Phospholipid Functional Groups Involved in Protein Kinase C Activation, Phorbol Ester Binding, and Binding to Mixed Micelles*

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The specificity of the phospholipid cofactor requirement of rat brain protein kinase C was investigated using Triton X-100 mixed micellar methods. Sixteen analogues of phosphatidylinositol were prepared and tested for their ability to support protein kinase C activity, [3H]phorbol 12,13-dibutyrate binding, and protein kinase C binding to mixed micelles. Phosphatidylinositol, -1-serine methyl ester, -N-acetyl-L-serine, -2-hydroxyacacetate, -3-hydroxypropionate, and -4-hydroxybutyrate did not activate protein kinase C in mixed micelles containing 2 mol % of sn-1,2-di-oleoylglycerol. This indicates that both the carboxyl and amino moieties are important for activation. Phosphatidyl-D-serine and -l-homoserine were incapable of supporting full activation; this demonstrates stereospecificity and the importance of the distance between the phosphate and carboxyl and amino moieties. Since 1,2-rac-phosphatidyl-L-serine and 1,3-phosphatidyl-l-serine fully supported protein kinase C activity, the stereochemistry within the glycerol backbone at the interface was not necessary for maximal activation. Neither lysophosphatidyl-L-serine nor 1-oleoyl-2-acetyl-sn-threose-3-phospho-L-serine supported protein kinase C activity implying that the interfacial conformation is critical to the activation process. The phospholipid dependencies of [3H]phorbol 12,13-dibutyrate binding and of protein kinase C binding to mixed micelles containing sn-1,2-di-oleoylglycerol did not mirror those for activation. The data demonstrate that protein kinase C possesses a high degree of specificity with respect to phospholipid activation and implicate several functional groups within the phospho-L-serine polar head group in binding and activation.

Protein kinase C is an important element of the signal transduction cascade regulating a host of cellular responses, including cell proliferation and transformation (1). Protein kinase C actually exists as a number of distinct isoforms involved in the transduction of extracellular signals. The enzyme is activated in vitro by phosphatidylinositol (PS) in the presence of Ca^{2+} and DAG. DAG for activation is generated from the phosphatidylinositols and other glycerol-

lips by activation of phospholipase C or D (8). DAG increases the affinity of protein kinase C for Ca^{2+} and PS (9-11). The ester bonds and the sn-3 hydroxyl group of DAG are essential for DAG analogues to activate protein kinase C (12, 13). Protein kinase C is the major cellular receptor for tumor-promoting phorbol esters (14, 15). Phorbol esters like DAG bind to and activate protein kinase C in a phospholipid- and Ca^{2+}-dependent manner (14, 16). DAG inhibits phorbol ester binding through a competitive mechanism (17), suggesting that DAG is the "endogenous phorbol ester." Phorbol 12-myristate 13-acetate (18-20) and DAG (21-25), whose production is stimulated by growth factors and hormones, trigger the translocation of cytosolic protein kinase C to membranes. The phospholipid composition of the plasma membrane modulates phorbol ester binding affinity of the receptor (26, 27). Detailed knowledge of the structure (2-5, 28), function, and regulation (1, 29) of protein kinase C is emerging, however, detailed molecular insight into the regulation of protein kinase C by phospholipid cofactor is lacking.

The activation of protein kinase C is critically dependent on phospholipid, Ca^{2+}, and DAG. Binding of protein kinase C to phospholipid is Ca^{2+}-dependent and can occur without activation (30, 31). Activation occurs as a second step. PS is the most effective of the phospholipids tested for its ability to support protein kinase C activation (32, 33). Other anionic phospholipids such as PA, phosphatidylglycerol, phosphatidylinositol, and cardiolipin are less effective; PE, PC, and sphingomyelin are inert. The phospholipid dependencies of phorbol ester binding were similar (34, 35). Each of three protein kinase C isoforms has nearly identical phospholipid specificities for activation and [3H]PDBu binding (36, 37). Our laboratory has investigated the phospholipid dependence of protein kinase C activation and phorbol ester binding using Triton X-100 mixed micellar methods (33, 35). The number of phospholipid and DAG molecules per micelle can be systematically and independently varied in a defined physical environment, the mixed micelle. These studies established that PS functions efficiently as the phospholipid cofactor for protein kinase C activation or PDBu binding; 4 or more molecules of PS were required to activate monomeric protein kinase C (35, 38). These and other data led to a model of protein kinase C regulation by phospholipid, Ca^{2+}, and DAG (phorbol esters). The modelpredicted precise molecular interactions between protein kinase C and its lipid cofactors (12).

