Activation of Protein Kinase C in Lipid Monolayers*

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The potential of lipid monolayers spread at an air-water interface was investigated as a well defined membrane model able to support protein kinase C (PKC) association and activation. PKC association to a mixed phospholipid film (phosphatidylcholine, phosphatidylserine) could be detected by an increase of the monolayer surface pressure. This association was strikingly dependent upon the presence of submicromolar concentrations of Ca\(^{2+}\). The effect of Ca\(^{2+}\) resulted in an increase of the PKC penetration into the lipid core at a given permissive surface pressure as well as in a marked increase of the critical surface pressure (29–38 dynes/cm) above which the enzyme was excluded from the membrane. Inclusion of diacylglycerol or tetradecanoyl phorbol acetate (TPA) did not modify the PKC-monolayer association in a detectable manner. PKC associated to the lipid layer exhibited the expected catalytic property and was fully activated when diacylglycerol or TPA was included in the membrane. PKC activity was highly dependent upon the surface pressure of the lipid monolayer, being optimal between 30 and 35 dynes/cm.

Study of the compression isotherm of various diacylglycerol structures revealed that all potent PKC agonists exhibited an expanded liquid phase behavior with collapse pressure below 40 dynes/cm, in contrast to weak activators which showed condensed isotherms with high collapse pressure (~60 dynes/cm). These observations showed that the lipid monolayer system is well adapted to the study of the molecular mechanisms involved in the regulation of PKC activity at a model membrane interface. They are in line with the suggestion of a major role of Ca\(^{2+}\) in the association (translocation) of PKC to membrane in living cell and suggest that diacylglycerol (and TPA) might activate membrane-associated PKC through local change in the surrounding lipid phase organization.

The phospholipid- and calcium-dependent protein kinase

C (PKC)\(^*\) is widely considered as a ubiquitous cell component involved in signal transduction after receptor-mediated activation of phosphoinositide hydrolysis. Subsequent generation of diacylglycerol (DAGs) and inositol trisphosphate is believed to trigger a Ca\(^{2+}\)-dependent association of PKC to plasma membrane concomitant with its activation (1–3). Several tumor-promoting agents (e.g. phorbol esters) are able to substitute for DAG in this activation process, and PKC is considered to represent the active phorbol ester receptor in the living cell (4). On the basis of reconstitution experiments using mixed micelles, a model of protein kinase C activation in the presence of phosphatidylserine (PS), DAG, and Ca\(^{2+}\) has been proposed, involving a supramolecular complex between one protein kinase molecule, one DAG, four PS molecules, and Ca\(^{2+}\) ions (5). However, little is known about the nature of the interactions among the components of the active complex. Although a negatively charged phospholipid species (e.g. PS, cardiolipin) is required for optimal PKC activation, it is not known whether the organization of the lipid layer might affect the interaction of protein kinase C with hydrophobic domains of phospholipids and its activation. On the other hand, activation of PKC may also be dependent upon the nature of the protein substrate (6) and its presentation under a micellar or aggregated form (7, 8). The best defined experimental approach that has yet been developed for studying PKC activation using a membrane model may be the mixed micelle system (9, 10). The physical properties and the stability of the liposome system are not easily controlled, especially during the time span of the reactions studied.

In this study, we investigated the usefulness of lipid monolayers at an air-water interface as a well defined and simple membrane model to examine PKC interaction with lipids and its activation at the membrane level.

MATERIALS AND METHODS

Beef brain phosphatidylcholine and phosphatidylserine, 1,2-diacyl-sn-glycerol were purchased from Serdar Research Laboratories (London, Ontario). Phorbol esters were purchased from Sigma. The lipids were purified further by thin layer chromatography using standard systems for neutral (hexane/ethyl acetate, 3:1 (v/v)) or polar (chloroform/methanol/NH\(_4\), 2:1:0.1 (v/v)) lipids.

PKC Preparations—Protein kinase C was purified from a rat brain soluble fraction using a four-step protocol adapted from (11) including DEAE-cellulose, ACA-4A gel filtration, phenyl-Sepharose, and protamine-agarose affinity chromatography. Protein kinase C activity was assayed as described previously (12).

