Activation of Protein Kinase C by Coexisting Diacylglycerol-Enriched and Diacylglycerol-Poor Lipid Domains†

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ABSTRACT: To test the hypothesis that activation of protein kinase C (PKC) is related to the interface between coexisting diacylglycerol- (DAG-) enriched and DAG-poor phases, the thermotropic phase behavior of the ternary mixtures dimyristoylphosphatidylcholine (DMPC)/dimyristoylphosphatidylserine (DMPS)/dioleoylglycerol (DO), DMPC/DMPS/1-palmitoyl-2-oleoylglycerol (PO), and DMPC/DMPS/dimyristoylglycerol (DM) was analyzed and compared with the ability of the lipid mixtures to support PKC activity. Differential scanning calorimetry (DSC) was used to monitor the gel-to-liquid crystalline phase transition as a function of the mole fraction of DO (χ_{DO}), PO (χ_{PO}), or DM (χ_{DM}) in DMPC/DMPS (1:1) multilamellar vesicles (MLVs) and of χ_{DO} in large unilamellar vesicles (LUVs). The addition of DAG at low mole fractions gave rise to the appearance of two or more overlapping transitions. The phase boundaries of the ternary mixtures deduced from the partial phase diagrams were $\chi_{DO} = \sim 0.10$ and ~ 0.3 for DMPC/DMPS/DO, $\chi_{PO} = \sim 0.05$ and ~ 0.4 for DMPC/DMPS/PO, and $\chi_{DM} = \sim 0.025$ and $\sim 0.5-0.6$ for DMPC/DMPS/DM. Above these mole fractions of DAG, the transitions again became very sharp. The ability of the lipid mixtures to support activity of PKC α and PKC η was examined below and above the gel-to-liquid crystalline phase transition. In the gel phase, PKC activity went through a maximum as a function of increasing mole fraction of each DAG and was restricted to lipid compositions in which coexisting phases were observed. Maximal activity decreased with increasing saturation of the DAG. In the fluid state, maximal PKC activity was shifted to higher DO mole fractions and the peak was much broader. Collectively, these data support a role for both the presence and nature of interface between compositionally distinct domains in activation of PKC.

Protein kinase C (PKC)[1] is a family of serine and threonine-specific protein kinases implicated in cell growth and differentiation [reviewed in Stabel Parker (1991) and Nishizuka (1988)] as well as in carcinogenesis [reviewed in Basu (1993)]. All eukaryotic cells contain some PKC family members but isozyme distribution varies widely. Most PKC isozymes are activated by diacylglycerol (DAG), and all are activated by phosphatidylserine (PS). The isozymes share conserved regions which are interspersed with regions that vary among isozymes and confer substrate specificity, cofactor dependence, rates of downregulation, and subcellular localization [reviewed in Jaken (1992)]. Cofactors for specific isozymes are Ca^{2+}, DAG, and PS for PKC α, β_I, β_{II}, and γ; DAG and PS for PKC δ, ϵ, η, μ, and θ, and only PS for PKC ξ and λ (Nakamura & Nishizuka, 1994). The conserved amino-terminal regulatory region interacts with membrane cofactors and, except for ξ and λ, PKC binds the DAG analog phorbol ester with a 1:1 stoichiometry. The regulatory region also contains a highly conserved pseudosubstrate site. Activity of PKC on the membrane surface apparently involves a structural rearrangement of the protein-lipid complex with removal of the pseudosubstrate from interaction with the catalytic carboxy-terminal portion of the protein.

Activation of PKC is commonly stimulated by the transient generation of DAG within the inner leaflet of the plasma membrane. DAG is often produced in an initial burst from the phospholipase C-catalyzed hydrolysis of phosphoinositide (PI). Prolonged elevation of DAG often occurs upon stimulation with growth factors acting through hydrolysis of phosphatidylcholine (PC) and is required for cell growth and differentiation (Nakamura & Nishizuka, 1994; Exton, 1990).

Since DAG concentrations required for PKC activation differ widely depending on the phospholipid composition of lipid vesicles used in *in vitro* assays (Bolen & Sando, 1992), we had hypothesized that DAG tends to form DAG-enriched domains within the cellular membrane upon a signal transduction event. DAG induces such phase separation in a variety of equilibrated PC/DAG binary mixtures (Quinn et al., 1995; Lopez-Garcia et al., 1994; Heimburg et al., 1992; Cunningham et al., 1989; De Boeck & Zidovetzki, 1989; Ortiz et al., 1988). PKC activation was found to correspond

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[1] Abbreviations: A23187, Ca^{2+} ionophore antibiotic; DAG, diacylglycerol; DSC, differential scanning calorimetry; DM, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; DO, 1,2-dioleoyl-*sn*-glycerol; DS, 1,2-distearoyl-*sn*-glycerol; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LUV, large unilamellar vesicles; χ, mole fraction; MOPS, 3-morpholinopropanesulfonic acid; MLV, multilamellar vesicles; PC, phosphatidylcholine; PI, phosphoinositide; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PO, 1-palmitoyl-2-oleoyl-*sn*-glycerol; PS, phosphatidylserine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

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with the formation of DAG-enriched domains in both the gel and fluid lipid state (Dibble et al., 1996). In the gel state, activity passed through a maximum under conditions where DO-poor and DO-enriched domains coexist over a range of compositions from χ_{DO} ~0.1 to χ_{DO} ~0.3 in (DMPC/DMPS,1:1)/DO mixtures (Dibble et al., 1996).

In the present work, we further hypothesize that the maximum observed in PKC activation in the gel state is related to the interface between coexisting DAG-enriched and DAG-poor phases. To test this hypothesis, the extent of phase separation in the PC/PS/DAG model membrane system was manipulated by altering acyl chain mismatch of the diacylglycerol. The model membrane system of DMPC/DMPS/DAG (Bolen & Sando, 1992; Boni & Rando, 1985) was selected for two reasons: (1) its high degree of saturated acyl chains enables the phase transition to be monitored by differential scanning calorimetry and, hence, its mixing to be characterized, and (2) because activation of PKC varies extensively over a wide range of DAG concentrations in this system.