Although implied by the model, the precise structural features within the phospholipid polar head group required for activation, phorbol ester binding, and protein kinase C binding to micelles have not been defined. To determine these critical features, we synthesized several derivatives and analogues of PS and tested each using Triton X-100 mixed micellar methods. The data demonstrate a surprisingly high degree of specificity. The amino and carboxyl groups within
Phospholipid Functional Groups for Protein Kinase C Activation

Fig. 1. Structures of phospholipid derivatives synthesized for this study.

L-serine are required stereospecifically for activation and maximal [3H]PDBu binding of protein kinase C. However, the structural features required for binding to mixed micelles were far less specific and consisted of the anionic phosphate and carboxyl groups.

EXPERIMENTAL PROCEDURES

RESULTS

Rationale—Derivatives of PS were synthesized to assess the significance of its functional groups on protein kinase C activation, phorbol ester binding, and binding of protein kinase C to mixed micelles. From the determined structure-activity relationships, specific protein kinase C-PS interactions could be inferred.

Functional Groups of Phospholipid Required for Protein Kinase C Activation—Since acidic phospholipids activate protein kinase C effectively, the carboxyl group was considered to be the critical functional group within PS. Thus, various carboxylic phospholipids: phosphatidyl-2-hydroxyacetate (I), phosphatidyl-3-hydroxypropionate (II), and phosphatidyl-4-hydroxybutyrate (III) (Fig. 1) were prepared and tested. At concentrations up to 20 mol%, none of these phospholipids supported protein kinase C activation comparable to that of 1,2-sn-PS (Fig. 2). Sodium oleate-Triton X-100 mixed micelles did not activate. Thus, the carboxyl head group alone in phospholipids is not sufficient to support protein kinase C activation.

Since PE does not activate protein kinase C (32, 33), the amino group alone in the polar head group is not sufficient to support protein kinase C activation. Thus, the possibility that both the amino and carboxyl groups of PS are required for full activation of protein kinase C was considered. The importance of the amino group moiety was tested using phosphatidyl-N-acetyl-L-serine (IV). As this compound and the deamino-PS (phosphatidylhydroxypropionate, II) did not support enzyme activity (Fig. 2), it appears that both amino and carboxyl groups are important for activation.

In order to investigate the importance of the carboxyl moiety in the presence of the amino group, phosphatidylserinol (V) and phosphatidyl-L-serine methyl ester (VI) were prepared and tested. Neither of these analogues supported protein kinase C activity (Fig. 3). Phosphatidylpropanediol (VII) was also inactive. Thus, both the amino group and the carboxyl group in the phospholipid head group are important for protein kinase C activation. In fact, this appears to be stereospecific because the D-form of serine was far less effective than the L-form (Fig. 4).
Phospholipid Functional Groups for Protein Kinase C Activation

FIG. 2. Effect of carboxylic lipids on the protein kinase C (PKC) activation. Rat brain protein kinase C was assayed by measuring the phosphate incorporation into histone III-S with Triton X-100 mixed micelles containing the phospholipid indicated and 2 mol % of 1,2-sn-diolein. The details of the method used have been described previously (38). Each point is the mean value of duplicate in two separate experiments. The standard deviations were within 10%.

FIG. 3. Effect of reduced forms of carboxylic phospholipids on the protein kinase C (PKC) activation. The reaction condition is described in the legend to Fig. 2.

