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THE MODULATION OF PROTEIN KINASE C BY HYDRATION AND MEMBRANE SPONTANEOUS CURVATURE

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Abstract

It has been shown previously that the biophysical properties of the membrane influence the ability of protein kinase C (PKC) to bind to it and become activated. The mechanism by which this occurs has not been firmly established. The following studies were undertaken to elucidate the roles of membrane structure in modulating PKC. This was accomplished by determining the effects that a defined change in membrane structure had on the binding and catalysis of PKC, by addition of phospholipids or other compounds to lamellar phase membranes, or through the use of non-lamellar phases. Also, the role of hydration in binding and catalysis was studied.

Unsaturated phosphatidylethanolamines (PEs) were found to be activators of the enzyme, when added to large unilamellar vesicles (LUVs). Relative activation caused by different PEs correlated with their bilayer to hexagonal phase transition temperatures (T_Hs), but the extent of activation was not always proportional to the effect on T_H since two PEs which caused similar levels of activation did not cause a similar change in T_H. Activation was found to correlate best with the interfacial polarity of LUVs containing the PEs. Other properties of the phospholipid, such as its intrinsic radius of curvature, and its bending modulus, as well as the actual curvature strain it imposes on the LUVs did not correlate with the activation seen.

Cubic phases were found to activate membrane-bound PKC to a greater extent than LUVs of similar composition which possessed a higher level of curvature strain. Hexagonal phases, containing the lowest level of curvature strain, supported levels of activation similar to that of lamellar phases.

Lipophosphoglycan from *Leishmania donovani*, was a potent inhibitor of membrane-bound PKC, which could exert its effects from the opposite monolayer of the bilayer to which the enzyme binds. Polyethylene-glycol linked PEs were also found to inhibit the membrane-bound form of the enzyme.

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List of Abbreviations

AKAP	A-kinase anchoring protein
ATP	adenosine triphosphate
CPD	cholesterylphosphoryldimethylethanolamine
CT	cytidylyltransferase
DAG	diacylglycerol
DANSYL	5-dimethylaminoaphthalene-1-sulfonyl
DEPE	1,2-dielaidoyl phosphatidylethanolamine
DOPC	1,2-dioleoyl phosphatidylcholine
DOPE	1,2-dioleoyl phosphatidylethanolamine
DPH	3-[-p-(6-phenyl-1,3,5-hexatrienyl)phenyl]
DPLPE	1,2-dipetroselinoylphosphatidylethanolamine
DTMAC	4-(n-dodecylthiomethyl)-7-(N,N-dimethyl-amino) coumarin
DVPE	1,2-divaccenoyl phosphatidylethanolamine
H_{II}	hexagonal phase
IP_3	inositol triphosphate
LPG	lipophosphoglycan
LUV	large unilamellar vesicle
MARCKS	myristoylated alanine rich C-kinase substrate
MO	mono-olein
NBD	4-nitrobenzo-2-oxa-1,3-diazole
NMR	nuclear magnetic resonance
PEG	polyethyleneglycol
PKA	protein kinase A
PKC	protein kinase C
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIP_2	phosphatidylinositolbiphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
POPC	1-palmitoyl, 2-oleoyl-phosphatidylcholine
POPE	1-palmitoyl, 2-oleoyl-phosphatidylethanolamine
POPS	1-palmitoyl, 2-oleoyl-phosphatidylserine

PS	phosphatidylserine
RACK	receptors for activated C-kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLV	sucrose loaded vesicle
TCA	trichloroacetic acid
T_H	bilayer to hexagonal phase transition temperature

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Chapter 1. Introduction:

1.01 Roles of Protein Kinase C

Since its discovery in the late seventies (Takai *et al.*, 1977. Inoue *et al.*, 1977). protein kinase C (PKC) has been the focus of many studies due to its role in intracellular signaling (reviewed in Nishizuka, 1986, 1992). Functions of PKC are numerous, and differ somewhat for various isozymes. Some roles include involvement in processes such as cell growth or proliferation, ion conductance, contraction of smooth muscle, gene expression, and tumorigenesis (reviewed in Nishizuka, 1986). Some of these functions overlap with one another.

Cell growth or proliferation is initiated by growth factors binding to their receptors. This induces dimerization and autophosphorylation of the receptor, recruiting phospholipase Cγ (PLCγ) to the membrane. Activated PLCγ cleaves PIP_2 to produce DAG and IP_3. Activated PKC then phosphorylates various substrates associated with cell growth. Some of these include vinculin, talin, integrin, MARCKS, and IκB (Borner and Fabbro, 1992). Vinculin, talin and integrins are involved in stabilization or formation of focal contacts between cells (Burridge *et al.*, 1988). Phosphorylation of these proteins by PKC may contribute to the reassembly of focal adhesions during the mitotic cycle (Herman and Pledger, 1985, Beckerle, 1990, Woodgett *et al.*, 1987). Phosphorylation of MARCKS by PKC causes release of the protein from calmodulin and disappearance of it from focal contacts (Rosen *et al.*, 1990,

Thelen *et al.*, 1991). RACK1 has been shown to bind to integrins after PMA stimulation, suggesting that PKC can localize to these regions (Liliental and Chang, 1998). Activation of cells by growth factors results in phosphorylation of IκB, and dissociation from the NFκB heterotetramer (Baeuerle and Baltimore, 1988). NFκB is a transcription factor that translocates to the nucleus to activate transcription of many genes, including those for cell proliferation (Lenardo and Baltimore, 1989).

Gene transcription can be activated by PKC through the control of the fos and jun family of transcription factors. For example, activation of T cells involves the enhanced expression of c-Fos. PKC mediates the phosphorylation of c-Fos, which complexes with c-Jun, to form the transcription factor AP1, which is then able to bind to DNA to enhance transcription of the IL-2 and IL-2Rα genes (reviewed in Hug and Sarre, 1993). PKC also acts through the MAP kinase pathway, by phosphorylation of Ras and/or c-Raf-1 (Schonwasser *et al.* 1998, Pelech *et al.* 1993, Satoh *et al.* 1992). This leads to phosphorylation and activation of MAP kinase kinase (MEK), then MAP kinase, which has many cellular targets, including transcription factors Jun, Fos and Myc (Pelech *et al.* 1993, Lin *et al.* 1993, Nishizuka, 1995).

PKC is involved in contraction of vascular smooth muscle as well. Opening of voltage-gated Ca^{2+} channels as well as release of Ca^{2+} from the sarcoplasmic reticulum raises the concentration of intracellular Ca^{2+} which enables the interaction of Ca^{2+} with calmodulin and activates calmodulin-dependent myosin light-chain kinase (MLCK). MLCK phosphorylates myosin, which activates actomyosin ATPase, causing cycling of myosin along actin filaments, producing contraction (reviewed in Lee and Severson,

1994). The PKC substrates and the exact mechanism by which PKC is involved in this process has not been fully determined. However, several potential targets for PKC exist. PKC may be able to inhibit the activity of myosin light chain phosphatase (Lee and Severson, 1994). Also, it may act through the phosphorylation of proteins such as calponin and caldesmon. These proteins bind actin to inhibit smooth muscle actomyosin ATPase, and phosphorylation by PKC inhibits this action so that the ATPase is no longer inhibited (Winder and Walsh, 1993, Walsh *et al.* 1994). It has also been suggested that this effect is due to PKC activation of the MAP kinase cascade, with MAP kinase being the one to phosphorylate caldesmon (Adam *et al.* 1992, Adam and Hathaway, 1993). PKC is thought to play a role in modulation of Ca^{2+} homeostasis through voltage-gated Ca^{2+} channels, which may account for some of the effects on muscle contraction (Lee and Severson, 1994).

PKC is believed to play a role in modulation of ion conductance through the action of Na^{+}, Ca^{2+}, and K^{+} channels (Knox and Kaczmarek, 1992). Voltage-gated Na^{+} channels are made up of an α and two different β subunits. PKC phosphorylates the α subunit, and this causes a decrease in the peak Na^{+} current (Costa and Catterall, 1984, Numann *et al*, 1991). Therefore, PKC may modulate the threshold and propagation of action potentials in neurons.

A role for PKC in memory has also been established (Pascale *et al.*, 1998). PKCγ in particular is involved in long-term potentiation (LTP), since mRNA encoding this isoform increases after LTP (Thomas *et al.*, 1994) and mice lacking this isoform show decreased LTP (Abeliovich *et al.*, 1993). There is an increase in phosphorylation of the

PKC substrate GAP-43/B-50 following LTP (Ramakers *et al.*, 1995). GAP-43/B-50 plays a role in the neurotransmitter release required for LTP and is one of the major PKC substrates in the nervous system (De Graan *et al.*, 1991, Gispen *et al.*, 1991). Age-related changes in PKC activity have been observed (Pascale *et al.*, 1998). The activity of PKC as well as its translocation to membranes is reduced in cortical structures of aged rats in comparison to those from younger ones (Friedman and Wang, 1989, Battaini *et al.* 1990, Pascale *et al.*, 1998). This has been found to correspond with reduced levels of RACKS1 found in aged cortical tissues (Pascale *et al.* 1996). Impairment of translocation has also been observed in tissues from patients with Alzheimer's disease in comparison to healthy tissues (Wang *et al.* 1994).

PKC plays a role in tumorigenesis. Tumor formation is initiated by genetic damage caused by carcinogens, radiation, or insertion of viral promoters near silent genes. Oncogenes (altered versions of normal genes involved in cell growth) are activated and tumor supressor genes are inhibited so that cells undergo uncontrolled cell proliferation. The promotion stage involves further DNA modification (eg. methylation), so that cells are no longer dependent on the tumor promoter for growth. Progression involves further DNA damage, leading to a malignant phenotype (reviewed in Borner and Fabbro, 1992). Activation of PKC by TPA or overexpression of PKC contributes to the selective outgrowth of cells in the initiation stage, and also enhances the susceptibility of fibroblasts to transformation by oncogenes (Dotto *et al.* 1985, Hsiao *et al.* 1984, 1989). Differences in effects exist depending on cell type and isozyme. Overexpression of PKCβI in rat fibroblasts resulted in cells that escaped

normal growth control and acquired a partially transformed phenotype (Housey *et al.* 1988). Basal phosphorylation of the MARCKS protein was increased slightly in these cells, and significantly when the cells were treated with PMA (Guadagno *et al.* 1992). Basal expression level of c-myc and c-jun were higher in these cells, as well as the levels of expression after induction by TPA (Borner and Fabbro, 1992). However, negative feedback regulation of the EGF receptor is also enhanced, therefore having the opposite effect, and limiting growth factor signalling. This may be overcome by production of an autocrine growth factor which creates a positive autocrine loop to enhance cell growth (Borner and Fabbro, 1992). Cellular transformation with v-ras, v-src, or v-fos increased the level of expression of PKCα, PKCδ, and decreased the expression level of PKCε (Borner *et al.* 1992).

1.02 Domain structure of Protein Kinase C

PKC is a family of enzymes of related structure and function (reviewed in Mellor and Parker, 1998). Eleven different mammalian isoforms have been identified. All isoforms consist of a single polypeptide chain and contain both a catalytic and a regulatory domain. However, they can be divided into three broad categories based on the structure of their regulatory domains (Figure 1). The conventional isoforms α, β, γ contain C1A, C1B, and C2 modules on their regulatory domains. The C1 modules are involved in DAG and phorbol ester binding, while the C2 is involved in Ca^{2+} and phospholipid binding. Novel PKCs δ, ε, θ, and η, contain a modified C2 domain, and therefore are not regulated by Ca^{2+}, but still maintain specificity for PS and DAG. The

atypical PKCs (ζ, λ, ι) contain a shortened C1 domain, but lack the C2 domain. Their regulatory mechanism has not been well established.