MATERIALS AND METHODS

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycerol (DO), 1-palmitoyl-2-oleoyl-*sn*-glycerol (PO) and 1,2-dimyristoyl-*sn*-glycerol (DM) were from Avanti Polar Lipids, Inc. (Birmingham, AL). All were greater than 99% pure as determined by chromatographing 0.1 mg on Adsorbosil-Plus thin-layer chromatography (TLC) plates (Alltech Associates, Inc., Deerfield, IL) using the following solvent systems: for PS, chloroform/methanol/acetic acid/water (50/30/8/4 v/v/v/v); for PC, chloroform/methanol/water (65/25/4 v/v/v); and for DAG, petroleum ether/ethyl ether/acetic acid (160/40/2, v/v/v) to determine purity and chloroform/acetone (95/5, v/v), to determine the extent of isomerization. Hydrated lipid was extracted for analysis before and after DSC and kinase assay experiments. Lipid breakdown did not exceed 1%. DAGs readily undergo acyl chain migration, forming the 1,3 isomer, which does not support PKC activity. We estimated the extent of isomerization of DAG in chloroform stock solutions to be <1% and of DAG in temperature-cycled vesicles to be <10−25%; isomerization is dependent on temperature, time, and type of glass used to store samples (Kodali et al., 1990a; Avanti Polar Lipids, personal communication). 3-Morpholinopropanesulfonic acid (MOPS), ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and potassium chloride were Chemika-grade and the magnesium chloride hydrate was puriss-grade from Fluka Chemical Corp. (Ronkonkoma, NY). Chloroform, methanol, and benzene were HPLC-grade (Fisher Scientific, Pittsburgh, PA) or Baker Analyzed (J. B. Baker, Inc., Bricktown, NJ). The Ca^{2+} ionophore antibiotic A23187 was from Calbiochem Corp. (La Jolla, CA). [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Histone (Type III-S) and phorbol esters were from Sigma Chemical Co. (St. Louis, MO). Grace's culture medium, yeastolate, lactalbumin, and fetal calf serum for the culturing of the Sf-9 insect cells were from Gibco (Grand Island, NY).

Preparation of Solutions. Water was double-distilled through glass. Before weighing, the potassium chloride was dried to a constant weight with gentle heating and the magnesium chloride hydrate was dried to a stoichiometric hydrate, $MgCl_2 \cdot 4H_2O$, by heating at 100 °C for 1 h. The primary standard phosphate solution in water was prepared from an analytical concentrate (J. T. Baker, Inc., Bricktown, NJ). The concentration of phospholipid stock solutions in chloroform was periodically determined by phosphorus analysis (Kingsley & Feigenson, 1979). All lipid stock solutions were stored in the dark, under an argon atmosphere, at −20 °C.

Preparation of Multilamellar Vesicles. Mixtures of DAG and phospholipid were prepared by aliquoting stock solutions of lipid in chloroform into borosilicate culture tubes using gastight syringes (Hamilton Co., Reno, NV). To all samples for DSC and PKC activity assays, the ionophore A23187 in chloroform was added at an ionophore/total phospholipid mole ratio of 1/1000 to facilitate the equilibration of Mg^{2+} across hydrated bilayers. Sample preparation, hydration of samples, temperature cycling, and addition of $MgCl_2$ were identical to the procedure in Dibble et al., 1996. All MLVs were stored in the dark under an argon atmosphere at room temperature.

Preparation of Large Unilamellar Vesicles. LUVs were prepared by extruding 0.5 mL of MLVs, at 50 mM total phospholipid, through a 0.1 mm pore size polycarbonate filter (Costar Scientific Corp., Cambridge, MA) 35 times at a temperature above the gel-to-liquid crystalline phase transition using a home-built handheld extruder. We repeatedly found that dispersions with $\chi_{DO} > 0.35$ were impossible to extrude [see Dibble et al. (1996)]. We attribute this phenomenon to the presence of a phospholipid-poor phase (see Results) when $\chi_{DO} > 0.35$. LUVs that were treated with Mg^{2+} were dialyzed against MOPS/KCl buffer containing 5 mM $MgCl_2$ using a 5000 molecular weight cutoff cellulose ester dialysis membrane in a microdialyzer (both from Spectrum Medical Industries, Inc., Houston, TX) with 0.5 mL capacity wells for a minimum of 12 h at 4 °C. All LUVs were stored at 25 mM total phospholipid in the dark under an argon atmosphere at 4 °C and were stable for many days as determined by quasi-elastic light scattering using a Nicomp Model 370 submicron particle sizer.

Differential Scanning Calorimetry. Heat capacity scans were obtained with a MicroCal MC-2 (Northhampton, MA). All were scanned at +10 °C/h. For LUVs, the phospholipid concentration of each sample was determined by phosphorus analysis after the DSC scans. From the heating scans, onset and termination temperatures (i.e., apparent solidus and liquidus temperatures) of the gel-to-liquid crystalline phase transition were estimated as the two temperatures at which the thermogram deviates from baseline.

PKC Purification. The Ca^{2+}-dependent isozyme PKC α and the Ca^{2+}-independent isozyme PKC η were purified from Sf-9 insect cells infected with recombinant baculovirus containing the specific isozyme construct by sequential chromatography on Q-Sepharose and phenyl-Superose (both from Pharmacia Biotechnology, Uppsala, Sweden). The baculovirus constructs were kindly provided by Drs. P. Parker (Protein Phosphorylation Laboratory, Lincoln's Inn Fields, London, U.K.) and D. Fabbro (Ciba-Geigy, Basel, Switzerland). Enzyme purity was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis followed by silver staining, and by Western blotting using isozyme-specific rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). PKC concentration was