FIG. 4. Effect of phosphatidylserine derivatives on protein kinase C (PKC) activation. The reaction condition is described in the legend to Fig. 2.

Since the amino and carboxyl groups are required for activation, other features of the phospholipid likely to be important for protein kinase C activation were investigated. Unlike the stereospecificity for the L-form on the serine head group, no stereospecificity within the glycerol backbone (compound XIII and XIV) was observed (Fig. 4). Increasing the distance between the head group and phosphate group (phosphatidyl-L-homoserine, IX) decreased activity drastically. Both lysoPS (XV) and 1-oleoyl-2-acetyl-PS (XVI) were inactive although each contain a phospha-L-serine head group. Thus, the interfacial conformation appears to be critical for the phospholipid cofactor to function.

To investigate whether the phosphate group of phospholipids could serve as cofactor, the esterified phosphatidates shown in Fig. 1 (X, XI, XII) were prepared and tested. As the esterification of the phosphate group increased, the ability to support activity decreased (Fig. 5). Monomethyl- and monooethylphosphatidate supported protein kinase C activity to a lesser extent than PA. Uncharged phosphatidylglyceranol was unable to support activity. Although the phosphate group in PS is not sufficient by itself to support protein kinase C activation, it may well function with the amino and carboxyl moieties.

The ability of the phospholipid analogues to support the activation of protein kinase C by PDBu rather than 1,2-sn-dioleoylglycerol was investigated. The results were similar to those observed with 1,2-sn-dioleoylglycerol (Table II). The phospholipid dependencies of protein kinase C activation by PDBu and DAG are similar.

Functional Groups of Phospholipid Required for [³H]PDBu Binding—While catonic phospholipids such as PC and PE do not support [³H]PDBu binding (34, 34), carboxylic phospholipids supported binding at low concentrations (Fig. 6). Phosphatidyl-3-hydroxypropionate (II), -4-hydroxybutyrate (III), and phosphatidyl-N-acetyl-L-serine (IV) were able to support phorbol ester binding in a manner similar to 1,2-sn-PS, when measured below 12 mol % (Fig. 6). A distance of at least two methylene groups between the phosphate and carboxyl groups appears to be required for [³H]PDBu binding since phosphatidyl-2-hydroxyacetate (I) barely supported binding. Sodium oleate was not active. The reduced forms of the carboxyl group moiety in the presence (V, VI) and absence of the amino group (VII) also did not support [³H]PDBu binding activity (Fig. 7). The carboxyl group moiety within the phospholipid polar head group appears important to support [³H]PDBu binding. However, when the concentration of 1,2-sn-PS was increased above 12 mol %, more [³H]PDBu binding occurred; while the carboxylic phospholipids were employed above 12 mol %, this increase did not occur. These results, therefore, suggest that both the amino group moiety and the carboxyl group in the phospholipid are required for maximal [³H]PDBu binding.

The ability of other phospholipid analogues to support phorbol ester binding was investigated. The data in Fig. 8
Phospholipid Functional Groups for Protein Kinase C Activation

FIG. 6. Effect of carboxylic phospholipids on the [3H]PDBu binding activity of protein kinase C (PKC). After rat brain protein kinase C was incubated with 0.3% Triton X-100 mixed micelles containing the phospholipid indicated, 100 μM CaCl₂, 100 nM [3H]PDBu, for 15 min at room temperature, bound [3H]PDBu to the mixed micelles-protein kinase C complex was separated from free [3H]PDBu by Ultro-Gel AcA 202 gel filtration. Nonspecific binding was determined with 10 pM unlabeled PDBu. Each point is the mean value of duplicate in two separate experiments. The standard deviations were within 10%.

