indicated. Other details were described under "MATERIALS AND METHODS." Concentrations of total dioctanoyl-glycerol (1,2-*sn*-dioctanoyl-glycerol plus 2,3-*sn*-dioctanoyl-glycerol) were 1.07 μg/ml, (○); 0.53 μg/ml, (△); 0.27 μg/ml, (▲); and 0.13 μg/ml, (●). Without dioctanoyl-glycerol, (x).

presence of various ratios of the enantiomers. The enzyme activity was directly proportional to the quantity of 1,2-*sn*-dioctanoyl-glycerrol, indicating no inhibitory action of 2,3-*sn*-dioctanoyl-glycerol.

To explore whether this stereospecificty observed *in vitro* may be extrapolated into intact cell systems, experiments were done with human platelets since an endogenous protein having a molecular weight of 47,000 (47K protein) was known to serve as a substrate specific to protein kinase C.[16, 39] Fig. 2B shows that, when added to intact platelet suspensions, again only 1,2-*sn*-dioctanoyl-glycerol could induce phosphorylation of this 47K protein, whereas both 2,3-*sn*- and 1,3-dioctanoyl-glycerol showed little activity. Another set of experiments indicated that the maximum

phosphorylation of 47K protein induced by the addition of 1,2-*sn*-dioctanoyl-glycerol was similar to that caused by thrombin. The result suggests that no enzymatic recemization of diacylglycerol occurs in intact cells.

Release of serotonin

The stereospecificity of diacylglycerol was observed also for its biological activity to induce release reaction in the simultaneous presence of Ca^{2+} and A23187. Fig. 4 summarizes the results of such experiments, showing that serotonin was released efficiently by the addition of 1,2-*sn*- but not 2,3-*sn*- nor 1,3-dioctanoyl-glycerol. Ca^{2+} was needed for this release reaction. Another set of experiments indicated that the maximum velocity of this release reaction obtained by the simultaneous addition of 1,2-*sn*-dioctanoyl-glycerol and A23187 was equivalent to that caused by physiological stimulants such as thrombin.

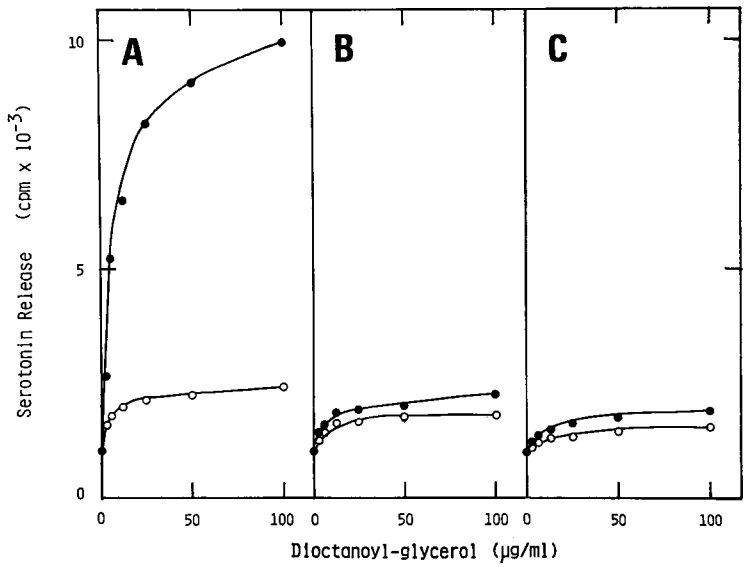

Fig. 4 Effects of various dioctanoyl-glycerol and A23187 on serotonin release from intact platelets. The radioactive platelets were stimulated with each dioctanoyl-glycerol indicated, in the presence or absence of A23187. Other details were described under "MATERIALS AND METHODS." *A*, With 1,2-*sn*-dioctanoyl-glycerol; *B*, 2,3-*sn*-dioctanoyl-glycerol; and *C*, 1,3-dioctanoyl-glycerol. In the absence of A23187, (○); in the presence of A23187, (●).