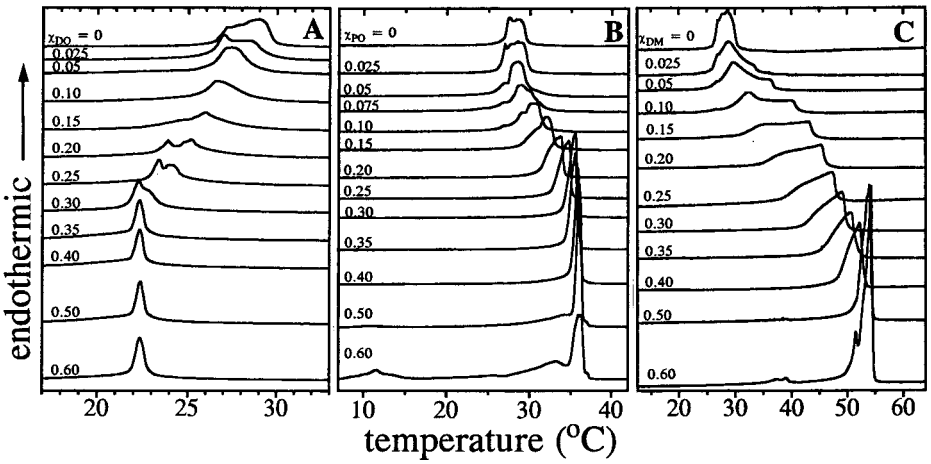

FIGURE 1: Excess heat capacity functions for (DMPC/DMPS 1/1)/DAG MLVs. Each sample had 10 mM phospholipid hydrated in 20 mM MOPS, 100 mM KCl, and 0.1 mM EGTA, pH 7.5. The fourth scan at each χ_{DAG} is shown. (A) Excess heat capacity functions for (DMPC/DMPS 1/1)/DO MLVs. The values of χ_{DO} are indicated within each figure. Thermograms were up-scans from 0 to 60 °C at 10 °C/h. This figure is identical to Figure 3A in Dibble et al., (1996) and is shown here for comparative purposes. (B) Excess heat capacity functions for (DMPC/DMPS 1/1)/PO MLVs. The values of χ_{PO} are indicated within each figure. Thermograms were up-scans from 10 to 70 °C at 10 °C/h. (C) Excess heat capacity functions for (DMPC/DMPS 1/1)/DM MLVs. The values of χ_{DM} are indicated within each figure. Thermograms were up-scans from 10 to 70 °C at 10 °C/h.

determined by a phorbol ester binding assay (Sando & Young, 1983). Purified enzyme was stored in 30% (w/v) glycerol and 20 mM MOPS, pH 7.5, at −80 °C. Enzyme was thawed and diluted with MOPS/KCl buffer just prior to the kinase assays. The enzyme remained 90% intact, as estimated by gel electrophoresis followed by silver staining, after incubation for 1 h at 35 °C.

Assay of PKC Activity. Kinase activity was measured as the ability of PKC α or PKC η to phosphorylate lysine-rich histone III-S. The reaction mixture of 75 μL total volume contained 4 or 5 nM PKC, 4.0 or 2.67 mM total lipid, 9−10 μM histone, 40 μM ATP spiked with [γ-^{32}P]ATP to 1.6−3.7 Ci/mmol, 5 mM $MgCl_2$, 20 mM MOPS, 100 mM KCl, 0.1 mM EGTA, pH 7.5, and $CaCl_2$, buffered with EGTA to 5 μM free Ca^{2+}. Ca^{2+} was omitted in assays using PKC η. Free Ca^{2+} (5 μM) was included in all assays with PKC α to act only as a cofactor for the enzyme. The effect of the low Ca^{2+} concentration on lipid physical properties was expected to be negligible in the presence of 5 mM $MgCl_2$ and 100 mM KCl. All lipid samples assayed for PKC activity had been temperature-cycled, equilibrated with 5 mM $MgCl_2$, and again temperature-cycled, as described in Dibble et al. (1996). Before being aliquoted, the lipid was heated above the gel-to-liquid crystalline phase transition and vortexed extensively. Lipid was equilibrated at the temperature of the assay, PKC was added, and the mixture was further incubated at the desired temperature. The reaction was initiated by adding a temperature-equilibrated substrate mixture: histone, [γ-^{32}P]ATP and Ca^{2+} in MOPS/KCl buffer containing 5 mM $MgCl_2$. Lipid and $MgCl_2$ were preincubated at the temperature of the assay before the addition of PKC for the $MgCl_2$ dependence studies. Substrate and enzyme concentrations were kept low throughout this work to avoid unnecessary perturbation of lipid phase behavior, which does occur at high histone/lipid ratios. Linear reaction kinetics were maintained at all temperatures at which the assay was performed; the reaction was allowed to proceed for 40 min at 2 °C or 45 s at 35 °C with the MLVs and for 15 min at 13 °C, 5 min at 24 °C or 2 min at 35 °C with the LUVs. The reaction was terminated by spotting 60 μL of the reaction mixture onto Whatman P-81 cation-exchanger paper (Whatman International Ltd., Maidstone, England). The papers were washed three times with 50 mM NaCl to remove unreacted ATP. ^{32}P transferred to histone was determined by measuring the Cerenkov radiation emitted from the dried papers. As a control to determine whether enzyme degraded over the duration of the assay, enzyme and lipid were incubated for various times and activity was assayed. Activity did not vary within error over a range of incubation times of 40−120 min at 2 °C and 2−45 min at 35 °C.

RESULTS

Thermotropic Phase Behavior of DMPC/DMPS/DO, DMPC/DMPS/PO, and DMPC/DMPS/DM MLVs. Differential scanning calorimetry was used to monitor the gel-to-liquid crystalline phase transition as a function of mole fraction of DO (χ_{DO}; Figure 1A), PO (χ_{PO}; Figure 1B), or DM (χ_{DM}; Figure 1C) in DMPC/DMPS (1/1) MLVs. The addition of DO, PO, or DM at low mole fractions to DMPC/DMPS gave rise to the appearance of two or more overlapping transitions. Eventually, a sharp component developed with increasing DAG until it dominated the transition curve at $\chi_{DO} > \sim 0.3$, $\chi_{PO} > \sim 0.4$ and $\chi_{DM} > \sim 0.5-0.6$. The phase boundaries of the ternary mixtures were deduced from the mole fractions of DAG over which the solidus line (the onset temperature) remained invariant. Gel−gel immiscibility was detected from $\sim 0.10 < \chi_{DO} < \sim 0.3$ for (DMPC/DMPS 1/1)/DO, from $\sim 0.05 < \chi_{PO} < \sim 0.4$ for (DMPC/DMPS 1/1)/PO, and from $\sim 0.025 < \chi_{DM} < \sim 0.5-0.6$ for (DMPC/DMPS 1/1)/DM (Figure 2). Little variance was observed in the fluidus line (the termination temperature) for the (DMPC/DMPS 1/1)/PO mixture. Fluid−fluid immiscibility may exist from $\sim 0.025 < \chi_{PO} < -0.6$ (Figure 2). Fluid−fluid immiscibility also is suggested in the (DMPC/DMPS 1/1)/DO mixture (Dibble et al., 1996).