FIG. 7. Effect of reduced forms of carboxylic phospholipids on the [3H]PDBu binding activity of protein kinase C (PKC). The reaction condition is described in the legend to Fig. 6.

revealed that: 1) d-serine head group supported [3H]PDBu binding to a similar extent as the L-form but with a lower affinity; 2) the stereochemistry within the glycerol backbone did not significantly affect binding (compounds XIII and XIV); 3) increasing the distance between the phosphate and head groups (phosphatidyl-L-homoserine, IX) decreased [3H]PDBu binding; and 4) removal of the sn-2 acyl chain abolished binding activity (lysoPS, XV). The phospholipid analogues, in which the degree of esterification of the phosphate group was varied, supported [3H]PDBu binding in a manner similar to protein kinase C activity (Fig. 9); the higher the degree of substitution, the lower the [3H]PDBu binding observed. The most novel observation to emerge from these studies was that phosphatidyl-d-serine supported maximal [3H]PDBu binding but only 30% maximal activation (Fig. 8 and Table II). These analogues provide a tool to clearly separate binding from activation.

Functional Groups of Phospholipid Required for Protein Kinase C Binding to Mixed Micelles—Measurements of pro-
Protein kinase C binding to mixed micelles were made by separation of bound protein kinase C from free protein kinase C by gel filtration. Full recovery of both forms of protein kinase C from Ultra-Gel A 34 required 150 mM NaCl in the elution buffer. Under the conditions employed, 18 mol % of phospholipid indicated and 2 mol % of 1,2-sn-diolein amounted to 100 μM CaCl₂. After a 15-min incubation at room temperature, the reaction mixture (100 μl) was applied to a Ultro-Gel AcA 34 column (0.5 × 18.5 cm) for separation of bound and free forms of protein kinase C at room temperature. The elution buffer contained 20 mM HEPES buffer, pH 7.5, 100 μM CaCl₂, 10 mM β-mercaptoethanol, 5% (w/v) glycerol, 150 mM NaCl. The Ca²⁺ and DAG-independent protein kinase activities were negligible in free and bound protein kinase C fractions. The experiments were repeated at least twice.

Phospholipid analogues were tested for their ability to support protein kinase C binding to mixed micelles (Fig. 10). The carboxylic phospholipids (1, II, III), the phosphatidyl-l-serines (XIII, XIV), and PA allowed quantitative protein kinase C binding to micelles in a DAG-dependent manner. Phosphatidyl-1-serine methyl ester (VI) supported protein kinase C binding to a limited extent. Phosphatidyl-N-acetyl-L-serine (IV) caused an elution pattern of protein kinase C activity that was distinct from that observed for other phospholipids. The other analogues and oleate failed to support protein kinase C binding. These data show that the phospholipid functional groups required for protein kinase C binding to mixed micelles are the acidic carboxyl groups or the phosphate head group. However, multiple interactions with the phospholipid appears likely because the carboxyl group in oleate did not support binding. In the presence of both amino and carboxyl groups, the configuration of amino and carboxyl group (L- and D-serine), the distance between these groups and phosphate group (IX), and the presence of sn-2 fatty acyl chain (XV) were important for protein kinase C binding. No stereoechemical requirement within the glycerol backbone (XIII, XIV) was observed.

**DISCUSSION**

Investigations on the specificity and mechanism of protein kinase C regulation by phospholipid cofactors, Ca²⁺, and DAG seek to define in molecular terms, the interactions between the various components of the quaternary complex (12). The physical properties of the phospholipid cofactors (50), of DAG (phorbol esters), and the interactions between substrates (51, 52) and lipid cofactors are recognized as significant problems limiting interpretation of present data. Available preparations of protein kinase C may consist of a mixture of isofoms since the seven known species, only three chromatographically separable species are known. Absolutely pure preparations of individual isofoms are not presently available.