*The abbreviations used are: PKC, protein kinase C; DAG(s), diacylglycerol(s); PC, phosphatidylcholine; PS, phosphatidylserine; TPA, tetradecanoyl phorbol acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenebis(oxyethylene-nitriU))tetrasuccinic acid.

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Liposome System for PKC Activity Assay—To optimize DAG-specific activation of PKC, we adapted a liposome assay system according to (13), using defined phospholipid vesicles (PC, PS, 4:1 (mol/mol)). Briefly, phosphatidylcholine, phosphatidylethanolamine, and diacylglycerol dissolved in chloroform were mixed and taken to dryness under nitrogen. Mixed micelles were formed at 30 °C by addition of buffer (100 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA and 100 mM β-octyl glucoside (Boehringer Mannheim)). The suspension was diluted 100-fold before use for detergent elimination from micelles and to allow formation of liposomes. Protein kinase C activity was measured using histone H2, as the protein substrate in a phosphorylation buffer made of 20 mM HEPES, pH 7.5, containing 2.5 mM EGTA, 5 mM free MgCl2, 1 μM free CaCl2, [γ-32P]ATP (0.5 Ci/mmol), and 10 μM ATP.

Lipid Monolayer and Related Assay Technique—The apparatus (KSV-Helsinki) and the data treatment used have been described in detail previously (14). The lipids dissolved in CHCl3 were carefully spread at the air-water interface from a Hamilton syringe. Surface pressure area isotherms were recorded using a compression rate of 120 mm/min. The aqueous phase was made of 150 mM NaCl and 2.5 mM EGTA. Surface pressure area curves were determined at the air-water interface using a 249.5-mm-long and 148.5-mm-wide Teflon trough. The surface tension was recorded using a Beckman LM500 electrobalance.

Measurement of PKC Interaction with the Lipid Monolayer—Monolayers were formed by applying the appropriate amount of lipids at the surface of the aqueous phase (4 mL in a 3-cm-diameter circular Teflon trough in order to obtain the desired surface pressure as indicated. The aqueous subphase was stirred continuously using a magnetic bar. Surface pressure measurements were performed following the Wilhelmy method (14) after injection of 5 μL of purified PKC into the subphase. The surface pressure increase was recorded continuously. The aqueous phase consisted of 20 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 5 mM MgCl2, and 2.5 mM EGTA. The Ca2+ concentration was adjusted using a microcomputer program adapted by Michel-Villaz (Biophysique Moléculaire et Cellulaire, Centre d'Études Nucléaires Grenoble, France) to obtain controlled free Ca2+ concentrations as indicated and as described in (15).

PKC Activity at the Monolayer Interface—Phospholipid monolayers containing various cofactors (DAG, phospholipase C as indicated) were spread at the air-water interface of a 4-ml trough filled with a subphase consisting of HEPES buffer, 20 mM, pH 7.5, containing 2.5 mM EGTA. The subphase was adjusted to 1 μM free Ca2+, 10 μM [γ-32P]ATP (2 Ci/mmol). The protein substrate (6 μg of histone H2) was first added into the subphase and the monolayer left to stabilize (30 min). Purified enzyme (20 ng) was then injected to initiate the phosphorylation reaction, which was stopped by adding an excess of unlabeled ATP (0.3 mM final concentration) into the subphase. The phosphorylated lipid was then removed by aspiration (16), and an equal volume of subphase was withdrawn. Both samples were treated with 12.5% trichloroacetic acid in the presence of casein (15 μg/ml) as a protein carrier. The precipitates were collected after centrifugation, washed twice, resuspended in 0.1 ml of 1 M NaOH, and the radioactivity associated with the lipids determined using a scintillation counter. The PKC activity associated with the lipid monolayer was calculated as the difference between the radioactivity incorporated into the protein recovered with the lipid layer minus that measured in an equal volume of the subphase. This difference constituted the interfacial excess of the phosphorylated protein and was thus referred to as the interfacial PKC activity. In this procedure, we assumed that the histone distribution between the subphase and interphase was stable during the assay. We showed (see Table II) that phosphorylation of histones probably does not induce dissociation of this substate from the lipid film by measuring the distribution between the subphase and the interface of prephosphorylated histones with various specific activity.