In the ternary mixtures of (DMPC/DMPS 1/1)/PO and (DMPC/DMPS 1/1)/DM, second and third cooperative transitions were observed when $\chi_{PO} > \sim 0.4$ at ∼12 and 33 °C and $\chi_{DM} > \sim 0.5$ at ∼37−39 and 52 °C, respectively. The second and third cooperative transitions may be related to the α and β' crystalline forms of pure DAG as shown by

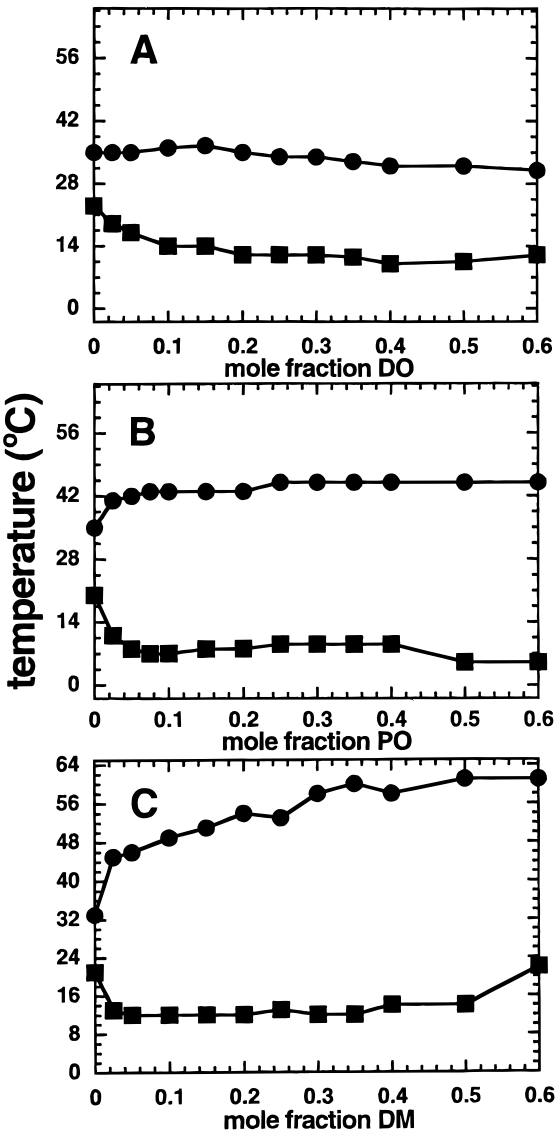

FIGURE 2: Phase diagrams for the ternary mixtures (DMPC/DMPS 1/1)/DO, (DMPC/DMPS 1/1)/PO and (DMPC/DMPS 1/1)/DM. Onset (■) and termination (●) temperatures of the gel-to-fluid phase transition from Figure 1, panels A−C, are plotted as a function of χ_{DO} (A), χ_{PO} (B), and χ_{DM} (C).

Kodali et al. (1990b). The metastable α form melts 15−20 °C lower than the β' crystalline form. The unsaturated DO will have transitions at or near 0 °C, making their detection unlikely [see Dibble et al. (1996) for further details]. Above $\chi_{DO} > \sim 0.3$, $\chi_{PO} > \sim 0.4$, and $\chi_{DM} > \sim 0.5-0.6$ there may be coexistence of a lamellar DAG-enriched phase of composition χ_{DO} ~0.3, χ_{PO} ~0.5, and χ_{DM} ~0.5−0.6 and a separate phase of nearly pure DAG. Similar phase coexistence has been detected in DPPC/DP and DMPC/DM binary mixtures (López-Garcia et al., 1994; Heimburg et al., 1992). It is not known whether this putative, nearly pure, DAG phase resides within the bilayer.

The addition of 5 mM $MgCl_2$ shifted the main transition a few degrees higher and broadened the curves as discussed previously [data not shown; see Dibble et al. (1996)].

Thermotropic Phase Behavior of DMPC/DMPS/DO LUVs. Differential scanning calorimetry was used to monitor the gel-to-liquid crystalline phase transition as a function of mole fraction of DO in DMPC/DMPS (1/1) LUVs. The compositions were limited from $\chi_{DO} = 0$ to $\chi_{DO} = 0.3$ because dispersions with $\chi_{DO} > 0.3$ were impossible to extrude, as discussed previously. As with MLVs, the general trend is for DO to greatly broaden the gel-to-liquid crystalline phase transition and to shift the transition to lower temperatures. Coexisting broad and sharp components within the thermogram were not as obvious with LUVs as with MLVs. The melting of the narrower appearing thermograms of the DO-rich phase is apparently much less cooperative when it is constrained to 100 nm diameter vesicles (data not shown). When LUV samples were equilibrated with 5 mM free Mg^{2+}, the gel-to-liquid crystalline phase transition was shifted a few degrees higher and broadened as with the MLVs (data not shown).