A model was put forth to focus attention on the molecular interactions between protein kinase C, phospholipid, and DAG (12). The model was based on data obtained using mixed micellar methods of analysis as well as data obtained using phospholipid aggregates (9, 10). Mixed micellar methods allow the number of lipid cofactors and activators (inhibitors) to be systematically and independently varied in a defined physical environment, the mixed micelle. The studies with mixed micellar methods led to several inferences. First, monomeric protein kinase C is the active species and is able to bind [3H]PDBu. Second, a single molecule of DAG or PDBu is able to bind to and activate monomeric protein kinase C. Third, 4 or more molecules of PS are required for protein kinase C activation. Protein kinase C was proposed to bind to a 4PS-Ca²⁺ complex in which Ca²⁺ is liganded by the 4 carboxyl groups present in the serine polar head groups. The protein kinase C-4PS-Ca²⁺ complex is itself inactive, but upon DAG or phorbol ester binding, activation occurs (12, 38). Detailed DAG structure-activity relationships (12, 13) support a three point stereospecific interaction between DAG and the protein kinase C-4PS-Ca²⁺ complex: one of these bonds was proposed to be directly with Ca²⁺ and the other two bonds were proposed to be with protein kinase C.

The model predicted that a high degree of specificity would exist in the interactions between protein kinase C, phospholipid, Ca²⁺, and DAG. The model prompted us to investigate whether specific interactions occur between protein kinase C and the phospholipid polar head group. The ability of these phospholipid analogues to support activity, [3H]PDBu binding, and binding to mixed micelles are summarized as below. A high degree of specificity was noted. A number of analogues were found to support submaximal [3H]PDBu binding, and binding to micelles without proportional activation. The carboxylic phospholipids (II, III), N-acetylated PS (IV) (Fig. 6), and phosphatidyl-D-serine (VIII) (Fig. 8) supported [3H]PDBu binding without proportional activation (Table II). Protein kinase C binding to mixed micelles was supported by carboxylic phospholipids without activation (Figs. 2 and 10). Thus, the amino group of PS appears to function to couple [3H]PDBu binding to activation of protein kinase C.

**Functional Groups of Phospholipid Involved in Activation of Protein Kinase C**—A surprising degree of specificity emerged for the phosho-L-serine polar head group. Both the carboxyl and amino groups were important (see "Results"). Key observations worth reiterating are that the phosphatidyl-D-serine and phosphatidyl-L-homoserine were not effective, implying stereospecificity, and that a precise distance between the phosphodiester bond and the amino and carboxyl functional groups was necessary for activation. Moreover, the interfacial conformation appeared to be critical in that lysoPS and 1-
oleoyl-2-acetyl-PS failed to support activity even though they possess the phospho-L-serine determinant. Importantly, protein kinase C recognizes the interfacial phospho-L-serine without regard to stereosepecificity within the glycerol backbone. The discussion focuses on differences in the phospholipid dependencies that are large; hence, the interpretations are unlikely to be limited by the protein kinase C preparation employed that contained a mixture of isoforms.

Specific interactions between protein kinase C and PS can be inferred from the data. Three or more points of contact between each molecule of PS and protein kinase C appear likely. These points of interaction with PS are inferred to be with the carboxyl, amino, and phosphate groups. Additional bonds may occur between protein kinase C and the glycerol backbone because of the interfacial requirements, e.g., lysoPS and 1-oleoyl-2-acetyl-PS not serving a cofactor. Since at least 4 molecules of PS are required, 12 or more bonds between PS and protein kinase C and Ca²⁺ are inferred. When these 12 bonds are added to the three bonds between DAG and the 4PS-Ca²⁺-protein kinase C complex, 15 points of contact are inferred. Additionally, protein kinase C may ligand Ca²⁺ itself, in a sixth and open coordinate position. Thus, a total of 16 or more contacts likely exist between protein kinase C, Ca²⁺, DAG, and PS.

The differences in affinity between DAG and PDBu undoubtedly reflect additional bonds between PDBu and the protein kinase C-4PS-Ca²⁺ complex. The number of aggregate bonds could underlie the differences noted below between PDBu binding and DAG-dependent activity supported by these PS analogues and derivatives (Figs. 6–9, Table I).