1.03 Regulation of different PKC Isozymes

Typically, activation of PKC involves stimulation of a receptor at the cell surface. which leads to activation of phospholipase C. This causes cleavage of PIP_2 to DAG and IP_3, leading to PKC activation. The DAG produced from PIP_2 is thought to exist transiently. A longer lasting DAG pool comes from the hydrolysis of PC, which is believed to be required for sustained cellular responses (in response to long acting signals such as growth factors and cytokines) (reviewed in Nishizuka, 1995). PC hydrolysis by PLD forms PA and choline. PA has been shown to activate PKC. but can also be converted to DAG by a phosphomonoesterase (Exton, 1990). Hydrolysis of phospholipids by PLA_2 produces free fatty acids and lysophospholipids. Free fatty acids such as oleic, linoleic, arachidonic and docosahexaenoic acid have been shown to enhance the DAG dependent activation of PKC (Shinomura *et al.* 1991. Seifert *et al.* 1988, Chen and Murakami, 1992).

The various PKC isozymes differ with respect to the phospholipids and other compounds which are able to activate them. For example, cardiolipin can activate PKCα, βI and ε (Kocks *et al.*, 1993, Saido *et al.*, 1992), arachidonic acid is a PKCα, βII, γand ε activator, but not for PKCβI or ε (Shearman *et al.*, 1989. Burns *et al.*, 1990). PKCη can be activated by cholesterol sulfate, as well as PIP_3 which also activates PKCε, δ and ζ (Nishizuka, 1992, Nakanishi *et al.* 1993). Conventional

isoforms α, β and γ have also been shown to be activated by PIP_3 (Singh *et al.* 1993). PKCε is activated by *cis*-unsaturated fatty acids, while PKCδ is only activated by them in the absence of DAG, and inhibited in the presence of DAG (Koide *et al.* 1992, Ogita *et al.* 1992). Only the conventional PKCs (PKCα purified from a bacculovirus system and rat brain PKC, which consists of mainly the α, β and γ isoforms) were used in this study, so they will be the main focus of future discussions.

1.04 Phosphorylation of Protein Kinase C

Conventional PKCs must be processed by three phosphorylation reactions before they are catalytically competent and can respond to activators (Tsutakawa *et al.*, 1995, Keranen *et al.*, 1995). PKC is synthesized in the cell in an inactive form and is associated with a cytoskeletal component. The first phosphorylation is a trans phosphorylation on T500 by PDK-1 (phosphatidylinositol dependent protein kinase), and occurs in the activation loop for conventional (Dutil *et al.*, 1998) as well as novel and atypical (Le Good *et al.*, 1998) PKC isozymes. This renders PKC catalytically competent. The other two phosphorylations are autophosphorylations, and occur on residues T641 and S660, on the C-terminus in PKCβ (Keranen *et al.*, 1995). The first maintains catalytic competence, so that the phosphate on T500 is no longer required, while the second causes the release of PKC into the cytosol where it can respond to signals (Keranen *et al.*, 1995).

1.05 Roles of cofactors

In the presence of Ca^{2+}, PKC binds to PS and DAG in the membrane (Hannun and Bell. 1986). Binding in the presence of all three cofactors causes the release of the autoinhibitory pseudosubstrate site from the substrate binding site activating the enzyme, so that it can phosphorylate its protein substrates (House and Kemp. 1987. Orr *et al.*, 1992). *In vivo*, all three cofactors are required for enzyme activation.

The pseudosubstrate site of PKC is an autoinhibitory domain. It sits in the active site when the enzyme is in the cytosol, thereby keeping PKC inactive (House and Kemp, 1987, Orr *et al.*, 1992). It resembles a PKC substrate, and contains basic residues, with an alanine in place of the usual serine or threonine, so that it cannot be phosphorylated (Kemp and Pearson, 1991). Mutation of alanine 25 of PKCα to glutamic acid RFARKGALRQKNV -> RFARKGELRQKNV resulted in an enzyme that showed cofactor independent activity (Pears *et al.* 1990). Binding to membranes in the presence of DAG causes a conformational change which releases the pseudosubstrate site from the active site. Arginine-rich proteins can also cause this release, so that PKC can phosphorylate these substrates in the absence of lipid and Ca^{2-} (Orr and Newton, 1994).

It has been shown that these peptides also bind to a regulatory site on PKC, which is separate from the active site (Bruins *et al.*, 1998).

The C1 domain is involved in binding of DAG and phorbol esters (tumour promoters which can activate PKC in the absence of DAG, in effect bypassing the need for an extracellular signal). The structure of the C1B domain of PKC delta has been

solved by x-ray crystallography, in the presence and absence of bound phorbol ester (Zhang *et al.*, 1995). This domain has a globular structure, with a conserved cysteine-rich motif that can co-ordinate Zn^{2+}. Two beta sheets form the ligand binding pocket. It was shown that the overall structure of the domain did not change upon binding ligand, but that the phorbol ester bound in a water-lined hydrophilic groove, so that only the hydrophobic surfaces on PKC are exposed in this area (Figure 2). Therefore, DAG and phorbol esters serve to increase the affinity of PKC for membranes by acting as hydrophobic anchors. This explains the increase in affinity due to DAG that has been shown by *in vitro* binding assays. The apparent binding constant of PKC for 3:1 PC/PS LUVs increased by 500 times with the addition of 1 mol% DAG (Mosior and Epand, 1993). Although the structures of C1A and C1B are similar, subtle differences in amino acid sequence may account for the non-equivalent roles in the two binding sites for phorbol esters, C1B being the predominant one for PKCδ, since mutation of C1B caused a 21-fold shift in the dose response curve for translocation while mutation of C1A caused no significant shift (Hunn and Quest, 1997, Szallasi *et al.*, 1996). However, the binding affinities of phorbol esters for C1A and C1B domains of PKCγ are similar (Quest and Bell, 1994).

The translocation of PKC in the presence of DAG has recently been shown to be possible even in the absence of acidic phospholipids and Ca^{2+} (Mosior and Newton, 1996). Another role of DAG and phorbol esters may be to increase the catalytic activity of the enzyme. DAG increases the catalytic rate constant of PKC (Hannun and Bell, 1990). The rate of PKC phosphorylation of histone as well as the MARCKS peptide

doubled in the presence of 1 mol% DAG (Mosior and Epand, 1993). This may be accomplished by stabilization of the active conformation.

The C2 domain is involved in binding of Ca^{2+}. The structure of the first C2 domain of synaptotagmin has been solved by X-ray crystallography (Sutton *et al.*, 1995). The C2 domain of PKCβ has been modeled against this structure (Edwards and Newton, 1997). It consists of a four-stranded beta sheet. Amino and carboxyl termini form loops containing conserved aspartate residues. This forms the binding pocket for Ca^{2+}. Just behind this pocket are several hydrophobic residues. Two of the beta strands contain a high density of positive charge (Edwards and Newton, 1997). The role of Ca^{2+} may be to structure the surrounding residues to make them competent for lipid binding. Evidence suggests that one Ca^{2+} ion can bind to PKC, and it has been shown that this binding site lies about 0.3 nm from the membrane surface when PKC is bound (Mosior and Epand, 1994). The affinity of PKC for Ca^{2+} is 3.5 fold higher in the presence of phospholipid than in its absence (Mosior and Epand, 1994). Ca^{2+} does not act as an electrostatic switch enabling binding of PKC, since mutation of several aspartates to arginines, which would mimic the effect of Ca^{2+} because of their positive charge, decreased the membrane affinity of PKC (Edwards and Newton, 1997). The concentration of Ca^{2+} that is required for activation is higher than the amount required for binding, and is different for different isozymes (Keranen and Newton, 1997).

The catalytic domain of the enzyme contains binding sites for ATP and a protein substrate. It shows 40% sequence homology to the catalytic domain of PKA, which has been used to model the structure of PKC (Orr and Newton, 1994). The changes are

limited to 3 random coil regions and 3 minor deletions/insertions on the surface. Most of the conserved residues are in the core of the structure, which is made up of two lobes. One important difference is the presence of the pseudosubstrate domain on PKC. which is held in place by acidic residues lining the entrance to the active site. *In vitro*. the substrate specificity is not high, which suggests targeting is important for in vivo functioning (Newton, 1997).

PKC becomes associated with targeting or anchoring proteins such as RACKs (receptors for activated C-kinase) and AKAP 79 (A-kinase anchoring protein) (Mochly-Rosen, 1995, Klauck *et al.*, 1996). These proteins are associated with the cytoskeleton and may serve to localize PKC to the proper target. It has recently been shown that PKC can be acylated in the C1 domain by palmitoyl CoA, and that this facilitated its interaction with membranes (Ford *et al.*, 1998). This may be an additional mechanism for translocation of PKC.

1.06 Interaction of different domains.

Different domains of PKC interact for further regulation of function. Inactive cytosolic PKC has its pseudosubstrate domain in the active site (House and Kemp. 1987, Orr *et al.*, 1992). In this conformation, the hinge region which joins the catalytic to the regulatory domain is inaccessible to proteolytic enzymes. The hinge joining the pseudosubstrate to the C1 domain is also inaccessible. Binding to the membrane through the C2 domain in the presence of Ca^{2+} and PS causes a conformational change that exposes the hinge region (which connects the catalytic and regulatory domains) to

proteolysis (Orr *et al.*, 1992). Binding in this form is mostly due to electrostatic interactions and does not result in fully active PKC unless DAG and excess PS are present. It is not specific for the lipid headgroup, besides requiring a negative charge. Binding to DAG releases the pseudosubstrate site and results in full activation of PKC. PKC can bind to lipid through the C1 domain alone in the absence of PS and Ca^{2-} in the presence of high concentrations of phorbol esters (Mosior and Newton, 1996). This interaction results in a low level of activation. Binding to DAG in the presence of Ca^{2-} and PS causes full activation of PKC that is stronger than the interactions of the two domains separately (reviewed in Newton and Johnson, 1998). Some of the extra energy may be a result of the pseudosubstrate site binding to anionic phospholipid which could contribute about 6 kcal/mol to the interaction (Mosior and McLaughlin 1991). Also, a site on the C-terminus is exposed to proteolysis only when PKC binds to the membrane in the presence of very low levels of Ca^{2+}, which are enough to sustain binding, but not activation. However, in the presence of activating levels of Ca^{2-}, this region is again inaccessible (Keranen and Newton, 1997). The above data suggest that there are interactions occurring between the different domains of PKC.

The site on PKC responsible for binding to PS is thought to be the C2 domain. However, it is unlikely that this is the only domain involved, since the specificity for PS is only observed in the presence of DAG (Orr and Newton, 1992a, 1992b). In its absence, PKC can bind with equal affinity to other anionic lipids. This suggests that the binding site for PS is only aligned properly when both the C1 and C2 domains are membrane bound (Newton and Johnson, 1998).

It has been debated as to whether the role of PS is to bind specifically to a site on PKC causing its activation, or whether PS structures the membrane so that DAG is optimally presented to PKC. Evidence for and against a specific binding site for PS has been provided. One study suggested there was a specific site in the C2 domain due to the presence of a putative PS binding motif (Igarashi *et al.*, 1995), but mutation of conserved residues in this site had no effect on PKCs affinity for PS or its activation (Johnson *et al.*, 1997). A recent study showed that there was no PKC activation or binding obtained when membranes containing enantiomeric forms (2,3 forms) of PS, DAG and PC were used to make LUVs (Johnson *et al.*, 1998). These membranes would be expected to have the same biophysical properties as 1,2 isomers, but no stereospecific PS binding site. Some binding of PKC to the membrane occurred if 2,3 DAG was used in the presence of 1,2 PS and PC, but very low activity was obtained. Therefore, this suggests that there is a stereospecific requirement for PS in order to obtain maximal activation.