Protein Kinase C Activation As Supported by DMPC/DMPS/DO, DMPC/DMPS/PO, and DMPC/DMPS/DM MLVs and DMPC/DMPS/DO LUVs. The ability of (DMPC/DMPS 1/1)DO (Figure 3A), (DMPC/DMPS 1/1)PO (Figure 3B), and (DMPC/DMPS 1/1)DM (Figure 3C) MLVs equilibrated with 5 mM $MgCl_2$ to activate the Ca^{2+}-dependent isozyme PKC α was evaluated in the gel state to examine the influence of interface on activation of PKC. Lipid domains are more stable in the gel state (Mouritsen & Jørgensen, 1994) and the diffusion of substrate and enzyme is much slower than in the fluid state. In each case activity remained near background at mole fractions of $\chi_{DO} < 0.08$, $\chi_{PO} < 0.05$, and $\chi_{DM} < 0.15$ and then increased to a maximum at $\chi_{DO} = 0.20$, $\chi_{PO} = 0.15$, and $\chi_{DM} = 0.37$, compositions that approximate the midpoint between the phase boundaries of $\chi_{DO} = \sim 0.10$ and ~0.3, $\chi_{PO} = \sim 0.05$ and ~0.4, and $\chi_{DM} = \sim 0.025$ and ~0.5−0.6 (Figure 2). Activity then decreased to levels approximately 20−25% of the maximum at $\chi_{DO} > \sim 0.3$ and $\chi_{PO} > \sim 0.4$. The limited activation supported by DMPC/DMPS/DM decreased to background by $\chi_{DM} > \sim 0.6$. The absolute maximum in activity in the gel phase decreased as the saturation of DAG increased in the order DO > PO >> DM, although the same total lipid, enzyme, and substrate concentrations were used in each case.

Analysis of PKC activation in the fluid state was limited to the ternary mixtures with DO. The high temperatures required to maintain a fluid state for the other ternary mixtures were incompatible with enzymatic assays because of protein denaturation. With (DMPC/DMPS 1/1)/DO MLVs, activity was near background when $\chi_{DO} < 0.08$ but activity increased with increasing χ_{DO} from $\chi_{DO} = \sim 0.08$ to ~0.40. Activity decreased slightly at $\chi_{DO} = \sim 0.60$, suggesting a very broad maximum in activity, shifted to higher χ_{DO} with respect to activity in gel-phase DMPC/DMPS (Dibble et al., 1996). A sharper peak has been observed in PKC activity supported by 1-stearoyl-2-oleoylphosphatidylcholine (SOPC)/1-stearoyl-2-oleoylphosphatidylserine (SOPS)/PO MLVs in the fluid state (data not shown).

To eliminate potential effects of Ca^{2+} on the lipid (Feigenson, 1986, 1989), the ability of the ternary mixture of (DMPC/DMPS 1/1)/DO LUVs to support PKC activity was investigated using a Ca^{2+}-independent isozyme, PKC η. The ability of (DMPC/DMPS 1/1)/DO LUVs equilibrated with 5 mM free Mg^{2+} to activate PKC η was examined below (13 °C), within (24 °C), and above (35 °C) the gel-to-liquid crystalline phase transition (Figure 4). At 35 °C, PKC activity was extremely low when $\chi_{DO} \leq 0.10$ but activity increased gradually with increasing χ_{DO} from χ_{DO} ~0.10 to χ_{DO} ~0.30. At temperatures below and within the gel-to-liquid crystalline phase transition, the activation of PKC η

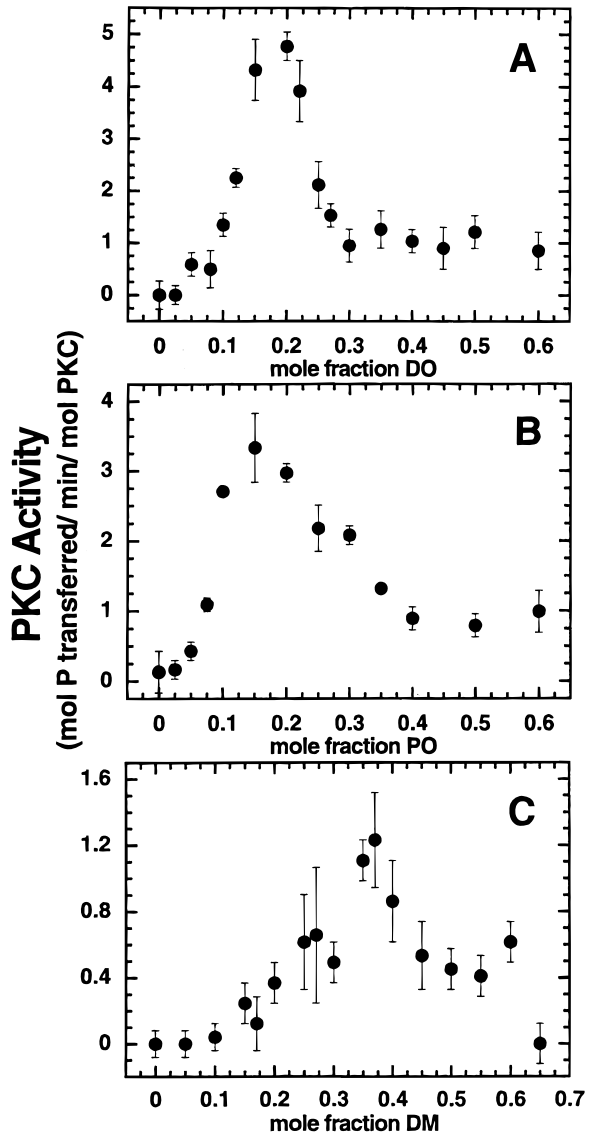

FIGURE 3: PKC α activity as a function of χ_{DAG} in (DMPC/DMPS 1/1)/DAG MLVs at 2 °C. Each sample contained 2.67 mM total lipid and 5 nM PKC α. The amount of ^{32}P transferred to histone was measured for 40 min at 2 °C. Each point represents the mean ± SD of triplicate determinations. A lipid-free background was subtracted from each point and the errors were added in all cases. (A) PKC α activity as a function of χ_{DO} in (DMPC/DMPS 1/1)/DO MLVs. Maximal phospholipid-dependent activity at $\chi_{DO} = 0.20$ was 106 000 ± 6000 cpm with a background of 23 000 ± 3000 cpm. Plot shown is representative of four independent assays. This figure is identical to Figure 7B in Dibble et al. (1996) and is shown here for comparative purposes. (B) PKC α activity as a function of χ_{PO} in (DMPC/DMPS 1/1)/PO MLVs. Maximal phospholipid-dependent activity at $\chi_{PO} = 0.15$ was 101 000 ± 15 000 cpm with a background of 17 000 ± 700 cpm. Plot shown is representative of two independent assays. (C) PKC α activity as a function of χ_{DM} in (DMPC/DMPS 1/1)/DM MLVs. Maximal phospholipid-dependent activity at $\chi_{DM} = 0.15$ was 30 000 ± 7000 cpm with a background of 12 000 ± 2000 cpm. Plot shown is representative of three independent assays.

