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STRUCTURAL AND CHEMICAL SPECIFICITY OF DIRADYLGLYCEROLS FOR PROTEIN KINASE C ACTIVATION

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SUMMARY -- The structural and chemical specificity of diacylglycerols, lipid components of the quaternary complex for protein kinase C activation, have been evaluated. The ether-linked analogs of the diacyl lipids, either dialkyl or alkyl acyl, were not effective activators of protein kinase C and thus had little influence on reducing the Ca^{++} requirement of the enzyme. Ester-linked compounds such as 1-palmitoyl-sn-2-butyrylglycerol were as effective as dioleoylglycerol in stimulating protein phosphorylation. Increasing the carbon number at the sn-2 position from two to four resulted in enhanced enzymatic activity, suggesting that the chain length at the secondary hydroxyl is also of importance. These data clearly establish the necessity of the sn-1 carbonyl group of ester-linked glycerolipids in the protein kinase C activation complex.

Protein kinase C, which is activated by Ca^{++}, phospholipid, and diacylglycerol (1-2), is now thought to play a major role in cellular signal transduction. Among the most intriguing of discoveries in this area has been the demonstration that tumor-promoting phorbol diesters activate protein kinase C directly. In doing so, the phorbol diesters replace the diacylglycerol required for enzyme activation (3-5). This circumvents the natural path of diacylglycerol production via receptor-activated hydrolysis of inositol-containing phospholipids by phospholipase C (6-8). It now appears that the biological activity of the lipophilic phorbol esters is in part related to their ability to partition into membranes and mimic the action of diacylglycerols. In an effort to characterize the neutral lipid requirement for protein kinase C activation, we have synthesized and tested a series of diradylglycerols that differ not only in the acyl carbon chain length at the sn-2 position, but additionally in the type of linkage (ester,ether) located at the sn-1 position of the glycerol backbone. Acyl linkages are by far the most

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ABBREVIATIONS: PS, phosphatidylserine

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prevalent in vivo, however, the ether-linked lipids represent an interesting group of cellular compounds (9,10) whose biological functions have just recently been approached via the discovery of alkylacetylglycerophosphocholine [PAF, platelet activating factor (11-13)]. This work was undertaken to explore the chemical and structural requirements of diradylglycerols for protein kinase C activation and; moreover, to determine whether ether-linked lipids in general or the neutral constituent of platelet activating factor, alkylacetylglycerol, could participate in the activation of protein kinase C. It constitutes the first instance revealing the ester vs. ether chemical specificity of protein kinase C for diradylglycerols.

MATERIALS AND METHODS

Hexadecyl-sn-glycerol (chimyl alcohol) was purchased from Analabs, North Haven, CT. L-α-lysophosphatidylcholine (palmitoyl), sodium deoxycholate, histone Type IIIS, fatty acid-poor bovine serum albumin, phosphatidylserine (PS)[1] (bovine brain), and phospholipase C (B. cereus) were purchased from Sigma Chemical Co., St. Louis, MO. Pancreatic lipase was purchased from United States Biochemical Corp., Cleveland, OH.; acetic anhydride was a product of Fisher (Atlanta, GA), and butyric and hexanoic anhydrides were from Eastman (Rochester, NY). Dioleoylglycerol (1,2-isomer) was purchased from Nu Chek Prep, Elysian, MN, and the hexadecylmethoxyglycerol was a gift from Dr. Fred Snyder (Oak Ridge Associated Universities).

Synthesis of Diradylglycerols

The diester compounds were synthesized by reacting L-α-palmitoyl-lysophosphatidylcholine (10 mg) with 0.5 ml of the respective anhydride (acetic, butyric) and 0.5 ml of toluene containing 5 mg of dimethylaminopyridine. After heating the sealed tube in a boiling water bath for two hours, water was added to the reaction and lipids were extracted by the method of Bligh and Dyer (14) modified with methanol containing 2% acetic acid. The sn-2 substitution of the lysolipid was verified by thin-layer chromatography on layers of silica gel G developed in chloroform/methanol/ammonium hydroxide/water (60:35:8:2.3,v/v). To remove the phosphobase moiety, the lipids were then incubated with phospholipase C (10 units, B. cereus) as previously described (15), and the products (diacylglycerols) were purified by boric acid-impregnated preparative thin-layer chromatography (16), to obtain the 1,2-isomeric forms used in the experiments. Lipids were extracted from boric acid-impregnated gel using diethyl ether. The diethyl ether was diluted with one part hexane, and the organic phase was washed three times with water to remove boric acid.