Diacylglycerol phosphorylation

The diacylglycerol is thought to be phosphorylated by the diacylglycerol kinase in a signal transduction system. By using the partial purified diacylglycerol kinase from rat brain, the stereospecificity of this enzymatic reaction was examined. As shown in Table 1, phosphatidic acid was rapidly produced when 1,2-*sn*-diacylglycerol was employed, indicating clearly the stereospecificity of diacylglycerol kinase.

A similar set of experiments with several other broken tissue preparations including rat brain homogenates revealed essentially similar to that given in Table 1, indicating that diacylglycerol kinases in mammalian tissues utilize preferentially 1,2-*sn*- but not 2,3-*sn*- nor 1,3-diacylglycerol.

Table 1 Phosphorylation of three isomers of dioctanoyl-glycerol by the partially purified diacylglycerol kinase. Other details were described under "MATERIALS AND METHODS."

Dioctanoyl-glycerol	^{32}Pi-incorporated (μmol/mg/min)
1,2-sn-Dioctanoyl-glycerol	0.9678
2,3-sn-Dioctanoyl-glycerol	0.1623
1,3-Dioctanoyl-glycerol	0.0753

Conversion to phosphatidic acid

Under physiological conditions the diacylglycerol once produced by the hydrolysis of inositol phospholipids in a signal-dependent manner rapidly disappears largely due to its conversion to phosphatidic acid. Analogously, as shown in a previous report from this laboratory,[17] an exogeneously added diacylglycerol, 1-oleoyl-2-acetylglycerol, was readily phosphorylated to phosphatidic acid presumably by the action of diacylglycerol kinase. The experiment given in Fig. 5 indicates that this conversion also shows stereospecificity with 1,2-*sn*-configuration. The human platelets preloaded with ^{32}Pi were treated by either one of the three isomers of dioctanoylglycerol, and then the phospholipids were extracted directly and subjected to thin layer chromatogra-

phy. A large spot found in *Lane C* (*spot a*) was identified as 1,2-dioctanoyl-*sn*-3-phosphorylglycerol by comparison with an authentic sample of the synthetic product. A faint band (*spot b*) was not identified but presumably represents the corresponding phosphatidylinositol. A small amount of the phosphatidic acid found in *Lane D* may be derived from 1,2-*sn*-dioctanoyl-glycerol that contaminated the 2,3-*sn*-dioctanoyl-glycerol preparation due to spontaneous racemization as mentioned above.