However, there is also evidence to support the lack of a stereospecific requirement for L-PS. D-PS is as effective as L-PS in activating PKC, if the MARCKS peptide is used as a substrate (Epand *et al.*, 1998). This peptide was able to induce formation of PS-rich domains equally with either D-PS or L-PS. However, histone induced domain formation more readily with L-PS, which was reflected in the higher level of PKC activation observed when histone was the substrate. Also, it has been shown that activation as well as binding of PKC to membranes containing DANSYL-PE is as effective as in the presence of PS (Mosior *et al.*, 1996). This suggests that there is not a

specific requirement for PS, since DANSYL-PE can substitute. Note that there is a requirement for either PS, or a an analogue which can substitute for PS to achieve high affinity binding, and not any anionic phospholipid will do.

Since DAG/phorbol esters and Ca^{2+} have been shown to act synergistically, in that addition of DAG lowers the concentration of Ca^{2+} required for activation, and an increase in the level of Ca^{2+} lowers the DAG requirement (Mosior and Epand, 1993), it has been suggested that there is an interaction between Ca^{2+} and DAG/phorbol ester binding sites (Wolf *et al.*, 1985). However, phorbol esters are able to recruit PKC to the membrane in the absence of PS through the C1 domain alone, and this interaction is not affected by Ca^{2+}. Therefore, it is likely that these binding sites do not interact with respect to binding of PKC. The lowering of the concentration of Ca^{2+} required in the presence of phorbol esters is likely due to a tighter binding in their presence (Mosior and Newton, 1996). However, it has also been shown that a deletion mutant lacking the C2 domain was still partially dependent on Ca^{2+} and PS for activity (Kaibuchi *et al.*, 1989). Reasons for this conflicting evidence are not clear.

It has been suggested that the C-terminus of the catalytic domain interacts with the C2 domain (Keranen and Newton, 1997). Two PKC isozymes, βI and βII, differ only in their last 50 amino acids. However, the effect of Ca^{2+} on their regulation differs. The Ca^{2+} requirement for activation of PKC βII is an order of magnitude higher than that required by PKC βI. Therefore, the C-terminus must somehow interact with the C2 domain, so that the regulation by Ca^{2+} is different for these isozymes.

1.07 Regulation of proteins by membrane structure

Phospholipids in biological membranes are usually present in the form of a planar bilayer. The composition of lipids present differ according to cell type, providing specificity in function, since the activity of proteins which are embedded within or bound to the membrane is affected by the types of phospholipids present. The type of phospholipid present can influence the stability of the bilayer, and can control how close a membrane is to the bilayer to hexagonal phase transition temperature. The hexagonal phase is made up of phospholipids forming a cylindrical structure with a water filled core, and cylinders are packed with hexagonal symmetry, so that each is surrounded by six others. A number of phospholipids present in biological membranes will form the inverted hexagonal phase at certain temperatures if isolated (Seddon, 1990).

The biophysical properties of membranes have been shown to regulate PKC activity, as well as binding (reviewed in Epand, 1992). In most cases, membrane additives which affect PKC activity can be classified as activators or inhibitors of the enzyme based on how they affect the bilayer to hexagonal phase transition temperature (T_H) of model membranes. Phospholipids or other compounds with a large hydrophobic area and small polar group tend to destabilize membranes (lower T_H) and promote the formation of the hexagonal phase (Figure 3). Compounds with the opposite shape are bilayer stabilizers and raise T_H. In general, uncharged or zwitterionic compounds which raise T_H are inhibitors of PKC, while those which lower T_H are activators. Also,

positively charged additives are usually inhibitors, while negatively charged ones are activators, regardless of their effects on T_H (Epand, 1992).

Since most mechanistic studies on the activation of PKC are done *in vitro* where reaction conditions are easily controlled, it is important to note that it is not the actual formation of the hexagonal phase in natural membranes that causes activation of PKC, but rather the tendency to form it due to an increased monolayer curvature strain caused by the additive (Epand, 1992). Another effect of bilayer destabilizers is to increase the order of the acyl chains (Lafleur *et al.*, 1996, also reviewed in Gawrisch and Holte, 1996).

Regulation of protein function by membrane structure is a general mechanism to control the activation of enzymes, opening of channels, and functioning of receptors (reviewed in Epand, 1990, 1996). For example, autophosphorylation and signaling by the insulin receptor has been shown to be affected by the presence of non-lamellar forming lipids in the membrane. Compounds which raised the bilayer to hexagonal phase transition temperature were inhibitors, while compounds that promoted formation of the hexagonal phase were activators (McCallum and Epand, 1995). The conductance and lifetime of alamethecin channels is decreased in the presence of substances which raise T_H (Keller *et al.*, 1993). Also, rhodopsin functioning is inhibited by bilayer stabilizers (Gibson and Brown, 1993).

Some enzymes involved in lipid metabolism are regulated by the proportion of bilayer to non-bilayer forming lipids in the membrane (reviewed in Cornell and Arnold, 1996). CTP:phosphocholine cytidylyltransferase (CT), an amphitrophic enzyme is

stimulated in the presence of H_{II} forming phospholipids, while the activation of PLC is inhibited by them. This difference has been explained based on the roles of the two enzymes, and the reactants and products involved in their functioning (Cornell and Arnold, 1996). CT is involved in the synthesis of phosphatidylcholine, a lipid which forms bilayers, while PLC activity produces DAG, a hexagonal phase forming lipid. Therefore, the activation of each enzyme is enhanced by phospholipids which induce the opposite effect on the bilayer as their products. PLA_2 also fits into this category, since it produces lysoPC, and is activated by H_{II} forming lipids. Phospholipase A_2 is also better able to bind and become activated in the presence of phospholipids which form defects at boundaries of microdomains (Jain *et al.*, 1984).

1.08 The role of curvature strain in PKC activation

The addition of hexagonal phase forming lipids to a bilayer will cause each monolayer to curve spontaneously to minimize the unfavourable energy resulting from mismatches in head group size and acyl chain interactions (packing energies) (Gruner *et al.*, 1984). However, in a bilayer, this curvature cannot be expressed, and results in a bilayer with curvature stress/strain. If this value becomes sufficiently large, the bilayer will spontaneously form an inverted phase, such as a hexagonal or cubic phase.

Curvature stress can be defined by two terms used to describe a phospholipid, R_o, the radius of a hexagonal phase cylinder formed by that phospholipid alone, and K_c, the elastic bending modulus according the equation $0.5\ K_c/R_o$ (Helfrich, 1973). The addition of hexagonal phase promoters to the bilayer has been suggested to activate

PKC due to the curvature strain that results (Slater *et al.*, 1994). However, PKC activation has been shown to be a biphasic function of parameters related to curvature strain, so that there is an optimal level which leads to maximal activation of the enzyme. This level varies with the type of activator and the conditions of the assay (Slater *et al.*, 1994).

1.09 Goals of project:

It is known that the biophysical properties of the membrane affect PKC binding and activation. The exact mechanism by which this occurs is not known. It has been suggested that the increase in activation seen with hexagonal forming compounds could be due to the instability and curvature strain associated with these bilayers. Also, the increase in headgroup spacing which occurs could facilitate the interaction of the enzyme as well as the structural rearrangement required to activate it once bound. Studies were undertaken with the general aim of elucidating some of the details involved in this activation process by determining the effect of changing the membrane in several defined ways, by the addition of unsaturated PEs, LPG, and by the use of hexagonal and cubic phase membranes.

1.10 Phosphatidylethanolamines

Phosphatidylethanolamines are activators of PKC, especially if they are unsaturated (Slater *et al.*, 1994). This is due largely to their effects on the biophysical properties of the membrane, although PKC can bind to PE in the absence of DAG.

Investigation of how subtle differences in PE structure lead to differences in PKC activation and binding can help to elucidate the mechanism by which membrane properties affect PKC, if the effect of these structural differences on membrane properties is known. Also, carp have been shown to alter the level of unsaturation of the phosphatidylethanolamines in their neural membranes in response to environmental temperatures (Buda *et al.*, 1994). The purpose of this regulatory mechanism may be to control the activation of membrane-bound enzymes. Therefore, examining the role of unsaturated PEs in PKC functioning may have physiological significance.

It has been shown that the levels of 1-oleoyl, 2-docosahexaenoyl phosphatidylethanolamine (18:1/22:6 PE) in the brains of carp rose from 2-5% of the total PE in warm water fish to 14% of the PE in cold water fish (Buda *et al.*, 1994). This phospholipid is a predicted bilayer destabilizer based on its inverted cone shape, and by consequence an activator of PKC. It was therefore interesting to examine the role of 18:1/22:6 PE in modulation of PKC and compare its effects to those of PC with the same acyl chains, as well as PEs with less unsaturation. Also, the effects of these phospholipids on the bilayer to hexagonal phase transition temperature were examined and compared to their effects on PKC (manuscript 1).

Three PEs which differ only in the position of their double bond were previously well characterized with respect to several properties associated with T_H, including R_o and K_c (Epand *et al.*, 1996). These PEs were used in PKC assays to determine if any of these previously characterized properties correlated with their effects on PKC. Correlation with any of these properties would help to determine the physical property

associated with the bilayer to hexagonal phase transition that can serve to activate PKC. The interfacial polarity (or accessibility of the hydrophobic interior) of membranes containing these PEs was also examined (manuscript 5).

1.11 Lipophosphoglycan

Some membrane components provide a protective role for the organism which produces them. One example is the lipophosphoglycan (LPG) found on the surface of *Leishmania donovani*. LPG is a negatively charged glycocongugate consisting of repeating phosphorylated disaccharide and monosaccharide units linked to a lysophosphatidylinositol membrane anchor (reviewed in Turco and Descoteaux, 1992). It is believed that LPG is crucial for the survival of the parasite in host macrophage cells. These cells undergo an oxidative burst, a process mediated by PKC, which produces toxic oxygen radicals to kill the parasite. It has been shown previously that LPG is a potent inhibitor of PKC activity *in vitro* as well as in phagocytic cells (McNeely and Turco, 1987, Descoteaux *et al.*, 1992). It is this inhibition that may allow the parasite to survive in macrophages and continue its infectious cycle. Knowledge of the mechanism by which LPG is able to inhibit PKC activity may provide insight into ways to control the spread of Leishmania. LPG is also unique in that it is structurally different than most inhibitors of PKC, since it is negatively charged. Most inhibitors are either positively charged, or uncharged overall (manuscript 2).

1.12 The role of hydration in macromolecule function

Other membrane-anchored polymers such as polyethyleneglycol-linked phospholipids affect membrane properties. Like LPG, these polymers stabilize the bilayer phase. Originally, this study was started with the purpose of looking at the role of PEG-linked PEs in inhibition of PKC, since they are similar to LPG in general structure. Similarities in their effects would allow general conclusions to be made about the effects of hydrophilic bilayer stabilizing polymers on PKC function. PEG-PEs are also of interest because they are often used to increase the circulation time of drug-carrying liposomes (Kilvanov *et al.*, 1990), so it is important to have an understanding of any effects that these compounds have on important cellular enzymes.

These studies were then extended to look at the role of hydration in PKC functioning (manuscript 3), since free PEG is often used as an osmotic stressing agent to control the activity of water around a macromolecule (reviewed in Parsegian *et al.*, 1995). Studies of this sort can help to establish what changes in structure are taking place when PKC binds to membranes and becomes activated.

1.13 Cubic and Hexagonal Phases

Studies with PKC have previously used lipid vesicles, in which the phospholipids are in the lamellar phase. However, certain phospholipids have been shown to exist as a bicontinuous cubic phase at particular temperatures (Keller *et al.* 1996). These phases have been observed in the infoldings of the plasma membrane, the smooth endoplasmic reticulum, nuclear, and mitochondrial membranes (Hyde *et al.*, 1997). An

interconnecting network of channels is formed, with the phospholipid headgroups lining these channels. These display a continuous gradation in curvature, so that PKC can potentially select regions with optimal lipid packing for binding and activation. Due to the curved morphology, these phases have very little curvature strain, unlike lamellar phases with added hexagonal phase promoters.