exhibited a maximum as a function of χ_{DO} and then decreased, as with PKC α. The maximum in activity occurred at χ_{DO} ~0.15 and ~0.20 at 13 and 24 °C, respectively. At 35 °C, no significant activity was detected below $\chi_{DO} = 0.05$, but PKC was activated from $\chi_{DO} = 0.10$ to 0.30, the highest DO content analyzed.

The Arrhenius plot of the phospholipid-dependent activity of PKC η of 38 ± 4, 64 ± 3, and 121 ± 5 [mol of

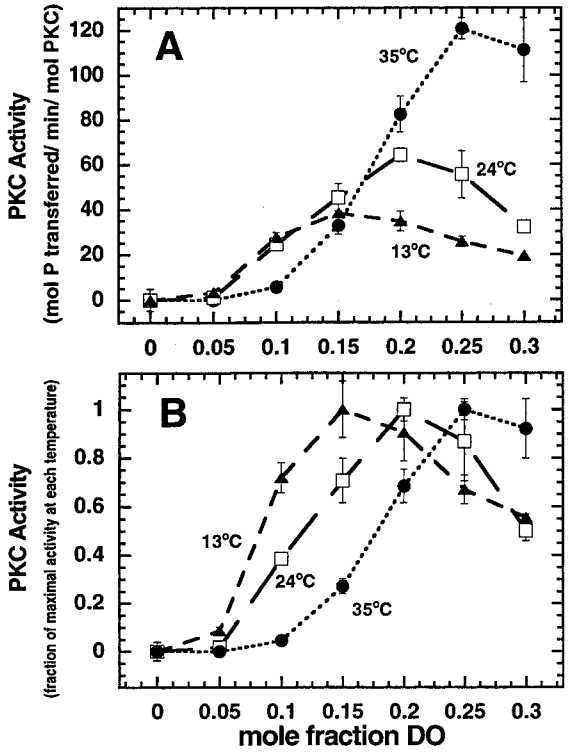

FIGURE 4: PKC η activity as a function of χ_{DO} in (DMPC/DMPS 1/1)/DO LUVs at 35, 24, and 13 °C. Activity is plotted as (A) moles of total phosphorus transferred to lysine-rich histone per minute per mole of PKC or as (B) fraction of maximal activity at each temperature. Each sample contained 4.0 mM total lipid and 5 nM PKCη. Other reaction conditions were as in Figure 3 with the exception of the omission of Ca^{2+}. The amount of ^{32}P transferred to histone was measured for 15, 5, and 2 min at 13, 24, and 35 °C, respectively. For panel A, maximal activity was 350 000 ± 40 000 cpm with a background of 28 000 ± 3000 cpm at 13 °C; 214 000 ± 10 000 cpm with a background of 29 000 ± 2000 cpm at 24 °C; and 151 000 ± 6000 cpm with a background of 28 000 ± 2000 cpm at 35 °C. For panel B, activity at each temperature was normalized to 1 at the composition with the highest activity. Each point represents the mean ± the range of duplicate determinations. A lipid-free background was subtracted from each point and the errors were added. Plot shown is representative of two independent assays.

phosphorus transferred to histone (mol of PKC)$^{-1}$] min^{-1} at the compositions providing maximal activity for each temperature, i.e., $\chi_{DO} = 0.15$ at 13 °C, 0.20 at 24 °C, and 0.25 at 35 °C, yielded an apparent activation energy, ΔE_a, of 9.5 kcal/mol (Figure 5). This activation energy is consistent with the interpretation that the observed temperature dependence for maximal PKC activity is simply due to an increase in the intrinsic reaction rate of the enzyme with temperature (Figure 4A). However, this does not explain the shift in the activity maximum to higher mole fractions of DO with increasing temperature (Figure 4B). This shift may be a consequence of dissipation of DO-enriched domains at higher temperatures, shifting the mole fraction of DO needed to induce the formation of DO-enriched domains to higher mole fractions.

To address the potential effects of Mg^{2+} on formation of DAG-enriched and DAG-poor domains, the effect of Mg^{2+} on activity was examined with varying χ_{DO} in (DMPC/DMPS 1/1)/DO MLVs. PKC requires Mg-ATP as a substrate and the K_D for Mg^{2+} dissociation from ATP is 100 μM, necessitating high concentrations of Mg^{2+} in the kinase assays (Hannun & Bell, 1990). PKC also may have low-

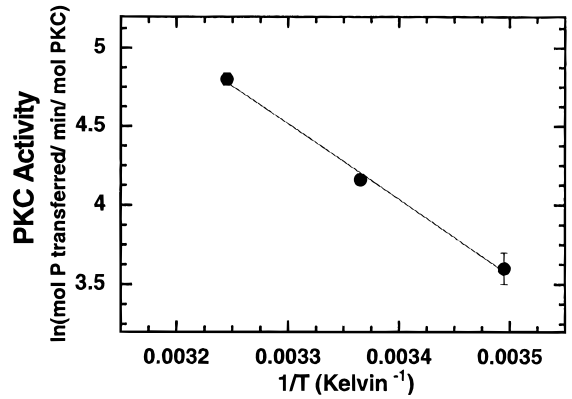

FIGURE 5: Arrhenius plot of the temperature dependence of reaction rate of PKC η activity as a function of χ_{DO} in (DMPC/DMPS 1/1)/DO LUVs at 35, 24, and 13 °C. The *ln* (maximal activity) is plotted versus inverse temperature (Kelvin^{-1}). Maximal phospholipid-dependent activity at 35 °C occurred at $\chi_{DO} = 0.25$ and was 121 ± 5 mol of phosphorus transferred to histone min^{-1} (mol of PKC)$^{-1}$; maximal activity at 24 °C occurred at $\chi_{DO} = 0.20$ and was 64 ± 3 mol of P transferred min^{-1} (mol of PKC)$^{-1}$; and maximal activity at 13 °C occurred at $\chi_{DO} = 0.15$ and was 38 ± 4 mol of P transferred min^{-1} (mol of PKC)$^{-1}$. The slope of the line when multiplied by the gas constant yielded an apparent activation energy, ΔE_a, of 9.5 kcal/mol.