Ether-linked diradylglycerols (sn-2 acetyl-, butyryl-, hexanoyl-substituted) were synthesized as per the ester compounds, using 1-hexadecylglycerol as the starting compound. This reaction produced the 1-alkyl-sn-2-3-disubstituted neutral lipid which migrated on thin-layer (chloroform/methanol, 98:1.5) with increasing R_f values (acetyl < butyryl < hexanoyl), the alkylhexanoylglycerol approaching the migration of commercial trioleoylglycerol. To obtain the ether-linked diradylglycerols, the triradylglycerols were incubated (in a final volume of 3.0 ml) with pancreatic lipase (5 mg), sodium deoxycholate (2.5 mg), $CaCl_2$ (45 mg), and 2.0 ml of 1.0 M Tris-HCl buffer, pH 8.0 for two hours at room temperature. The enzyme reaction was terminated and lipids were extracted by the addition of 6.0 ml chloroform/methanol (1:1). The 1,2-isomers were purified by boric acid-impregnated thin-layer chromatography (16) using chloroform/methanol

(98:1.5). The diradylglycerols migrated between hexadecylglycerol and dioleoylglycerol, in ascending order (acetyl, butyryl, hexanoyl).

Protein Kinase C Assay

Protein kinase C was partially purified by DE52 chromatography from mouse brain cytosol (17) and was a gift of Dr. Arco Jeng (N.C.I.). Enzyme (25 µl) was mixed with 25 µl kinase assay mixture on ice. The reaction was initiated by placing the tubes in a 30°C water bath for five minutes. The tubes were then placed on ice, and an aliquot (25 µl) was pipetted onto phosphocellulose paper and washed according to the method of Witt and Roskoski (18). The final concentration of reagents in the assay mixture was 50 mM Tris-Cl (pH 7.4), 0.5 mM dithiothreitol, 7.5 mM magnesium acetate, 0.325 mg/ml histone, 0.25 mg/ml bovine serum albumin, 2.5 mM ATP, and $CaCl_2$ and PS as indicated. All solutions were prepared in 50 mM Tris-Cl which had been passed over a Chelex 100 column (Bio-Rad, Richmond, CA) to remove Ca^{++}. 32P-ATP (2Ci/nmol, New England Nuclear, Boston, MA) was added to a final concentration of approximately 100 cpm/pmol ATP. Liposomes were prepared by mixing the diradyl lipids with PS in chloroform. After removal of solvent in vacuo, the mixtures were sonicated in 0.5 ml of chelexed 50 mM Tris-Cl (pH 7.4) six times for five seconds each time at five second intervals using the micro-tip of a sonicator (Heat Systems-Ultrasonics, Inc.). All assays were done in duplicate; variations from the mean were less than 10%.

RESULTS

In order to establish the structural requirements for the sn-2 position of diacylglycerol in the activation of protein kinase C, we compared the activities of 1-acyl-2-acetylglycerol and 1-acyl-2-butyrylglycerol. In each case the sn-1 position was occupied by palmitate (C-16:0), the most common phospholipid substituent at the primary position of glycerol (19). In vivo, diacylglycerols are thought to activate protein kinase C by reducing the enzymatic requirement for Ca^{++} (2). We therefore measured the Ca^{++}-dependence for enzyme activity at given concentrations of phospholipid and diacylglycerol to establish relative potencies of the different diacylglyercols. Under the assay conditions used, dioleoylglycerol, acylacetylglycerol, and acylbutyryl-glycerol all completely activated the kinase in the absence of added Ca^{++} when assayed at concentrations of 20 µg/ml PS and 0.8 µg/ml diacylglycerol. To assess relative potencies, the concentrations used were reduced to 4 µg/ml PS and 0.16 µg/ml diacylglycerol. The results shown in Fig. 1 indicate that increasing the carbon number at the sn-2 position from two to four increases the efficacy of the diacylglycerol to that of dioleoylglycerol, a potent activator of protein kinase C (2,20). Half-maximally effective Ca^{++} concentrations were estimated graphically in two experiments and were 12 $\pm$ 2 µM (dioleoylglycerol), 12 $\pm$ 2 µM (acylbutyrylglycerol), and 21 $\pm$ 2 µM (acylacetyl-glycerol). In the absence of diacylglycerol, 4 µg/ml PS stimulated kinase activity to only 30 percent of the levels measured in the presence of diacylglycerol, and the half-maximally effective Ca^{++} concentration was roughly estimated to be 100 µM.