Hexagonal phases have not been shown to exist in biological membranes. However, they are still of interest for use in PKC assays, due to their properties. The phospholipid headgroups that line the water filled channels have a highly curved structure, with no curvature strain. Determining the relative PKC activation in cubic and hexagonal phases provides insight into the roles of curvature strain versus curved morphology in this process (manuscript 4).

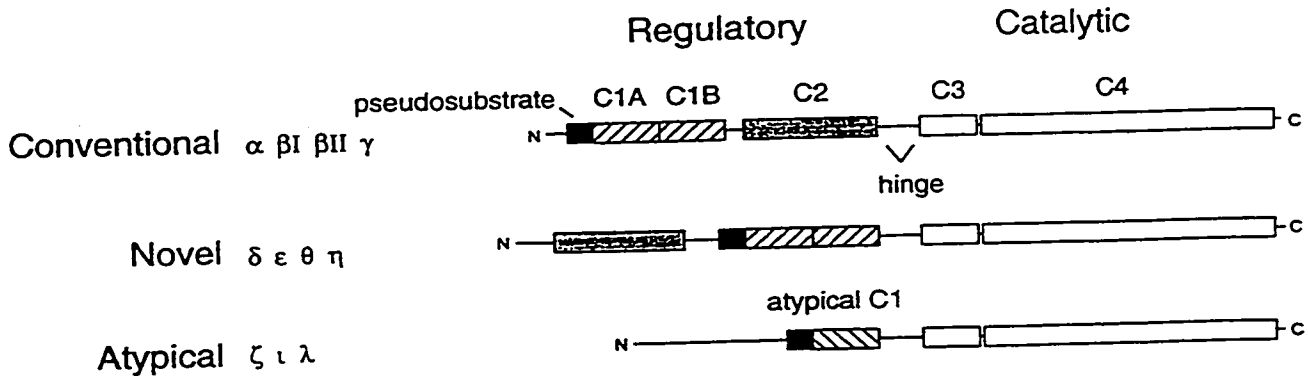


Figure 1. Domain structure of PKC isozymes. Schematic representation of the domains of conventional, novel and atypical PKC isozymes, showing N-terminal regulatory and C-terminal catalytic domains. C1 domain (hatched box) binds DAG and phorbol esters in conventional and atypical PKCs. C2 domain (grey) binds anionic lipids and Ca^{2+} (in conventional PKCs). C3 and C4 domains (unfilled boxes) bind ATP and substrate respectively. (Figure from Newton and Johnson, 1998).

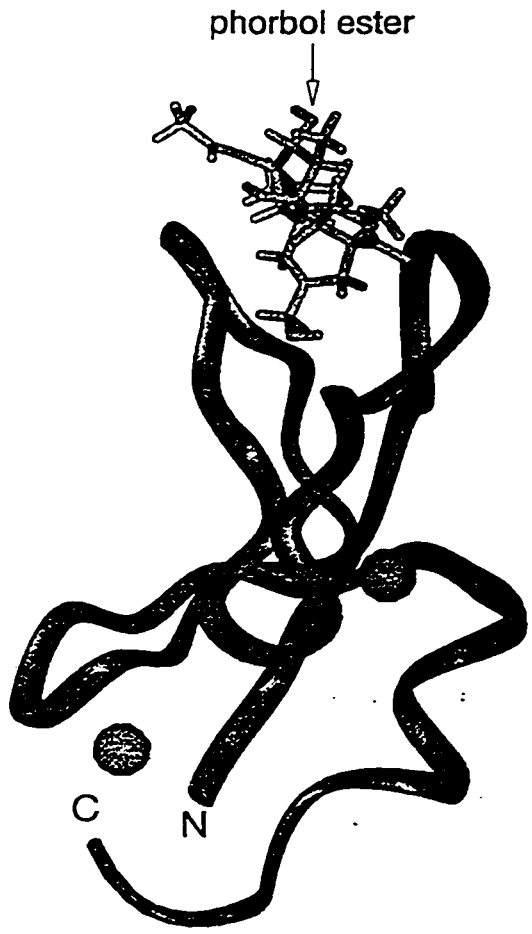
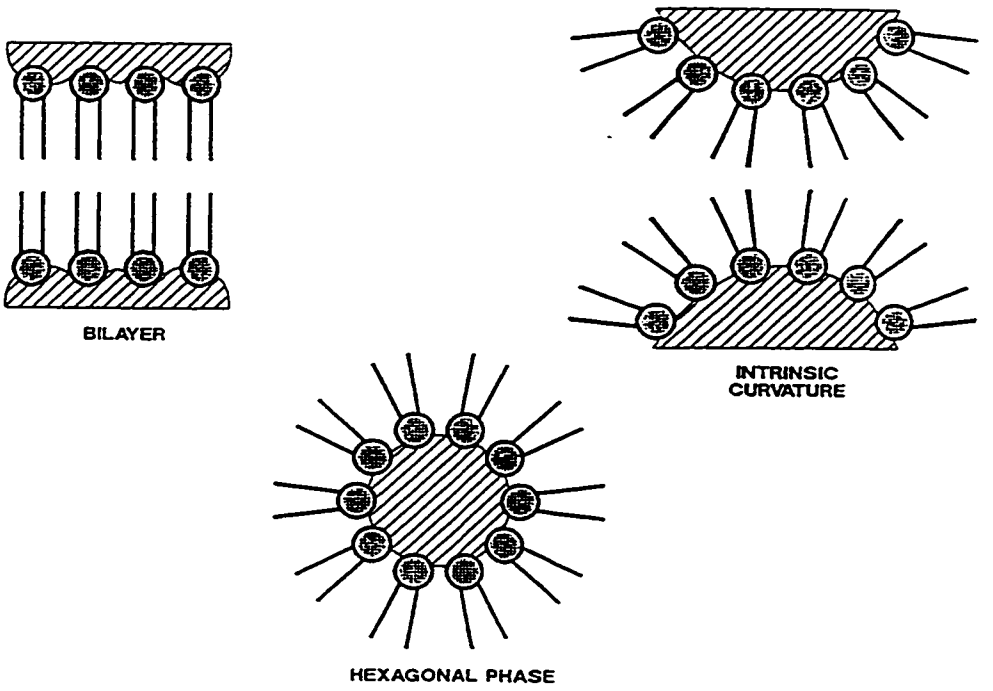


Figure 2. Structure of C1B domain of PKCδ. Ribbon diagram showing the X-ray crystallographic structure of residues 231-280 in the C1B domain of PKCδ bound to phorbol-13-acetate. Based on the coordinates of Zhang *et al.*, 1995. The grey balls represent zinc atoms. (Figure from Newton and Johnson, 1998).



Reprinted from "Protein kinase C, current concepts and future perspective", eds. D.S. Lester and R.M. Epand. Ellis Horwood, Chinchester England, pgs. 135-156, 1992.

Figure 3. Schematic diagram of phospholipids in a planar bilayer, a hypothetical bilayervoid of curvature strain, and a hexagonal phase cylinder. The hypothetical state is one in which each monolayer expresses its intrinsic radius of curvature so that it is free of packing constraints and the acyl chains are in contact with a non-polar solvent. The hexagonal phase is represented by a cross section of one hexagonal phase cylinder. Diagonal lines represent water. (Figure from Epand, 1992).

Chapter 2 Materials and Methods

Methods are outlined in each manuscript. However, a more detailed description of the assays used to test binding and activation of PKC is given below. Any alterations in this method are stated in each manuscript. Also, materials used for SDS-PAGE in testing the purity of PKC preparations are given.

2.01 Materials for SDS-PAGE

SDS and TEMED were purchased from Biorad, ammonium persulfate and acrylamide and bisacrylamide were from BRL. All materials were at least analytical grade.

2.02 SDS-PAGE

The purity of a PKC preparation from rat brain was monitored by running samples on a polyacrylamide gel. Samples were run on a 7% polyacrylamide gel which was then silver stained. Protein concentration was determined by the BCA assay.

2.03 PKC activity assay

The method of Mosior and Epand (1993) was used. A standard assay is described. Any changes are noted in methods sections of individual manuscripts. A total volume of 150 µl was used. Substrates were either protamine sulfate, histone H1, or the

MARCKS peptide (acetyl-FKKSFKL-amide), and used at a final concentration of 0.2 mg/ml for histone or protamine, and 90 μM for the MARCKS peptide. The reaction mixture was 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-Cl (pH 7.0), 200 μM Ca^{2-}, 0.3 mg/ml BSA, 100 μM lipid in the form of 100 nm LUVs, 100 ng/ml PKC (as measured by the BCA assay), and 20 mM [γ^{32}P] ATP (0.003 μCi).

All reactants were added to test tubes and vortexed prior to addition of radiolabelled ATP to initiate the reaction. Samples were vortexed and incubated with shaking at 25°C for 10 minutes. 2 ml of ice cold 25% (w/v) trichloroacetic acid was added to stop the reaction. Samples were placed on ice for 10 minutes, then filtered by suction through GF/C Whatman filters. Filters dried at 37°C for 30 minutes and counted using Cherenkov counting in a Beckman LS 3801 scintilation counter, set to counts per minute (CPM) on channel 3 (lower limit 0, upper limit 1000). The CPM were corrected for the age of the radioactive ATP, by the use of a "P" number which ranged from 2.0 to 6.0, depending on the number of days past the reference date. This was done so that the counts obtained would be the same regardless of the age of the ATP on the day of the experiment. Radioactive ATP was only used for a maximum of 20 days past its reference date.

2.04 Sucrose Loaded Vesicle binding assay

The method of Mosior and Epand (1993) was used. A standard assay is described. Any changes are noted in methods section of individual manuscripts. PKC was incubated with SLVs (100 ng of PKC with same relative amount of lipid as used in

activity assay) in a buffer of 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris (pH 7.0), 200 μM Ca^{2+} and 0.3 mg/ml BSA. This 750 μl was centrifuged at 100 000g for 30 minutes at 25°C in a Beckman TLA 100 centrifuge, TLA 100.2 rotor. The top 550 μl was separated from the bottom 200 μl, and each was diluted to 750 μl with buffer. The activity of these fractions was determined using protamine as the substrate in the assay described above. The activity of PKC obtained with protamine as a substrate is dependent on the amount of enzyme present, and independent of the amount bound to vesicles. Therefore, activities of top and bottom fractions are proportional to the amount of PKC present in them. The percentage of membrane bound enzyme was determined using the following formula:

$$Av = \frac{\beta Ab + (\beta-1)At}{\alpha + \beta - 1}$$

$$\%bound = \frac{Av}{Ab + At}$$

where: Av is the vesicle associated kinase activity
Ab is equal to the activity of the bottom fraction,
At is equal to the activity of the top fraction
α is the fraction of sedimented vesicles, typically 95% as determined by incorporation of trace tritium label.
β is the fraction of kinase activity found in the bottom fraction in the absence of vesicles

2.05 Purification of PKCα

PKCα was purified from the bacculovirus system. Insect cells overexpressing PKCα were provided by Robert Burns and Nancy Rankl of the Sphinx Pharmaceutical

Co. Frozen insect cells pellets were lysed in 2 mM benzamidine, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 40 µg/ml leupeptin and 200 µM phenylmethyl-sulfonylfluoride (PMSF). Cells were homogenized in a homogenizer. The mixture was centrifuged at 40 000g and the supernatant was applied to a Q-Sepharose column (Pharmacia:2.5 cm x 20 cm) equilibrated with 1 mM EGTA, 20 mM Tris-HCl, pH 7.5, and 10% v/v glycerol. Elution was carried out using 140 ml of Tris buffer alone, then 140 ml of each of Tris buffer containing 0.05 M, 0.15 M, 0.45 M, and 1.0 M KCl. PKCα was eluted with the 0.15 M KCl. Fractions were collected and every third was analyzed for PKC activity in a micellar assay (Hannun *et al.*, 1985, described below). Fractions with the highest PS and Ca^{2+} dependant activity were pooled, adjusted to 1.5 M KCl, and loaded onto a phenyl-Sepharose column (Pharmacia: 1.0 cm x 10 cm) equilibrated with 1.5 M KCl, 1mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5, and 10% glycerol. A salt gradient from 1.5 M to 0 M KCl was used to elute the enzyme. A gradient maker was used, starting with 50 ml of buffer containing 1.5 M KCl in one compartment, and an equal amount of 0 M KCl buffer in the other. Collected fractions were tested for activity as above. Fractions containing the highest activity were pooled and concentrated to ~ 0.40 mg/ml by filtration through a Diaflo (Amicon, Danvers, MA) 30 kDa cutoff filter and stored at -70°C in 50% glycerol. All purification steps except assaying for enzymatic activity were carried out at 4°C or on ice.