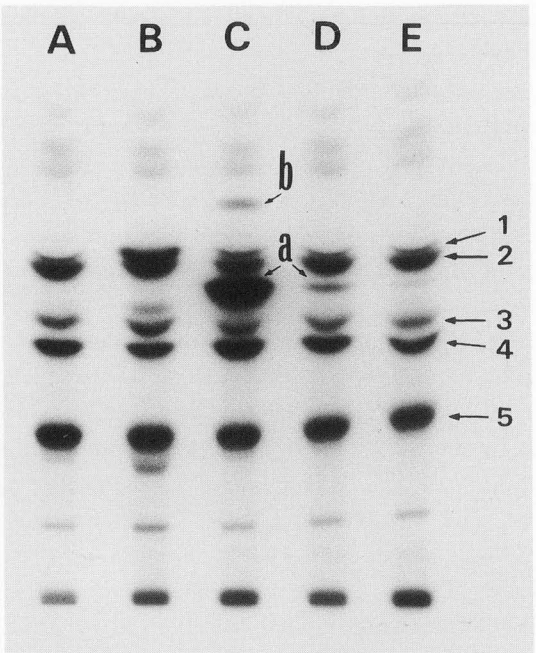

Fig. 5 Autoradiograph of thin layer chromatography of ^{32}P-labeled phospholipid of intact platelets. The radioactive platelets were stimulated by thrombin (2 units/ml) or each dioctanoyl-glycerol (125 µg/ml) as indicated, and the phospholipids were analysed as described under "MATERIALS AND METHODS." *lane A*, control; *lane B*, stimulated by thrombin; *lane C*, stimulated by 1,2-*sn*-dioctanoyl-glycerol; *lane D*, stimulated by 2,3-*sn*-dioctanoyl-glycerol; and *lane E*, stimulated by 1,3-dioctanoyl-glycerol. *spot 1*, phosphatidylinositol; *spot 2*, phosphatidic acid; *spot 4*, phosphatidylinositol 4-phosphate; *spot 5*, phosphatidylinositol 4,5-bisphosphate; and *spot a*, 1,2-dioctanoyl-*sn*-3-phosphorylglycerol. These spots were identified by comparison with authentic samples. *spot b* may correspond to 1,2-dioctanoyl-*sn*-3-phosphorylinositol.

DISCUSSION

The evidence for the linkage of inositol phospholipid turnover with activation of protein kinase C in signal transduction comes mainly from experiments with platelets. Under basal conditions, diacylglycerol is virtually undetectable in platelet membranes. Then, to explore further the link between inositol phospholipid hydrolysis and protein kinase C activation in stimulus-response coupling, a synthetic, membrane-permeable diacylglycerol such as 1-oleoyl-2-acetyl-glycerol was used to activate platelet protein kinase C directly.[17]

In this paper 1,2-*sn*-dioctanoyl-glycerol was shown to be an effective permeable diacylglycerol. Receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate generates two intracellular messengers, inositol-1,4,5-trisphosphate and 1,2-*sn*-diacylglycerol.[6, 34] However, there seem to be still some unsolved problems as to the role of diacylglycerol in the activation of protein kinase C, since the evidence available thus far is indirect for establishing the firm link between inositol phospholipid breakdown and protein kinase C activation. For instance, in cells and tissues diacylglycerol may often occur from triacylglycerol by the action of several lipases and also as an intermediate in the phospholipid biosynthesis. Extending the *in vitro* studies by Rand and coworkers,[8, 37] the results presented here suggest that diacylglycerol which serves as the activator of protein kinase C within cells contains the 1,2-*sn*-configuration, and other isomers are not involved for tranducing extracellular signals into the cell. Using triacylglycerols differentially labeled with radioactive fatty acids at various positions, it has been proposed that lipoprotein lipases from cow milk, rat adipose tissue, and rat post-heparin plasma preferentially cleave the fatty acyl moiety at position 1, and metabolize triacylglycerol through 2,3-*sn*-diacylglycerol.[1, 30, 31, 32, 35] However, this stereospecificity of lipoprotein lipases has not been unequivocally established.[3] On the other hand, hormone-sensitive lipase from rat adipose tissue has been proposed to metabolize triacylglycerols rapidly to accmulate mainly 2-monoacylglycerol *via* 1,2-*sn*- or 2,3-*sn*-diacylglycerol and not *via* 1,3-isomer.[12] In fact, the hydrolysis products of triacylglycerols by the partially purified

hormone-sensitive lipase have been shown to activate protein kinase C,[13] but the precise configuration of these intermediate diacylglycerols have not been examined. In contrast, many digestive lipases such as pancreatic lipase and lysosomal lipase do not appear to show stereospecific cleavage of triacylglycerol, and metabolize it rapidly by way of 1,2-*rac*-diacylglycerols.[2]

In animal cells diacylglycerol kinases have been found in various subcellular fractions including cytosol[18, 25, 36] and microsomes.[15, 18, 25, 36] Recent studies with antisera have revealed that there are multiple species of diacylglycerol kinases in various subcellular compartments in many tissues.[19] It has been suggested earlier[18] that diacylglycerol kinases purified partially from rat liver cytosol and microsomal fractions may utilize preferentially 1,2-*sn*-diacylglycerol, since the reaction velocity towards 1,2-*rac*-diacylglycerol is approximately a half of that towards the 1,2-*sn*-enantiomer. The present studies provide another evidence that diacylglycerol kinase(s) may be specific to the 1,2-*sn*-configuration, and that other stereoisomers are not involved in stimulus-response coupling through protein kinase C-dependent pathway.

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