RESULTS

Association of Protein Kinase C with a Lipid Monolayer—Association of a protein with a lipid monolayer spread previously at a given initial surface pressure (initial pressure, π0) may induce an increase in this surface pressure (Δπ). This increase is due mostly to the interaction of the protein with the hydrophobic domains of the lipids. Proteins that penetrate into the hydrocarbon region of the membrane should generate marked surface pressure changes whereas proteins that only absorb at the surface of the polar headgroups should cause small or undetectable surface pressure increases (17–19).

The interaction of PKC with a mixed phospholipid (PC/PS) monolayer was examined by recording the surface pressure change induced in the membrane upon injection of the kinase into the aqueous subphase. The experiments were conducted at different initial surface pressures (from 12 to 30 dynes/cm) and in the presence or absence of calcium. Fig. 1A shows that PKC penetration into the lipid layer (as reflected by the amplitude of the corresponding surface pressure increase) was inversely related to the value of the initial surface pressure of the membrane, as might have been expected. The major observation was that at all permissive initial surface pressures examined, the presence of Ca2+ (1 μM free) strikingly enhanced PKC insertion into the lipid film.

Graphic treatment of the data (Fig. 1) allowed extrapolation to zero surface pressure change, which yielded the critical surface pressure above which the protein can no longer penetrate the lipid monolayer (20). In the case of protein kinase C, the presence of Ca2+ into the subphase induced a marked increase in this critical surface pressure (29 dynes/cm in the presence of EGTA and 38 dynes/cm in the presence of 1 μM free Ca2+). These observations mean that in the absence of Ca2+, PKC could not penetrate into the lipid layer exhibiting a surface pressure higher than 28 dynes/cm. By sharp contrast, the presence of 1 μM Ca2+ confers to the enzyme the ability to associate into the membrane at high surface pressure.

In another set of experiments, DAG or tetradecanoylphorbol acetate (TPA) was included in the PC/PS phospholipid monolayer. As illustrated in Fig. 1B, critical surface pressures obtained in the presence of Ca2+ for PC/PS/DAG and PC/PS/TPA monolayers were 37 and 39 dynes/cm, respectively.

PKC Activation at the Lipid Monolayer Interface—Having at hand an experimental system able to mimic PKC association to biological membranes, we then investigated the activation of PKC upon its association with the lipid monolayer. As illustrated in Table I, PKC was optimally activated in the presence of Ca2+ (1 μM) and DAG (or TPA), provided that the lipid layer contained PKC. Phosphatidylcholine monolayers were unable to support PKC activation, even in the

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Interaction of protein kinase C with phospholipid monolayers, as detected by membrane surface pressure increase. The monolayer, constituted of different lipids, was adjusted to various initial surface pressures (π0). Purified protein kinase C (5 μg) was injected into the aqueous subphase beneath the lipid layer, and the increase in surface pressure (Δπ) was monitored until stabilization and recorded. Δπ was plotted against π0, yielding a linear relationship that allowed extrapolation to obtain the critical initial surface pressure excluding PKC from the monolayer. A, PC/PS (4:1) monolayers in the absence (○) or in the presence (△) of 1 μM Ca2+ in the subphase. B, PC/PS/DAG (4:1:0.5) monolayer in the absence (○) or in the presence (△) of 1 μM Ca2+. PC/PS/TPA (4:1:0.05) monolayer (*) in the presence of 1 μM Ca2+. In all cases the subphase was 2.5 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 2.5 mM EGTA.
**Activation of Protein Kinase C in Lipid Monolayers**