2.06 Purification of Rat Brain PKC

Brains were isolated from 24 male 200g Sprage-Dawley rats. The rest of the procedure was done on ice or at 4°C. Brains were washed and homogenized in 150 ml of 50 mM Tris/HCl buffer, pH 7.5 containing 2 mM benzamidine, 1% (w/w) Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM DTT (added just before use), 40 µg/ml leupeptin, and 200 µM PMSF. The lysate was centrifuged at 100 000 x g for 1 hour in a Beckman SW28 rotor. The supernatant was applied to a Q-Sepharose fast flow column (2.5 x 20 cm, 70 ml), equilibrated in DEAE buffer (1 mM EDTA, 1 mM EGTA, 20 mM Tris/HCl pH 7.5, and 10% (v/v) glycerol). The column was washed with 200 ml of DEAE buffer, then with 150 ml of 0.05 M KCl DEAE buffer, then with 150 ml of 0.15 M KCl DEAE buffer. Fractions were collected at this point, and assayed for PKC activity in a mixed micelle assay (Hannun *et al.*, 1985). Fractions displaying highest PS and Ca^{2+} dependent activity were pooled and concentrated with a YM30 Diaflo Ultrafiltration Membrane in an Amicon Ultrafiltration Cell to one half the original volume. Concentrate was adjusted to 1.5 M KCl and loaded onto a phenyl Sepharose column (1.0 x 10 cm, 10 ml) which was equilibrated in 1.5 M KCl DEAE buffer. The column was washed with 100 ml of 1.5 M KCl DEAE buffer. A linear gradient from 1.5M to 0.0 M KCl DEAE was applied (100 ml total). Fractions were collected throughout this gradient and assayed as above. Pooled fractions were concentrated as above, to about 3 or 4 ml total, brought to 50% glycerol (v/v) and stored in 50 µl aliquots at -70°C.

The mixed micelle assay (Hannun *et al.*, 1985) was used to assay collected fractions for PKC activity. The reaction consisted of 25 mM Tris/HCl. pH 7.5. 10 mM $MgCl_2$, 0.2 mg/ml histone, 3 mM EGTA or 300 μM $CaCl_2$, 0.3% (v/v) Triton X-100 or 0.3 mg/ml PS/DG (4:1, w/w) in 0.3% (v/v) Triton X-100. The total volume was 240 μl. 5 μl of the fraction to be assayed was added, and the reaction was initiated with 20 μl of 200 μM [γ^{32}-P] ATP (0.04 μCi). The reaction was carried out for 10 minutes at 30°C and stopped by the addition of 2 ml of ice cold trichloroacetic acid 25% (w/w). The stopped reactions were filtered through GF/C Whatman filters, which were washed 3 times with ice cold TCA. Filters were dried for 30 minutes at 37°C and counted as above. The micellar assay was only used to test activity of collected fractions during purification. It was not used for any of the experimental assays. Generally, the level of PKC activity obtained with the micellar assay was lower than that obtained using the LUV assay.

2.07 Preparation of LUVs and SLVs

Phospholipids were dissolved in 2:1 v/v chloroform methanol, and added to glass test tubes in appropriate amounts. Test tubes were vortexed and solvent was evaporated under a stream of nitrogen to leave a thin film of phospholipid deposited on the bottom and sides of the test tube. Films were desiccated under vacuum for 2 hrs. Films were hydrated with standard buffer (20mM Tris-Cl pH 7.0, 5mM $MgCl_2$, 100 mM KCl). votexed, and subjected to 5 freeze thaw cycles in liquid nitrogen. This procedure produces MLVs (multilamellar vesicles). MLVs were extruded by passing them 20

times through two polycarbonate filters with 100 nm pores using a hand held microextruder from Avanti (Alabaster AL).

SLVs were prepared simlarly to LUVs. Instead of standard buffer, a sucrose buffer was used to hydrate the films. Sucrose buffer consisted of 20 mM Tris-Cl pH 7.0, 170 mM sucrose, 5 mM $MgCl_2$. Freeze thaw cycles and extrusion were performed as with LUVs. 200 µl of SLVs were suspended in 800 µl of standard buffer and centrifuged at 100000g for 30 minutes at 25^{o}C to dilute out the sucrose. Pelleted SLVs were resuspended in standard buffer.

It is not expected that the presence of sucrose inside the vesicles will affect the phospholipids in the vesicle. If by any chance sucrose were to induce flip flop of phospholipids, this would be expected to occur at an extremely low rate, taking several days. All assays were performed between ~ 1 and 15 hours of preparation of the SLVs. Also, flip flop would by random, so that the symmetry of both monolayers would be maintained. An input of energy would be required for unidirectional flip flop of a selected phospholipid. In the case of LPG where SLVs were asymmetrical, this is not expected to be a problem since LPG is too large and hydrophilic to flip from one monolayer to another.

It should be mentioned that the Western Blot performed in manuscript 4 to test whether one isoform of PKC was preferentially binding to cubic phase membranes over another, involved using antibodies specific for each isoform, and basing the conclusions on the apparent darkness of the signal obtained in each lane. Antibody titrations were not done, and a densitometer was not used.

Chapter 3. Unpublished Results

3.01 Purity of PKC preparations

The following silver stained gels were run by Robert Bruins. They show the purity of PKC preparations obtained from the bacculovirus system (Figure 4a) and from rat brain (Figure 4b). PKCα was overexpressed in the bacculovirus system, so that it is the only isoform present. Rat brain PKC consists of several isoforms, but the major isoforms are the conventional ones (Epand, 1994). It is not known if any of the novel or atypical isoforms are present in this preparation. This would have required a Western blot using antibodies specific for those isoforms. If they are present, it is likely that they are in much lower quantities than the conventional isoforms. Also, PKC δ, ε and η display almost no activity towards histone, myelin basic protein, or protamine (Liyanage *et al.*, 1992, Dekker *et al.*, 1992). Most experiments used histone or protamine as substrates, so if other isoforms are present, their effect on the overall activity would not be significant. (Binding is also tested by activity of top and bottom fractions towards protamine, so their effects on binding would also not be detected). The unfractionated mixture from rat brain has overall similar properties to each of the individual isoforms (Epand, 1994). Some slight differences would be expected since the mixture may show less specificity towards Ca^{2+} than a preparation containing solely conventional isoforms. Also, subtle differences between different conventional isoforms. For example, PKCβI and βII differ by ten-fold in the amount of Ca^{2+} required

for activation (Keranen and Newton, 1997). However, under the conditions used, the presence of multiple isoforms should not be a problem, since all assays were measuring a comparison between two conditions, using the same enzyme preparation. The results will reflect the effects of membrane properties on a mixture of isozymes, rather than on a single isozyme. Even if some isoforms are more likely to bind to the membrane under certain conditions than are other isoforms, the end result is a measure of the binding of the mixture as a whole. All isoforms require binding to become activated, so that no activation is observed with unbound PKC and the mixture as a whole behaves in the same way.

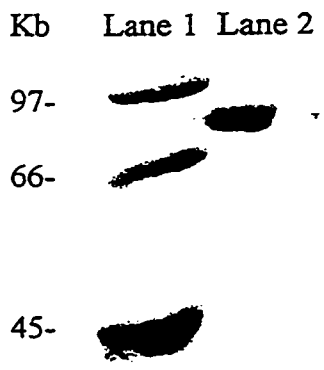


Figure 1a. This is an example of a silver stained gel used to test the purity of PKCα after purification. Lane 1: molecular weight markers. Lane 2: PKCα obtained after purification on Q-sepharose and phenyl-sepharose columns.

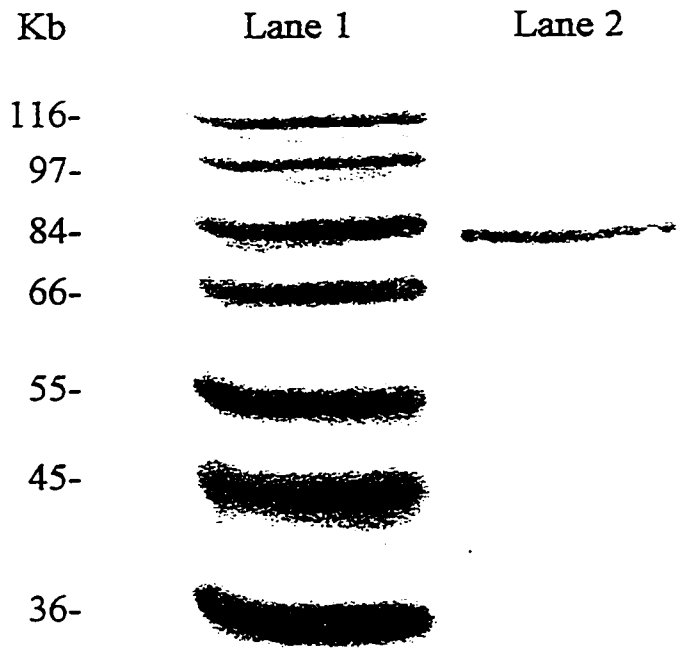


Figure 1b. An example of a silver stained polyacrylamide gel used to test the purity of rat brain PKC after purification. Lane 1: molecular weight markers. Lane 2: Rat brain PKC after purification on Q-sepharose, phenyl-sepharose columns.

Chapter 4: Manuscripts.

Manuscripts are presented in order of publication. Preceding each manuscript, the contributions from each author are outlined. Also, a brief introduction is given, in which the main findings and significance of the work are highlighted.

Manuscript 1 "Role of Phospholipids Containing Docosahexaenoyl Chains in Modulating the Activity of Protein Kinase C".

The purpose of this investigation was to discover the effects of polyunsaturated phosphatidylethanolamines on the binding and activation of PKC. The rationale for this was based on the finding that 18:1/22:6 PE in the brains of carp rose from 2-5% of the total PE in warm water fish to 14% of the PE in cold water fish (Buda *et al.* 1994). The purpose of this regulatory mechanism may be to control the activation of membrane-bound enzymes. Since this phospholipid is a predicted bilayer destabilizer based on its inverted cone shape and therefore and therefore an activator of PKC, it was interesting to determine the effects of this phospholipid on PKC binding and activation. Investigation of how subtle differences in PE structure lead to differences in PKC activation and binding can help to elucidate the mechanism by which membrane properties affect PKC.

The major findings of this investigation were that 18:1/22:6 PE was the more effective in lowering the transition temperature of 16:1/16:1 PE than other less

unsaturated PE species. This phospholipid was also the most effective in enhancing the activity of PKC. The increase in activity seen is a consequence of increasing the amount of enzyme bound to the membrane.