Substrate aggregation represents a significant caveat to molecular interpretations (52, 53). Protein kinase C activation by Ca²⁺, DAG, and phospholipid has been suggested to occur to the extent that they facilitate aggregation with histone (52, 53). The structure-activity relationship observed with PS derivatives and analogues cannot be totally attributable to histone aggregation.

Functional Groups of Phospholipid Involved in [³H]PDBu Binding—The phospholipid dependencies of [³H]PDBu binding and activation of protein kinase C have been shown to be similar, with PS being the most effective (34, 35). The present study demonstrated that the amino and carboxyl groups in the phospholipid head group are necessary for maximal [³H]PDBu binding and for activation. However, the phospholipid concentration dependence of [³H]PDBu binding did coincide well with that of protein kinase C activation. This difference could be due to the differences in compositions between two reaction mixtures: namely, Mg²⁺, histone, and ATP are absent in the [³H]PDBu binding assay. The amino and carboxyl functional groups of the L-phosphoserine polar head group are required for activation and [³H]PDBu binding; these requirements for [³H]PDBu binding clearly do not reflect substrate aggregation.

Some differences in the abilities of the phospholipid analogues to support [³H]PDBu binding and protein kinase C activity were observed. The carboxylphospholipids (II, III) (but not phosphatidyl-2-hydroxyacetate) and N-acetylated PS (IV) supported [³H]PDBu binding to protein kinase C similar to 1,2-sn-PS at levels up to 12 mol % (30% maximal binding) (Fig. 6); activation of protein kinase C by these analogues did not occur at these concentrations (Fig. 2). Thus, these carboxyl group containing PS analogues supported substantially [³H]PDBu binding but not proportional activation. An inactive protein kinase C-PS analogue-Ca²⁺-PDBu complex is inferred. These data also imply that the PS amino moiety is necessary to form an active complex. The amino group appears to couple [³H]PDBu binding to activation. This bifunctional PS amino group (for maximal [³H]PDBu binding and activation of protein kinase C) is also inferred for its stereosepecificity, e.g., the fact that phosphatidyl-d-serine supports [³H]PDBu binding (Fig. 8) but not activation (Fig. 4).

The precise configuration of amino and carboxyl groups in the phospholipid is necessary for coupling of [³H]PDBu binding to activation of protein kinase C. It is possible that [³H]PDBu binding supported by these analogues could be to ε-protein kinase C which does not phosphorylate histone (54).

The interfacial conformation of the phospholipid appeared to be important as evidenced by low [³H]PDBu binding supported by lysoPS. These PS derivatives and analogues are new tools for dissection of the mechanism of [³H]PDBu binding and activation.

Functional Groups of Phospholipid Involved in Protein Kinase C Binding to Mixed Micelles—Previous studies on protein kinase C translocation to membranes have generated contradictory data with respect to the role of DAG or phorbol ester. Ca²⁺ alone can induce protein kinase C translocation to membranes (53) or Ca²⁺ can act synergistially with phorbol esters (30, 31, 55, 56). The method employed in this report, a direct measurement of protein kinase C activity bound to mixed micelles, allows the number and type of phospholipids present per mixed micelle to be systematically and independently varied. The method is, however, time consuming, and requires 150 mM NaCl in elution buffer. Under these conditions, DAG is involved in the binding of protein kinase C to mixed micelles containing PS in the presence of 100 μM CaCl₂. The binding of protein kinase C to mixed micelles in the presence of Ca²⁺ alone was not detected.

These direct binding studies further showed that the carboxyl group or unsubstituted phosphate group of phospholipids is necessary for the protein kinase C binding to the micelles in the presence of 2 mol % of 1,2-sn-dioleoylglycerol. Since these carboxylphospholipids cannot support protein kinase C activation, the data show clearly that the binding of protein kinase C to the micelles is not sufficient for activation. The amino group moiety of phospholipid polar head group appears to have a role of coupling the binding to the activation of the enzyme.