**Potency of phospholipid monolayers of different compositions to support PKC activity at the membrane interface**

Protein kinase C activity associated with the lipid monolayer was assayed as described under "Materials and Methods." The protein substrate (histone H_i) was injected beneath the lipid monolayer prior to the introduction of purified PKC (20 ng). Interfacial PKC activity was calculated as the difference in trichloroacetic acid-precipitable 32P radioactivity between a withdrawn monolayer sample and an equal volume of the subphase. DAG, 1,2 dioleoyl-sn-glycerol.

| Lipid monolayer components | PKC activity | Subphase containing |
|----------------------------|--------------|---------------------|
|                            | cpm          | Calcium (1 μM) Histone H_i (2.5 μg/ml) |
| PC                         | 209          | +                   |
| PS                         | 1418         | +                   |
| PS/DAG                     | 6989         | + +                |
| PS/TPA                     | 6157         | + +                |
| PS/4n-phorbol              | 632          | + +                |
| PS                         | 395          | 0 +                |
| PS/DAG                     | -67          | + 0                |

**TABLE II**

**Distribution of histones between the interface and the subphase after their injection below a monomolecular lipid film**

Histone H_i is phosphorylated by PKC under standard conditions for 25 min and then dissociated from free ATP by filtration on a Sephadex G-50 column. Various amounts of the phosphorylated labeled histone are completed with nonphosphorylated histones in the ratio given in the Table. 6 μg of these solutions was then injected below a lipid film (PS/DAG, 2:1) formed at the surface of a 5 ml trough containing buffer as described for PKC activity assay in monolayers. The surface pressure of the film was adjusted to 30 dynes/cm. After stabilization of the surface pressure, equal volumes of the interface and the subphase were aspirated and counted in liquid scintillation. Data shown are the radioactivity detected at the surface and in the subphase for a standard volume of 100 μl.

| Phosphorylated histones | Interface | Subphase |
|-------------------------|-----------|----------|
| %                       | %         | %        |
| 100                     | 2021      | 77.6     | 583 | 22.4 |
| 75                      | 1458      | 81.1     | 137 | 18.8 |
| 50                      | 560       | 76.5     | 171 | 23.5 |
| 25                      | 324       | 79.6     | 83  | 20.4 |

The presence of Ca2+. Inactive phorbol esters (e.g. 4n-phorbol) were inactive as compared with TPA. The influence of the phospholipid monolayer surface pressure on the activation of PKC as assayed by histone phosphorylation was examined with a phosphatidylserine completed (or not) by DAG or TPA monolayer. For the catalytic assay of PKC, we assumed that histones remain associated at the film after their phosphorylation. As shown in Table II, the state of histone phosphorylation does not affect their interaction with the film. As illustrated in Fig. 2, a progressive increase of PKC activity was noted when the surface pressure was increased up to an optimal level of 35 dynes/cm. Above that pressure, the enzymatic activity was sharply reduced. From Figs. 1 and 2, it is clear that optimal PKC activity was obtained at a surface pressure (35 dynes/cm) close to its critical surface pressure (38 dynes/cm). By comparing data from Figs. 1B and 2, one might notice that a small but significant PKC activity was detected at surface pressures above critical pressures. In the case of PKC assay in monolayers (Fig. 2), histones added to the film could slightly modify the critical surface pressures of PKC penetration into the monolayer.

Compressn Isotherms of Various Dioctylglycerols and PKC Activation—Differences in the potency of various DAGs as activators of PKC have been reported previously (21) in

**FIG. 2.** Protein kinase C activation at the lipid monolayer interface, as a function of the surface pressure of the membrane. The monolayer containing different lipids as indicated was spread over the aqueous subphase made of 20 mM HEPES buffer, pH 7.5, containing 10 μM magnesium, 10 μM ATP, and [γ-32P]ATP (final specific activity 2 Ci/mmol). Histone H_i (6 μg) was injected beneath the lipid layer. Following a 30-min equilibration time, PKC (20 ng) was injected into the subphase; the surface pressure was recorded during the reaction period and plotted against the interfacial PKC activity assayed as described under "Results" and in the legend of Table I. The monolayers were PS/DAG (2:1), PS/TPA (2:0.1), and PS.