All experiments were performed by Jennifer Giorgione, with the exception of the differential scanning calorimetry in Table 1, which was performed by Richard Epand. The manuscript was written by Jennifer Giorgione and edited by Richard Epand. The original paper describing the phospholipid composition of carp neural membranes was performed in the lab of Tibor Farkas (Buda *et al.* 1994). Richard Epand approached Tibor Farkas with the idea of studying the effects of these phospholipids on PKC activation. Csaba Buda was the primary author for the 1994 paper (Buda *et al.* 1994).

Manuscript 1, as published in The Proceedings of the National Academy of Sciences of the USA. 92, 9767-9770 (1995).

Role of Phospholipids Containing Docosahexanoyl Chains in Modulating the Activity of Protein Kinase C

Key words: (thermal acclimatization/phosphatidylethanolamine/docosahexanoic acid)

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Classification: Biological Sciences: Biophysics

ABSTRACT It is known that the phospholipids of the brain cells of fish are altered during cold adaptation. In particular, the 1-monounsaturated-2-polyunsaturated phosphatidylethanolamines increase 2- to 3-fold upon adaptation to cold. One of the most striking changes is in the 18:1/22:6 species of phosphatidylethanolamine (PE). We determined how this lipid affected the bilayer to hexagonal phase transition temperature of 16:1/16:1 PE. We found that it was more effective in lowering this transition temperature than were other, less unsaturated, PE species. In addition, it was not simply the presence of the 18:1/22:6 acyl chains which caused this effect, since the 18:1/22:6 species of phosphatidylcholine had the opposite effect on this transition temperature. Zwitterionic substances that lower the bilayer to hexagonal phase transition temperature often cause an increase in the activity of protein kinase C (PKC). Indeed the 18:1/22:6 PE caused an increase in the rate of histone phosphorylation by PKC which was greater than that caused by other, less unsaturated, PE. The 18:1/22:6 phosphatidylcholine had no effect on this enzyme. The stimulation of the activity of PKC by the 18:1/22:6 PE is a consequence of this lipid increasing the partitioning of PKC to the membrane.

A detailed analysis of the phospholipid species in the brains of fish taken from summer (25°C) or from winter (5°C) waters revealed that the lipid species which underwent the largest change in relative amount was 1-oleoyl-2-docosahexanoyl-PE (18:1/22:6 PE) (1). This lipid species, which is in relatively low abundance (2-5%) in warm water fish, increases to approximately 14% in cold-adapted fish (1). This occurs despite the fact that there is little change in total fatty acid composition with change in growth temperature. The finding is of particular interest because PE with unsaturated acyl chains do not form stable bilayers, but rather form inverted hexagonal phases (H_{II}) at ordinary conditions. It has been shown (2) that micro-organisms adjust the fraction of non-lamellar lipids in their membranes as a consequence of their growth temperature. It is possible that a similar modulation of the physical properties of the membranes of cells from vertebrates also occurs. This would suggest that the 18:1/22:6 PE has a particularly strong influence on lipid polymorphism.

One of the consequences of a change in the relative proportion of bilayer and non-bilayer forming lipids is a modulation of the activity of certain membrane-bound enzymes. The activity of PKC is modulated by the physical properties of the bilayer (3,4). PKC is also relatively abundant in brain tissue, which is the organ in which differences in phospholipid species, with environmental temperature, have been detected. In this work we therefore also study the role of 18:1/22:6 PE in modulating the activity of PKC.

MATERIALS AND METHODS

Materials. Lipids were purchased from Avanti Polar Lipids, Pelham, AL. Histone H1 was from Gibco BRL, Grand Island, NY. Bovine serum albumin fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma Chemical Co., St. Louis, MO. [γ-^{32}P]ATP and [9,10,^{3}H]dipalmitoyl phosphatidylcholine were from NEN, Montreal, QC.

Differential Scanning Calorimetry (DSC). Lipid films were made from 16:1/16:1 PE dissolved in chloroform/methanol (2/1, v/v) either with or without the addition of a second phospholipid component, present at 5 mol % or less. After solvent evaporation with nitrogen, final traces of solvent were removed in a vacuum chamber for 90 min. The lipid films were suspended in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.0 by vortexing at 45°C for 30 sec. The final lipid concentration was 10 mg/ml. The lipid suspension was degassed under vacuum before being loaded into an MC-2 high sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A heating scan rate of 39°C/h was generally employed. The observed phase transitions were independent of scan rates between 10 to 60°C/h. The bilayer to hexagonal phase transition was fitted using parameters to describe an equilibrium with a single van't Hoff enthalpy and the transition temperature (T_H) reported as that for the fitted curve.

PKC Purification. Insect cells overexpressing PKC-α in the baculovirus system were kindly supplied by Robert Burns and Nancy Rankl of the Sphinx Pharmaceutical Co. The isolation procedure was a modification of that previously described by Stabel *et*

al. (5). Briefly, insect cell pellets were lysed in 2 mM benzamidine, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 40 μg/ml leupeptin, and 200 μM phenylmethyl-sulfonylfluoride (PMSF). After homogenization and centrifugation at 40,000 rpm, the supernatant was applied to a Q-Sepharose column (Pharmacia: 2.5 cm x 20 cm) equilibrated with 1 mM EGTA, 20 mM Tris-HCl, pH 7.5, and 10% (v/v) glycerol. Elution was carried out using 140 ml of Tris buffer alone, then 140 ml of each of Tris buffer containing 0.05 M, 0.15 M, 0.45 M, and 1.0 M KCl. PKC- was eluted with the 0.15 M KCl. Fractions were collected and analyzed for PKC activity in a previously described micellar assay (6). Fractions with the highest activity were pooled, adjusted to 1.5 M KCl, and loaded onto a phenyl-Sepharose column (Pharmacia: 1.0 cm x 10 cm) equilibrated with 1.5 M KCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5, and 10% glycerol. A salt gradient from 1.5 M to 0 M KCl was used to elute the enzyme. Collected fractions were tested for activity as above. Fractions containing the highest activity were pooled and concentrated to 0.40 mg/ml and stored at -70°C in 50% glycerol. Purified PKC-α had a specific activity towards histone in the micelle assay (6) of 1-2 μmoles/mg/min, with the phospholipid independent activity not exceeding 4% of the total kinase activity. The enzyme displayed a single band on a silver-stained electrophoresis gel.

PKC Binding Assays. A modification of the procedure of Rebecci *et al.* (7) was used as previously described (8). Briefly, PKC-α was incubated with sucrose-loaded large unilamellar vesicles (SLVs), then centrifuged at 100,000 g for 30 min at 25°C to separate the membrane-bound enzyme. The pellet and supernatant fractions were

assayed under identical conditions for activity towards protamine sulfate, in a buffer of 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.0, 1-100 μM $CaCl_2$ (depending on the experiment) and 0.3 mg/ml BSA.

PKC Activity Assays: The activity assay towards histone was performed as previously described (8,9). Histone was used at a final concentration of 0.2 mg/ml, phospholipid in the form of SLVs were 85 μM, [γ-^{32}P]ATP (0.2 mCi/ml) was 20 μM, and PKC was 100 ng/ml. The total volume was 250 μl, using the same buffer as in the binding assays. The results are expressed as fold activation. Fold activation is defined as the ratio of the activity in the presence of additive to that in its absence (POPC:POPS 7:3) after correcting for the non-specific activity measured in the absence of DAG and Ca^{2+}. An analogous expression for binding was used to calculate the fold binding.

Phospholipid Vesicles: Lipid films were made by dissolved phospholipids in 2:1 (v/v) chloroform:methanol and drying under a stream nitrogen followed by desiccation under vacuum for 2 hrs. Films were suspended in sucrose buffer (0.170 M sucrose in 5 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.0) and subjected to 5 freeze-thaw cycles. Vesicles were extruded through two 0.1 μm polycarbonate filters in a microextruder. The vesicles were suspended in the buffer used for binding assays and centrifuged at 100,000 g to dilute out the sucrose. Argon was bubbled through all solutions and vesicle preparations to protect lipids from oxidation.

RESULTS

The T_H of PE is particularly sensitive to the presence of certain additives (10). In the present work we use 16:1/16:1 PE as the host lipid since it has a relatively low T_H, so as to avoid decomposition of the polyunsaturated acyl chains. In order for one PE, at a low mol fraction, to affect the T_H of another PE, it must have a markedly different intrinsic radius of curvature (11). The extent to which the added lipid shifts the T_H of the host 16:1/16:1 PE can be evaluated from the slope of a plot of T_H versus mol fraction of the added lipid. Various mol fractions of added lipid between 0 and 0.05 were used. Addition of several unsaturated PEs to the 16:1/16:1 PE shows that they can all lower T_H. The 18:1/22:6 PE is the most effective among them (Table I). In contrast, the 18:1/22:6 phosphatidylcholine has the opposite effect and raises T_H (Table I).

SLVs consisting of 30 mol % POPS, 68 mol % POPC, plus 2 mol % of either DAG, 1,2-dioleoyl-PE (18:1/18:1 PE), 1-stearoyl-2-docosahexaenoyl-PE (18:0/22:6 PE), or 1-oleoyl-2-docosahexaenoyl-PE (18:1/22:6 PE) caused an activation of the PKC- catalyzed phosphorylation of histone at 2 μM Ca^{2+} compared to SLVs consisting of 30 mol % POPS and 70 mol % POPC (Fig. 1A and B). The greatest activation in POPS/POPC vesicles was observed with DAG (14.6-fold), followed by 18:1/22:6 PE (1.49-fold). 18:0/22:6 PE and 18:1/18:1 PE were equally effective, while 2 mol % of 1-oleoyl-2-docosahexaenoyl-3-phosphatidylcholine (18:1/22:6 PC) had no significant effect. Note that 2 mol % 18:1/22:6 PE appears weakly synergistic with 2 mol % DAG.

The activation of PKC-α catalyzed phosphorylation of histone by 18:1/22:6 PE was dependent on the concentration of calcium (Fig. 2). Replacement of a portion of POPC

in SLVs with 2 mol % or 10 mol % PE, showed the largest enhancement of activity at 1 μM Ca^{2+} (1.78-fold and 3.54-fold increase in activity, respectively). with the effect decreasing as the Ca^{2+} concentration was increased to 100 μM (1.18-fold and 1.44-fold increase, respectively). This effect correlated well with binding assay results (Fig. 3). 2 mol % and 10 mol % 18:1/22:6 PE increased the percentage of PKC- that was bound to SLVs of 30:70 mol % POPS/POPC by 5.5- and 10.9-fold, respectively, at 1 μM Ca^{2-}. This effect dropped to a 1.11-fold and 1.03-fold increase at 100 μM Ca^{2-}.

DISCUSSION

We have measured the effects of several forms of PE, containing unsaturated fatty acids, as well as 18:1/22:6 PC in affecting T_H of 16:1/16:1 PE. The 18:1/22:6 PE is moderately effective in lowering T_H. More hydrophobic substances such as diacylglycerols or alkanes are about 5-7 times more effective in this regard (10). Nevertheless, considering that the 18:1/22:6 PE is being added to another unsaturated PE. the finding that there is still a moderate lowering of T_H illustrates the greater H_{II}-forming tendency of 18:1/22:6 PE compared with either 18:0/22:6 PE, 16:1/16:1 PE or 18:1/18:1 PE. The effect on T_H is a consequence of the properties of the added phospholipid and not just on the presence of certain acyl chains as illustrated by the opposite effects of 18:1/22:6 PE and 18:1/22:6 PC on T_H (Table I). Therefore, the 9-12% increase in the 18:1/22:6 PE content of neuronal membranes of cold adapted fish (1) would be expected to have a significant effect on the physical properties of these membranes. One possible consequence of this change in phospholipid composition is to alter the functioning of membrane-bound enzymes such as PKC.