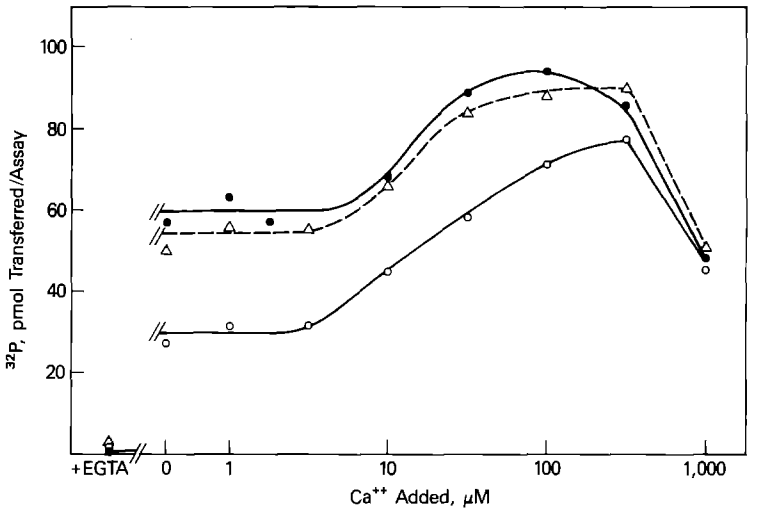

Fig. 1. Effect of diacylglycerols on the Ca^{++}-dependence of protein kinase C. Mixed liposomes containing PS and each diacylglycerol were prepared as described in Methods such that the final concentration in the assay was 4 μg/ml PS and 0.16 μg/ml diacylglycerol. Where indicated, EGTA was added to a final concentration of 1 mM. o, acylacetylglycerol; Δ, acylbutyrylglycerol; •, dioleoylglycerol.

A second set of diradylglycerols was designed to test the requirements of the acyl linkage at the sn-1 position of the glycerol backbone. For this purpose, a series of compounds with an ether linkage to the 16 carbon saturated

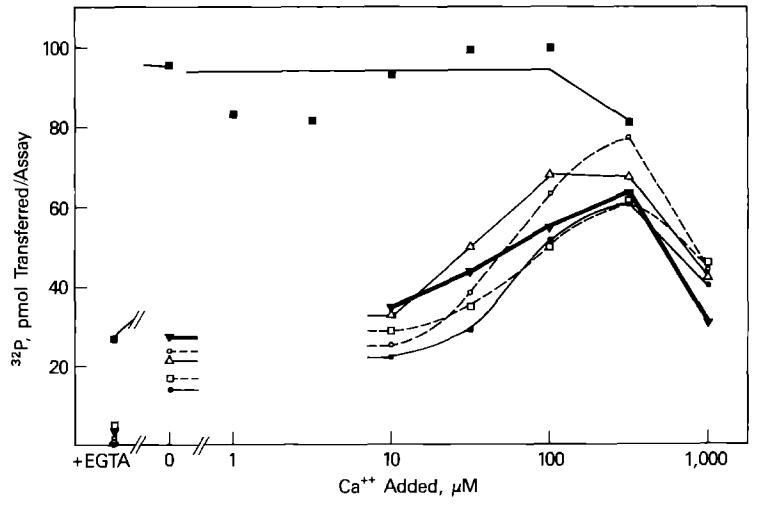

Fig. 2. Effect of ether-linked diradylglycerols in activation of protein kinase C. Mixed liposomes were prepared as described in Methods such that the final concentration in the assay was 20 μg/ml PS and 0.8 μg/ml of each glycerol derivative. Where indicated, EGTA was added to a final concentration of 1 mM. The bold line (▼) describes the Ca^{++} dependency with PS alone. Other symbols represent 1,2 substitutions of glycerol as follows: •, alkylacetyl; o, alkylbutyryl; Δ, alkylhexanoyl; □, alkylmethoxy; and ■, dioleoyl.