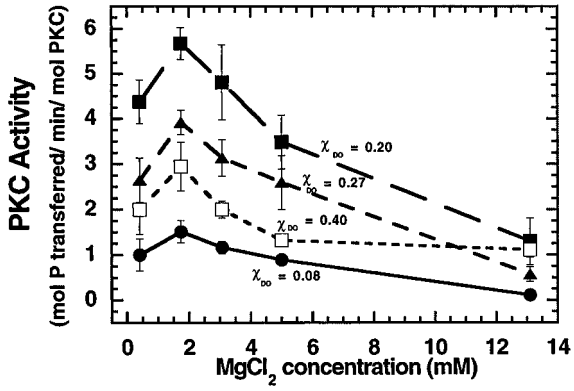

FIGURE 6: PKC α activity as a function of $MgCl_2$ in (DMPC/DMPS 1/1)/DO MLVs at 2 °C at $\chi_{DO} = 0.08$, 0.20, 0.27, and 0.40. Each sample contained 2.67 mM total lipid and 5 nM PKC α. Maximal phospholipid-dependent activity occurred at 1.74 mM $MgCl_2$ and at $\chi_{DO} = 0.08$ was 56 000 ± 9000 cpm, at $\chi_{DO} = 0.20$ was 210 000 ± 13 000 cpm, at $\chi_{DO} = 0.27$ was 145 000 ± 10 000 cpm, and at $\chi_{DO} = 0.40$ was 109 000 ± 20 000 cpm with a background of 16 000 ± 2000 cpm which was $MgCl_2$ concentration independent. Each point represents the mean ± SD of triplicate determinations from 2−4 independent experiments depending on the point. A lipid-free background was subtracted from each point and the errors were added.

affinity Mg^{2+} binding sites. Activity increased initially with $MgCl_2$ concentration, peaked at 1.74 mM, and then decreased at all DO mole fractions (Figure 6). The DO dependence of PKC activity was independent of Mg^{2+} concentration. The decrease in activity with increasing Mg^{2+} may be due to charge neutralization on the membrane surface [reviewed in McLaughlin (1989)] and loss of PKC and histone accumulated on the surface. However, the fact that the Mg^{2+} concentration required for maximal PKC activity was identical in all lipid mixtures argues that the dominant effect of Mg^{2+} is related to Mg-PKC or Mg-substrate effects and that Mg^{2+} effects on lipid domain formation, if present, are secondary. This is consistent with the DSC results with Mg^{2+}-equilibrated MLVs of (DMPC/DMPS 1/1)/DO where Mg^{2+} broadened the transitions, independent of the mole fraction of DO, arguing primarily for an ionic strength effect on the lipid behavior and not a specific interaction (Dibble et al., 1996).

DISCUSSION

We have tested the hypothesis that activation of PKC is related to the presence and nature of the interface between coexisting DAG-enriched and DAG-poor phases. Previous observations strongly suggested a role for interface in activation of PKC: (1) PKC activity was supported by DMPC/DMPS/DO under conditions where DO-poor and DO-enriched phases coexisted in the gel lipid state and where coexisting DO-enriched and DO-poor domains were implied in the fluid lipid state and (2) a maximum in activity was observed at mole fractions of DO that approximated the midpoint of the phase coexistence region in the gel state (Dibble et al., 1996). The simplest model to account for these observations implies a role for interface in activation of PKC. If PKC activity is enhanced by the amount of interface, activity would exhibit a maximum near the percolation threshold where the amount of interface is also maximum. To test the hypothesis that lipid mixtures with a poorly ordered interface would support PKC activity quantitatively differently than lipid mixtures with a highly ordered interface, the thermotropic phase behavior of three PC/PS/DAG mixtures was analyzed to determine their regions of phase coexistence and compared to the ability of the ternary lipid mixtures to support PKC activity. The DAGs were varied in each mixture to systematically increase the acyl chain matching and saturation of the ternary mixtures.

Interface within the gel-to-liquid crystalline phase transition in both one component (Papahadjopoulos et al., 1975) and in binary mixtures (Clerc & Thompson, 1995) is related to the permeability of phospholipid bilayers to small water-soluble molecules. The permeability is attributed to defects between the phases and theoretical models suggest that the interfacial region is poorly ordered due to an abundance of partially disordered acyl chain conformations (Cruzeiro-Hansson & Mouritsen, 1988). Similarly, the coexistence of a longer, unsaturated DAG-enriched phase with a primarily shorter-chained, saturated phospholipid phase such as in the DMPC/DMPS/DO mixture is assumed to generate a poorly ordered interface because of acyl chain mismatch and from the kink introduced by the *cis* double bond within the acyl chains of DO (Applegate & Glomset, 1991). In lipid mixtures with matched acyl chains, van der Waals interactions between chains will be more attractive than in mismatched mixtures. A protein, such as PKC, may not be able to intercalate into the interface region in matched mixtures because of the van der Waals interaction energy of the highly ordered acyl chains (Jørgensen et al., 1991).