PA May Be a Special Case—PA is effective in serving as a lipid cofactor for activation (60% of the maximal activity seen with PS; Fig. 5 and Table II), [³H]PDBu binding (40% of that observed with PS, Fig. 9), and binding to mixed micelles in a Ca²⁺- and DAG-dependent manner (Fig. 10). Interestingly, PA does not activate protein kinase C without preincubation with the substrate histone in the assay mixture. However, under both conditions (with and without preincubation with histone), PA can supplement the phospholipid cofactor to support the full protein kinase C activation at low concentrations of PS. PA may be acting with histone to perturb the structure of protein kinase C such that activation occurs in vitro in a nonphysiological manner. Thus, of the cellular phospholipids, PA like PS possesses the ability to support (or potentiate) activation and [³H]PDBu binding. Unlike PS which has not been shown to change in concentration in vitro.

1) The aggregation of histone by PS does not depend on the presence of CaCl₂ and DAG, but activation of protein kinase C by PS absolutely required CaCl₂ and DAG.

2) The aggregation of histone by PS increases linearly by increasing the PS concentration up to 20 mol % in the enzyme assay conditions, but the activity is saturated at 10 mol %. 3) Phosphatidyl-2-hydroxyacetate, phosphatidylpropionate, phosphatidylinositol-4-phosphate, and phosphatidyl-4,5-bisphosphate do not activate protein kinase C, but cause aggregation with histone.

response to signal transduction processes, PA does, in fact, increase during signal transduction processes (57-59). PA increases in response to extracellular agents via activation of a GTP, G-protein-dependent phospholipase D (59). Therefore, the possibility exists that PA may physiologically regulate protein kinase C.

Other Lipids Supporting Protein Kinase C Activation—Unsaturated fatty acids (60-63), retinoic acid (64), and short chain PCs (65) have been shown to activate protein kinase C to activities comparable to those obtained with PS. These reports appear contradictory to the data presented here. While the concentrations of unsaturated fatty acids, retinoic acid, and short chain PC reported to support activity were below their critical micellar concentration, the mechanism of activating lipids as monomers or premicellar aggregates may be different from those in mixed micelles or vesicles. Thus, the structure of protein kinase C may be perturbed in a nonphysiological manner and become active. These agents may cause the pseudo-substrate region of protein kinase C to be released from the active site and induce an active conformation by acting on sites which do not function in physiological activation. More study is required to define physiologically relevant mechanisms of protein kinase C activation.

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REFERENCES

EXPERIMENTAL PROCEDURES

**Materials:**
- 1,2-Di(n-decyl)-glycerol-3-phosphorylcholine (DOPC), 1,2-dipalmitoyl-PC (DPPC), 1,2-distearoyl-PC (DSPC), and 1,2-dioleoyl-PC (DOTPC)
- Transferrin, bovine serum albumin, and bovine plasma cholesterol
- N,N′-dioctadecyl-3,3',3″-tetramethylindocarbocyanine dihydrochloride (DiIC18)
- Imaging buffer (IB): 4mM HEPES, pH 7.5, 0.5% (w/v) Triton-X-100, 50mM NaCl

**Preparation of Lipid-Transferrin Complex:**
Mixtures of lipids were mixed with 100 µg of transferrin in a 2:1 molar ratio at a lipid concentration of 1mM. The mixture was applied to a Sephadex G-25 gel filtration column equilibrated with 50mM NaCl and 0.5% Triton-X-100. The column was eluted with the same buffer at a flow rate of 1ml/min. The fractions containing the lipid-transferrin complex were pooled, concentrated, and stored at -20°C.

**Gel Filtration:**
The protein kinase C complex was purified by gel filtration on Sephadex G-25 at room temperature. The column was eluted with the same buffer at a flow rate of 1ml/min. The fractions containing the protein kinase C complex were pooled, concentrated, and stored at -20°C.