chain was synthesized. The data of Fig. 2 show the diradylglycerol chemical specificity of protein kinase C as tested with diethers and alkylacylglycerols. The Ca^{++} requirement for enzymatic activity using alkylmethoxyglycerol (ether linkage to both the sn-1 and sn-2 positions) was not different than for PS alone. The half-maximally effective Ca^{++} concentrations were estimated to be 40 $\pm$ 10 μM and 37 $\pm$ 8 μM in the absence and presence of alkylmethoxyglycerol, respectively (n=2). Next, the diradylglycerols having only one ether linkage (sn-1) were evaluated in the protein kinase C reaction system. Neither alkylacetylglycerol nor alkylbutyrylglycerol significantly affected the Ca^{++} dependence for enzymatic activity. Additionally, the Ca^{++} activation curves for isomeric 1,2- and 1,3- alkylacetylglycerol were not different (1,3- isomer data not shown). Increasing the carbon chain length to six (alkylhexanoylglycerol) marginally decreased the Ca^{++} requirement. The half-maximally effective Ca^{++} concentrations, estimated in two experiments, were 38 $\pm$ 13 μM (alkylacetylglycerol), 27 $\pm$ 13 μM (alkylbutyrylglycerol), and 20 $\pm$ 5 μM (alkylhexanoylglycerol). This is in contrast to the Ca^{++} dependence in the presence of dioleoylglycerol; in this case maximal activity occurred with less than 1 μM added Ca^{++}. Increasing the concentrations of the ether-linked compounds to 3.2 μg/ml, in the presence of 20 μg/ml PS, did not increase the protein kinase C activity measured at 1 μM added Ca^{++}.

DISCUSSION

The results show that ether-linked diradylglycerols are not effective cofactors for the activation of protein kinase C. None of the ether compounds tested shifted the Ca^{++} requirement for enzymatic activity toward the low levels observed with dioleoylglycerol (Fig. 1). The low levels of Ca^{++} present in cell cytosol imply that physiological activators of protein kinase C should reduce the Ca^{++} requirement for enzymatic activity to less than 1 μM. Our data clearly established the necessity of the 1-acyl bond because 1-acyl-2-butyrylglycerol was as effective as dioleoylglycerol in reducing the Ca^{++} requirement (Fig. 1), whereas 1-alkyl-2-butyrylglycerol did not significantly change the Ca^{++} requirement from that observed with phosphatidylserine alone (Fig 2). Additionally, the acyl chain need not be unsaturated as in the case of the monoenoic species (20). Watson et al. (21) have also shown that the saturated diacylglycerol, 1,2-didecanoylglycerol stimulates protein kinase C of platelets. The presence of a carbonyl group at the primary position of the glycerol backbone appears to be an important chemical requirement for protein kinase C activation. These results indicate that diacylglycerol analogs could be employed for studying cellular differentiation in a culture system. A case in point would be the TPA-induced differentiation of HL-60 cells (22-23). Such diacylglycerol treatment of cultured cells may promote activation of protein

kinase C as do phorbol esters. However, the metabolic fate of the inducer must play a crucial role in the ability of these agents to act. Lipases have recently been described that hydrolyze phorbol esters as well as diacylglycerols (24-25). It is interesting to note that 1-O-hexadecyl-2-acetyl-sn-glycerol has recently been used to stimulate differentiation of cultured human leukemia cells (26). However, our data show that the ether-linked diradylglycerols are poor activators of protein kinase C, thus pointing out the complexity encountered between whole cell and cell-free systems as regards the mechanism of protein kinase C activation.

Our results also suggest that there may be an optimal chain length for the sn-2 substituent within a series of sn-1 (16:0) derivatives. Whereas acylbutyrylglycerol was more effective than acylacetylglycerol, dipalmitin has been shown to be a poor activator (20). Therefore, a certain minimal chain length may be important in the accessibility of the diacylglycerol to the kinase; however, this requirement may be overshadowed by decreased fluidity at some longer chain lengths. Along these lines, it is relevant to point out that detergents such as sodium dodecylsulfate and Triton X-100 can also activate protein kinase C (27,28).

Naturally occurring glycerophospholipids and neutral glycerolipids exist in which the acyl groups are replaced by alkyl moieties (9). Because of the unique ether bond these lipids have been useful in model systems to study membrane physiochemical properties (29). Our observation on the inability of the ether analogs of diacylglycerol to stimulate protein kinase C activity may be linked to phase transition temperatures (30) affecting fluidity parameters, or more importantly, to the lack of carbonyl oxygen, which is hypothesized to interact with other neighboring lipids (31-33) and membrane proteins, as in the case of glucose-6-phosphatase (34). It is believed that these studies may aid understanding the physiochemical interactions of the quaternary complex essential to protein kinase C activation and shed light on the chemistry of phorbol ester action.

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