**FIG. 3.** Relative potency of DAG structures with various fatty acyl side chains, as activators of protein kinase C. Protein kinase C activity was determined using the liposome assay as described under "Materials and Methods" in the presence of different DAG structures at increasing concentrations. 1, 1,2-dioleoyl-sn-glycercol; 2, 1,2-dioleoyl-sn-glycercol; 3, 1,2-dioleoyl-sn-glycercol; 4, 1,2-dioleoyl-sn-glycercol; 5, 1,2-dimyristoyl-sn-glycercol; 6, 1,2-dipalmitoyl-sn-glycercol.

search of structure-activity relationships (13). Fig. 3 illustrates the relative ability of different DAG structures to activate PKC in a mixed micelle assay system. The most active compound tested was the unsaturated long acyl chain
pressure correspondingly increased up to a saturation point where the monolayer was collapsed. On one hand, long and unsaturated chain DAG (typically 1,2-dioleoyl-sn-glycerol) showed an expanded liquid film behavior whereas the surface pressure was in the high range values (~11 dynes/cm). This increase is larger than those observed under similar conditions with polypeptides such as myelin basic protein, prothrombin, polylysine (14), or cardiotoxin (20).

The other known positive effectors of PKC (i.e., DAG and TPA) exhibited no detectable additional effect upon interfacial PKC binding. Although DAG may exert topological effects on the lipid layer organization related to PKC activity and not detectable by the present method, these observations are in agreement with previous reports using fluorescence energy transfer and light-scattering intensity measurements (6, 7, 26).

Calcium appeared to be the key factor governing optimal PKC penetration into the lipid core, even at high surface pressure (>30 dynes/cm). For instance, at 30 dynes/cm, the increase in the surface pressure was about 4 dynes/cm when PKC was injected in the subphase at 1 μg/ml concentration (~11 μM). This increase is larger than those observed under similar conditions with polypeptides such as myelin basic protein, prothrombin, polylysine (14), or cardiotoxin (20). The monolayer experimental device allowed the study of the PKC-membrane interaction at various initial surface pressures and the determination of a critical pressure above which the kinase is excluded from the membrane. The data pointed to the fact that a permissive initial surface pressure is required for the kinase to be able to associate with the lipid layer. The corresponding values were 29 and 38 dynes/cm, in the absence and in the presence of 5 mM Ca2+, respectively (8). It may be recalled that membrane surface pressure in living erythrocytes has been estimated to be 31 dynes/cm (27, 28). Such a value would be compatible with optimal association of PKC to living cell membrane and is in support of the proposal that Ca2+ may be the key intracellular messenger triggering PKC translocation to membrane, together with its association with the hydrophobic core of the phospholipids, thus resulting in a "primed" state of the kinase prior to its full activation at the membrane level (29).
In the present study, the lipid monolayer system was shown to represent a convenient support for examining PKC activity at the membrane interface. Kinase activation assayed in this system obeyed its established requirements (i.e., charged phospholipid such as PS, presence of Ca²⁺) and could be expressed fully when DAG or TPA was included in the lipid membrane. These data are in line with those obtained with other forms of lipid supports such as mixed micelles (8, 10) and confirm that a bilayer organization is not necessary to support full PKC activation (21). Our data are in contrast with the report that PKC was unable to phosphorylate histone H₁ at a phospholipid monolayer interface (8). The negative results reported by these authors may simply be explained by the fact that in their work the kinase was introduced in the aqueous subphase prior to its protein and nucleotide substrates. It has been well documented that preincubation of PKC in the presence of its lipid cofactors and in the absence of ATP induces an irreversible inactivation of the kinase (30). In addition, the authors introduced the protein substrate at a very high concentration with regard to that of the monolayer lipids. A high protein/phospholipid ratio, especially as PS and histones bear opposite charges, may result in the impairment of local phase transition in the membrane lipid organization, which could contribute to PKC activation (31). This hypothesis would be in line with studies showing that PKC-activating components are membrane bilayer destabilizing agents whereas PKC-inhibiting components behave as membrane stabilizers (32, 33).