Activation of PKC in the gel state was found to be most pronounced at mole fractions of DAG over which phase coexistence was detected and maximal PKC activation occurred at the approximate midpoint between the phase boundaries. Activation of PKC by fluid-state lipid was shifted to higher mole fractions of DO in comparison with gel-state lipid. This shift may be a consequence of higher temperatures tending to dissipate DO-enriched domains, shifting the mole fraction of DO needed to induce the formation of DO-enriched domains to higher mole fractions. Maximal activation of PKC in the gel state decreased as

saturation and acyl chain matching of the diacylglycerol with the phospholipids was increased. Similarly, half-maximal activation occurred at a much lower mole fraction of an unsaturated DAG (Figure 3A,B) compared with a saturated DAG (Figure 3C). In addition, Bolen and Sando (1992) have shown that unsaturation of either the bulk lipid or the DAG enhances the activity of PKC in the fluid state. This is the likely reason that many studies using highly unsaturated lipid systems report activation of PKC at significantly lower mole fractions of diacylglycerol compared to the range reported here. We thus conclude that the nature and extent of interface influences activity of PKC.

Not all types of lateral phase separation (and interface) appear to be sufficient for activation of PKC. Senisterra & Epand (1993) examined gel/fluid, PC/PS, and phospholipid/cholesterol phase-separated lipid systems in the absence of DAG and found no enhancement of PKC activity. Goldberg et al. (1995) examined Ca^{2+}-induced phase separation of PS in the presence of DAG and found a decrease in its ability to activate PKC upon phase separation.

In addition to some type of interface, the need of most PKC isozymes for PS and DAG argues that specific interactions with these lipids also are required for activity. The requirement for the 1,2-*sn*- vs 1,3-*sn*-isomer of DAG (Rando, 1988; Rando & Young, 1984) and the need for the L stereoisomer of PS for maximal activation of PKC (Lee & Bell, 1989; Newton & Keranen, 1994) support the idea that specific PKC−lipid interactions are required. Specific DAG and phorbol ester binding sites have been found in the cysteine-rich regions of the C1 domain, a conserved segment within the regulatory region (Burns & Bell, 1992).

PKC may represent a more complex variation on a general motif for activation of proteins by the membrane. In the cytosol, the stable conformation of some membrane-associating proteins may have its membrane-binding surface buried within amphipathic secondary structure (Dunne et al., 1996). The interface between lipid domains may provide a surface upon which a protein may undergo a conformational change from a membrane-associated or cytosolic, inactive protein into an active, membrane-bound form. Proteins such as CTP:cytidyltransferase and PLA_2 have been found to have enhanced activity within the gel-to-liquid crystalline phase transition (Cornell, 1991; Op den Kamp et al., 1974, 1975). Lateral pressure also may influence activity of membrane-bound protein in a manner reminiscent of interfacial activation. PLA_2 requires a greater osmotic pressure gradient for maximal hydrolytic activity as saturation of the phospholipid acyl chains of its substrate is increased. Osmotic swelling reduces the lateral packing of lipid and chain−chain van der Waals interactions (Lehtonen & Kinnunen, 1995). Conversely, a loss in activity in PLC β1, γ1, and δ1 with an increase in surface pressure in monolayers has been reported (Boguslavsky et al., 1994; Rebecchi et al., 1992). PKC may have a similar underlying mechanism of interaction with membrane interface and may, in addition, have specific lipid-binding sequences. Lipid-binding consensus regions within enzymes may ensure site specificity for activation, limited to cellular membranes enriched in particular lipids.

We speculate that PKC activation at domain interfaces may share features with activation by short-chain PC micelles (Walker et al., 1990; Walker & Sando, 1988), by highly unsaturated phospholipids (Bolen & Sando, 1992) in the presence of DO, and by branched-chain analogues of distearin (DS) with bulky derivatives (Zhou et al., 1988). Short-chain PC micelles have a spacing between the lipid components more similar to a monolayer in the liquid-extended phase than to a bilayer (McConnell, 1991). Such spacing may enable PKC to partially penetrate into the lipid in a manner analogous to the activation proposed between coexisting phases or domains. Mixtures of highly unsaturated lipids also may contain domains in the fluid phase, and domain formation may be induced by the tendency to form nonbilayer phases (Sackmann & Feder, 1995; Sackmann 1994). Such domain formation in DAG-containing mixtures has been observed by Goldberg et al. (1994). Regions of high curvature existing within a bilayer may likewise allow interfacial activation of PKC at the edges of the lipid protrusions. Substitution of a bulky derivative onto the saturated diacylglycerol distearin may promote activity by creating regions of poor lipid packing (Zhou et al., 1988).

Cellular membranes are composed of complex mixtures of lipid and proteins whose dissimilarities create distinct organized domains (Vaz, 1992; Edidin, 1990), even within fluid membranes (Mouritsen & Jørgensen, 1994; Hinderliter et al., 1994; Huang et al., 1993). Cellular membranes exist at compositions whose mixing of lipid components is at a threshold for activation of membrane proteins (Kinnunen, 1991). Slight perturbations in concentration of lipid and proteins, surface pH, concentration of divalent cations, or electrostatics may trigger or modify local regions of membrane immiscibility and domain formation [for a review see Kinnunen (1991)]. The sensitivity of biomembranes to physical as well as chemical perturbations or stimuli leads to regions of local order and discrete compositions that occur over a wide range of time scales. PKC appears to require compositionally distinct DAG-containing domains for activity (Dibble et al., 1996) and domains in which substrates and PS are both localized (Yang & Glaser, 1995). The organization within and activation by lipid domains may be a general feature of enzymes involved in signal transduction, including phospholipase C and many phospholipase A_2s (Zidovetzki et al., 1992; Dawson et al., 1983, 1984). The nature of the domains, their shape, extent, and continuity in the plane of the membrane, will influence the diffusion of components within and between the domains (Vaz, 1992, 1995). Localization of the components of an enzymatic reaction within one domain vs into separate domains can also influence the reaction kinetics (Thompson et al., 1992; Melo et al., 1992). Activation at a domain interface may thus represent a means by which the temporal generation of diacylglycerols within the plane of the membrane activates PKC and then deactivates the enzyme when the domain dissipates or the diacylglycerols are metabolized. These domains may be further modified by the partitioning of PKC, substrate, and other signal transduction enzymes within the domains (Buser et al., 1995; Kim et al., 1991). The effect of substrate and enzyme on domain composition and enzymatic activity are yet to be investigated.

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