**Affinity Chromatography:**
The protein kinase C complex was purified by affinity chromatography on a phospholipid-Sepharose column. The column was equilibrated with 50mM NaCl and 0.5% Triton-X-100. The column was eluted with the same buffer at a flow rate of 1ml/min. The fractions containing the protein kinase C complex were pooled, concentrated, and stored at -20°C.

**Electrophoretic Mobility Shift Assay:**
The protein kinase C complex was analyzed by electrophoretic mobility shift assay on a native polyacrylamide gel. The gel was run at room temperature and the sample was loaded at the top of the gel. The gel was stained with Coomassie brilliant blue and photographed.

**Phosphorylation Assays:**
The protein kinase C complex was assayed for its ability to phosphorylate different substrates. The reactions were performed in a buffer containing 50mM Tris-HCl, pH 7.5, 5mM MgCl2, 1mM DTT, and 1µM ATP. The reactions were started by the addition of the protein kinase C complex. The phosphorylation reactions were terminated by the addition of SDS-sample buffer and boiled for 5min. The samples were resolved on a SDS-polyacrylamide gel and analyzed by autoradiography.

**Inhibitory Assays:**
The protein kinase C complex was assayed for its ability to inhibit different PKC isoforms. The reactions were performed in a buffer containing 50mM Tris-HCl, pH 7.5, 5mM MgCl2, 1mM DTT, and 1µM ATP. The reactions were started by the addition of the protein kinase C complex. The reactions were terminated by the addition of SDS-sample buffer and boiled for 5min. The samples were resolved on a SDS-polyacrylamide gel and analyzed by autoradiography.

**Effect of Lipid Composition:**
The protein kinase C complex was assayed for its ability to phosphorylate different substrates in the presence of different lipids. The reactions were performed in a buffer containing 50mM Tris-HCl, pH 7.5, 5mM MgCl2, 1mM DTT, and 1µM ATP. The reactions were started by the addition of the protein kinase C complex. The reactions were terminated by the addition of SDS-sample buffer and boiled for 5min. The samples were resolved on a SDS-polyacrylamide gel and analyzed by autoradiography.

**Conclusion:**
The protein kinase C complex was purified by affinity chromatography on a phospholipid-Sepharose column. The purified protein kinase C complex was characterized by electrophoretic mobility shift assay and phosphorylation assays. The purified protein kinase C complex was used for further studies on its role in cell signaling pathways.

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*Note: The text is partially redacted due to the nature of the content.*
Phospholipid Functional Groups for Protein Kinase C Activation

Table I

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<td>339, 404, 702(M+Na)+, 801(M+2Na)+</td>
</tr>
<tr>
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<td>.08</td>
<td>339, 404, 702(M+Na)+, 801(M+2Na)+</td>
</tr>
<tr>
<td>XV</td>
<td>.08</td>
<td>339, 404, 702(M+Na)+, 801(M+2Na)+</td>
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<tr>
<td>XVI</td>
<td>.03</td>
<td>339, 404, 702(M+Na)+, 801(M+2Na)+</td>
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Table II

<table>
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<tr>
<th>Compounds</th>
<th>Protein Kinase C activity (pmol/min) at</th>
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<tr>
<td>1</td>
<td>5 mol%</td>
</tr>
<tr>
<td>1,2-pg-PA</td>
<td>4.3</td>
</tr>
<tr>
<td>I</td>
<td>2.4</td>
</tr>
<tr>
<td>II</td>
<td>7.1</td>
</tr>
<tr>
<td>III</td>
<td>5.0</td>
</tr>
<tr>
<td>IV</td>
<td>3.4</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>3.6</td>
</tr>
<tr>
<td>VII</td>
<td>4.4</td>
</tr>
<tr>
<td>VIII</td>
<td>4.4</td>
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<tr>
<td>IX</td>
<td>1.8</td>
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<tr>
<td>X</td>
<td>2.3</td>
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<tr>
<td>XI</td>
<td>1.9</td>
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<tr>
<td>XV</td>
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<tr>
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<td>6.4</td>
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Palmitate 6.4  4.1  4.7  0.1