As stated earlier, the activity of PKC is modulated by the bulk biophysical properties of the membrane bilayer (3,4). Uncharged or zwitterionic additives which lower the bilayer to hexagonal phase transition temperature (therefore destabilize) model PE bilayers are activators of PKC (12). It is possible that the destabilized bilayer facilitates binding of PKC and rearrangement to an active form (13). It has also been suggested that there is an optimal intrinsic bilayer curvature for the activation of PKC (14). The effect of the various species of PE on PKC activity (Fig. 1a) is in close

agreement with their effects on T_H (Table I). The 18:1/22:6 PC which raises T_H has essentially no effect on the activity of PKC. This is also expected since the added lipid is replacing POPC in the PKC assay. Both the POPC and the 18:1/22:6 PC would form stable bilayers, having little negative curvature strain. It has previously been shown that the stimulatory effect of PE on PKC is related to their T_H (15). This would suggest that, as expected, the 18:1/22:6 PE which has a particularly low T_H activates PKC more than the other phospholipids tested. However, in comparison to DAG, the effect of 18:1/22:6 PE is small (Fig. 1B).

DAG is likely to have a much larger effect on PKC because it is a better hexagonal phase promoter (10) as well as the fact that it appears to bind specifically to a site on PKC (16). There appears to be a small degree of synergism between the effects of DAG and of 18:1/22:6 PE (Fig. 1B). A synergism between DAG and unsaturated fatty acids is also known to occur (17,18).

A number of factors including the total lipid concentration, mol fraction of phosphatidylserine, Ca^{2+} concentration and the presence of diacylglycerol promote the activation of PKC. To the extent it is known, these factors act largely independently of one another (8). In most physiological circumstances all three cofactors, i.e. phosphatidylserine, Ca^{2+} and DAG are required for activation of PKC. However, this enzyme can also be activated in the absence of phosphatidylserine with PE at high concentrations of Ca^{2+} (19). It is also well known that in the presence of DAG, lower concentrations of Ca^{2+} are required to activate PKC (20). Similarly, lower concentrations of Ca^{2+} are required in the presence of 18:1/22:6 PE (Fig. 2). Thus 18:1/22:6 PE, like

other activating substances, can lower the requirements for other cofactors such as Ca^{2+}. Similarly high Ca^{2+} concentration can conversely lower the activation of PKC by 18:1/22:6 PE.

While both DAG and 18:1/22:6 PE lower the Ca^{2+} requirement for PKC activation. the effect of DAG is more than an order of magnitude greater. However. the potency of these two activators should not be compared directly, as they are likely to serve quite different functions physiologically. DAG can act as a signal to turn on PKC activity. This occurs at low concentrations of DAG and the DAG itself is rapidly metabolized. mostly to phosphatidic acid, so that the activation of PKC is transient, as should occur for a signalling mechanism. In contrast 18:1/22:6 PE can build up to substantial concentrations in the membrane, about an order of magnitude greater than that of DAG. Furthermore, once a level of 18:1/22:6 PE is established, as a consequence of the environmental conditions, this steady state level is maintained over a long period of time. The 18:1/22:6 PE does not act as a signal to activate PKC to maximal or close to maximal levels, but rather modestly increases PKC activity both in the presence and absence of signals. The fact that this modest increase in activity occurs over a long period of time likely has important physiological consequences.

There are two mechanisms by which changes in membrane physical properties can affect the activity of PKC. One is by promoting the translocation of the enzyme from the aqueous phase to the membrane where it becomes activated and the second is by activating the membrane-bound form of the enzyme. DAG can activate PKC by both mechanisms, although it acts predominantly by affecting membrane partitioning (8). The

tumor promoter pristane is also able to activate PKC by increasing the proportion of membrane-bound enzyme (21). The authors conclude that this is a result of the effects of pristane on the biophysical properties of the membrane. The activation caused by 18:1/22:6 PE (Fig. 2) is also largely accounted for by increased partitioning of the enzyme to the membrane (Fig. 3). Another amphitropic enzyme whose activity is stimulated by the presence of non-lamellar forming lipids is CTP:phosphocholine cytidylyltransferase (22). It has recently been shown that incorporation of docosahexanoic acid into fetal lung tissue results in the stimulation of this enzyme (23). It may thus be a general phenomenon that incorporation of 22:6 into certain phospholipids increases the negative curvature strain of the membrane and leads to an activation of specific enzymes.

ACKNOWLEDGEMENTS

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FOOTNOTE

Abbreviations used: PE, phosphatidylethanolamine; 18:1/22:6 PE, 1-oleoyl-2-docosahexanoyl PE (other PE used are also designated by the nature of the acyl chains, *i.e.*, length: number of *cis* double bonds; H_{II}, inverted hexagonal phase; PKC, protein kinase C; T_H, bilayer to hexagonal phase transition temperature; DAG, 1,2-dioleoyl-sn-glycerol; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PC, phosphatidylcholine; SLV, sucrose-loaded large unilamellar vesicles.

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Table I

Effect of several phospholipids on the T_H of 16:1/16:1 PE

Lipid Additive	Shift of T_H (Degrees/mol fraction additive)
18:0/22:6 PE	-84 ± 6
18:1/18:1 PE	-72 ± 19
18:1/22:6 PE	-143 ± 8
18:1/22:6 PC	+265 ± 8

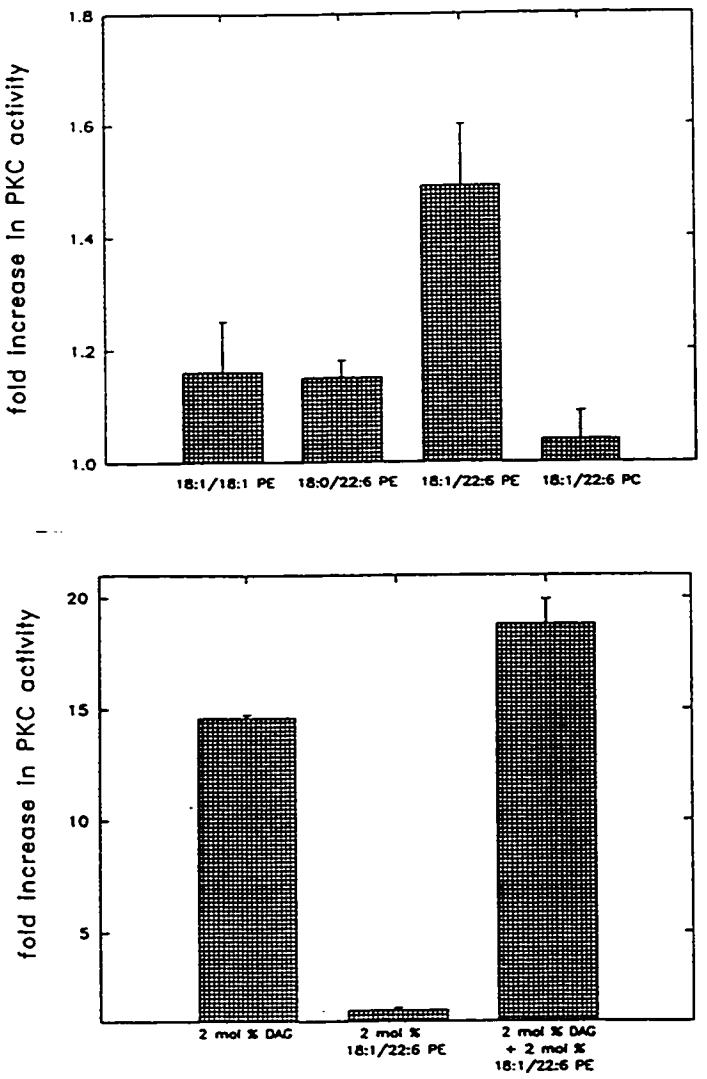

FIG. 1A & B. **Fold Increase in Activity of PKC-α Towards Histone for LUVs Containing 2 mol% of Various Phospholipids and/or DAG.** The LUVs were composed of 30 mol% POPS, 2 mol% of each additive, with the remainder POPC. The final Ca^{2+} concentration was 2 μM. The activity is expressed as a fold increase obtained with LUVs containing no additive. Data is expressed as the mean of triplicates ± S.D.

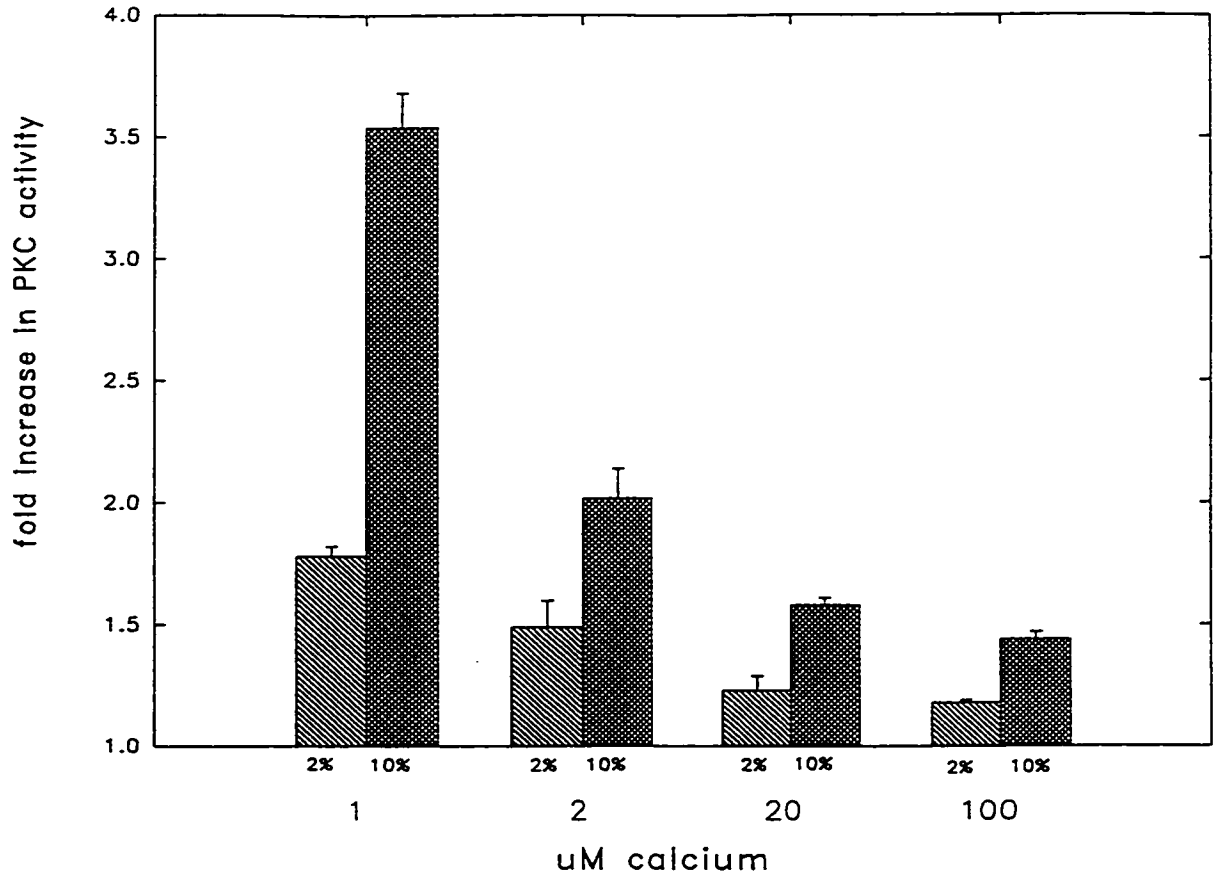

FIG. 2. **Fold Increase in Activity of PKC-α Towards Histone for LUVs Containing 2 mol% of 10 mol% 18:1/22:6 PE at Various Calcium Concentrations.** The LUVs were composed of 30 mol% POPS with 2 or 10 mol% 18:1/22:6 PE, the remainder being POPC. The activity is expressed as a fold increase in the activity obtained with LUVs containing no additive. Data is expressed as the mean of triplicates ± S.D.

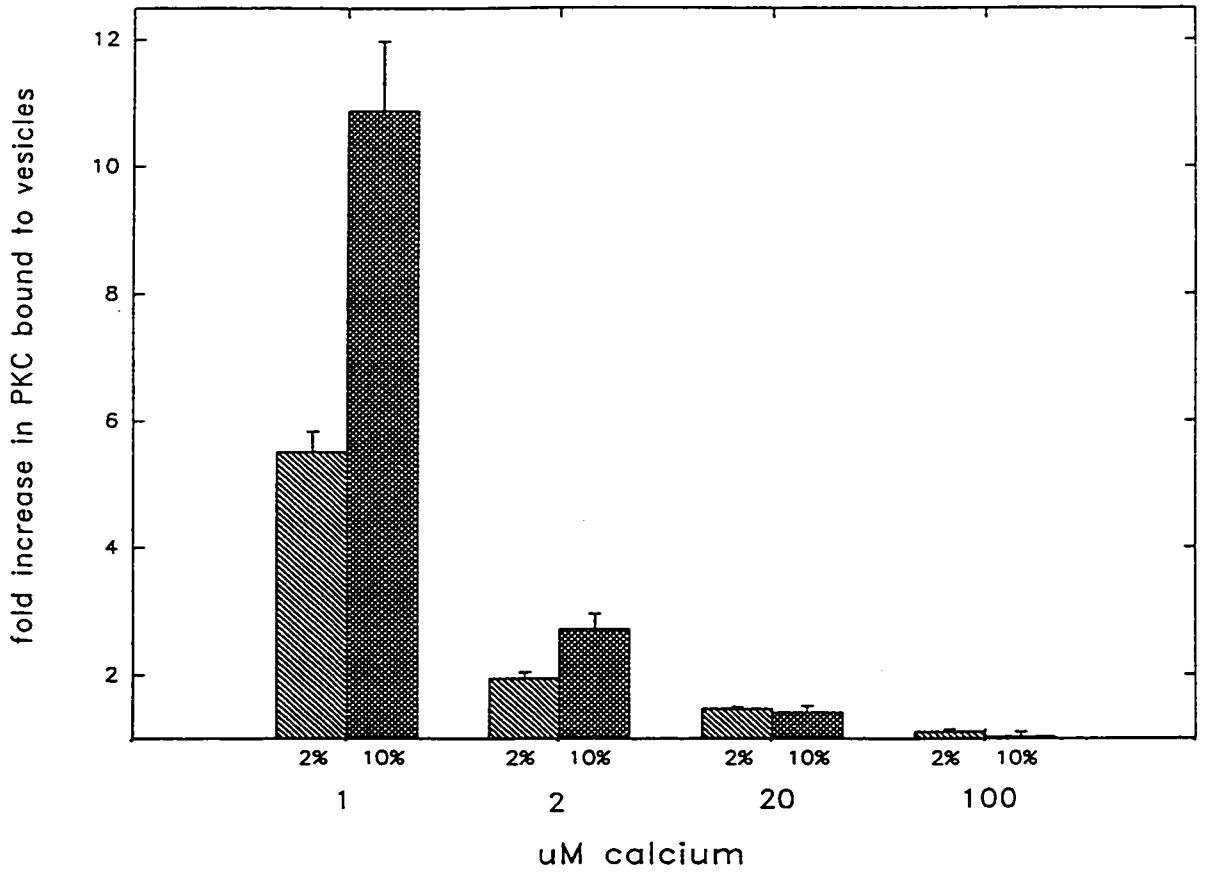

FIG. 3. **Fold Increase in Binding of PKC-α to SLVs Containing 2 mol% or 10 mol% 18:1/22:6 PE at Various Calcium Concentrations.** The SLVs were composed of 30 mol% POPS, with 2 or 10 mol% 18:1/22:6 PE, the remainder POPC. The binding is expressed as a fold increase in % of PKC-α bound to SLVs containing no additive. Data is expressed as the mean of triplicates ± S.D.

Manuscript 2 "Transbilayer Inhibition of PKC by the Lipophosphoglycan from *Leishmania donovani*".

The rationale for this investigation was to study the effects of LPG on the binding and activation of PKC. Since the role of LPG in the survival of *leishmania* is believed to involve the inhibition of PKC, it was interesting to determine the mechanism by which this hydrophilic polymer accomplishes this. LPG is an inhibitor that is structurally different than many other inhibitors of PKC since it is negatively charged. LPG was found to be able to inhibit the membrane bound form of the enzyme due to its bilayer stabilization. The interesting outcome of this study was that LPG was able to accomplish this from the opposite monolayer to which the enzyme binds. This is the first example of a PKC inhibitor which functions this way. Also, the LPG and PKC are found on opposite monolayers *in vivo,* so this inhibition may also occur in macrophage cells.

The experiments in this study were carried out by Jennifer Giorgione. The manuscript was written by Jennifer Giorgione, and edited by Richard Epand. Salvatore Turco provided the purified LPG, and LPG fragments. He also contributed some background information on LPG for the introduction of the manuscript.

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Transbilayer Inhibition of Protein Kinase C by the Lipophosphoglycan from *Leishmania donovani*

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Running Title: Lipophosphoglycan inhibition of PKC

Key words: (PKC/sucrose loaded vesicles/membrane partitioning/ PKC activation/enzyme kinetics)

Classification: Biological Sciences: Biophysics

Abbreviations:

Lipophosphoglycan, LPG; protein kinase C, PKC; sucrose-loaded vesicle, SLV; large unilamellar vesicle, LUV; Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Tyr, R-peptide; 1,2-diolein, DG; bilayer to hexagonal phase transition temperature, T_H; phosphatidylserine, PS; phosphatidylcholine, PC; 1-palmitoyl, 2-oleoyl phosphatidylserine, POPS; 1-palmitoyl, 2-oleoyl phosphatidylcholine, POPC; dielaidoyl phosphatidylethanolamine, DEPE.

Abstract

Lipophosphoglycan (LPG), the predominant molecule on the surface of the parasite *Leishmania donovani*, has previously been shown to be a potent inhibitor of protein kinase C (PKC) isolated from rat brain. The mechanism by which LPG inhibits PKC was further investigated in this study. LPG was found to inhibit the PKCα-catalyzed phosphorylation of histone in assays using large unilamellar vesicles composed of 1-palmitoyl, 2-oleoyl phosphatidylserine and 1-palmitoyl, 2-oleoyl phosphatidylcholine either with or without 1% 1,2 diolein added. The results also indicated that while PKC binding to sucrose loaded vesicles was not substantially reduced in the presence of LPG at concentrations of 1-2%, the activity of membrane-bound PKC was inhibited by 70%. This inhibition of the membrane-bound form of PKC is not a consequence of reduced substrate availability to the membrane. However, Km shifted from ~ 31 ± 4 μM to 105 ± 26 μM in the presence of 5% LPG. LPG caused PKC to bind to membranes without inducing a conformational change as revealed by the lack of an increased susceptibility to trypsin. An LPG fragment containing only one repeating disaccharide unit was not as effective as the entire LPG molecule or of larger fragments in inhibiting the membrane-bound form of the enzyme. The shorter fragments were also less potent in raising the bilayer to hexagonal phase transition temperature of a model membrane. LPG is also able to inhibit the membrane-bound form of PKCα from the inner monolayer of LUVs, the opposite monolayer to which the enzyme binds in our assay. Inhibition is likely a result of alterations in the physical properties of the

membrane. To our knowledge, this is the first example of a membrane additive that can inhibit the membrane-bound form of PKC in the presence of other lipid cofactors.

Introduction

Lipophosphoglycan is the major glycoconjugate found on the surface of the promastigote form of *Leishmania donovani*, a protozoan parasite that causes human leishmaniasis (reviewed in refs. 1 and 2). The structure of the *L. donovani* LPG consists of an average of 16 repeating units of Gal(β1,4)Manα1-PO_4 units linked to a glycan core of 3 Gal, 2 Man, 1 Glc-PO_4, and one unacetylated glucosamine residue. The entire polysaccharide portion of LPG is anchored by a 1-O-alkyl-2-lyso-phosphatidylinositol, resulting in a highly negatively charged molecule of average molecular weight of 9 kDa. LPG is expressed on all species of *Leishmania* with conservation of the lipid anchor and glycan core and with some variation in the repeating units. A number of interesting functions have been proposed for LPG (1), including protection of the parasite against microbicidal activities in *Leishmania*-macrophage interactions. Macrophages undergo an oxidative burst during phagocytosis, which leads to the production of oxygen radicals. Protein kinase C, an enzyme involved in signal transduction, is thought to be a mediator of the oxidative burst (3) and other cellular processes. PKC acts by phosphorylating serine and threonine residues on its protein substrates. The classical isoform of PKC used in this study, is regulated by calcium, phosphatidylserine and diacylglycerol.

In previous studies, purified LPG was shown to be an inhibitor of PKC activity *in vitro* as well as an inhibitor of PKC-mediated events in living phagocytic cells (4-9). Thus, it appears that an effective inhibition of PKC activity achieved by LPG may represent a critical step for successful establishment of *Leishmania* promastigotes within host macrophages. In this study, we undertook an investigation to better understand the

relationship between LPG and inhibition of PKC activity. Using PKC purified from rat brain, as well as PKCα purified from a bacculovirus expression system, we have found that LPG inhibits the membrane-bound form of PKC.

In vivo, LPG and PKC are found on opposite sides of the membrane when a *Leishmania* parasite approaches a host cell. LPG is intercalated on the macrophage cell surface during phagocytosis of the parasite into the macrophage, as observed by immunofluorescence using anti-LPG monoclonal antibodies (10), while PKC binds to the cytoplasmic side of the plasma membrane. We demonstrate that despite the positions of LPG and PKC on opposing monolayers, inhibition is still observed. This demonstrates that inhibition of PKC by LPG may be of biological importance in the initial stages of infection by *Leishmania* and that this inhibition must be a consequence, at least in part, of effects of LPG on membrane physical properties which can be transmitted across the bilayer.

Experimental Procedures

Materials--Lipids were purchased from Avanti Polar Lipids, Alabaster, AL. Histone H-I was from Gibco BRL, Grand Island NY. Bovine serum albumin fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma Chemical Co., St. Louis MO. [γ-^{32}P]ATP and [9,10-^{3}H] dipalmitoyl phosphatidylcholine were from NEN, Montréal, Que. The peptide acetyl-Phe-Lys-Lys-Ser-Phe-Lys-Leu-amide was purchased from NRC Canada. The peptide Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr (R-peptide) was synthesized at the peptide facility of the University of

Kentucky, Lexington, Kentucky. LPG was isolated and purified from *Leishmania donovani* as previously described (11).

LPG fragments--LPG was cleaved at acid-labile phosphodiester bridges (12). Purified LPG was treated with 0.02 M HCl at 60°C for 1 min. The sample was then neutralized with NaOH and subjected to gel filtration chromatography on Sephadex G150 (1x100 cm) equilibrated with 0.04 N NH_4OH and 1 mM EDTA; 0.6 ml fractions were collected and aliquots were assayed for carbohydrate by phenol-sulfuric acid (13). Three pools were taken based on relative sizes due to partial depolymerization of the repeating units. The pools were desalted by dialysis. The resulting LPG fragments contained an average of 1, 6, or 12 repeating Gal(α1,4)Manα1-PO_4 units.

PKC purification--Rat brain PKC was purified by a modification of the procedure of Huang et al. (14), as described elsewhere (15). Purified PKC displayed a single band on the silver-stained electrophoresis gel. The specific activity of the enzyme for histone in a micellar assay (16) was 1-2 mmol/mg min. The phospholipid independent activity in this assay did not exceed 2% of the total kinase activity. Some experiments were performed with rat brain PKC purchased from Molecular Probes (Eugene, OR).

PKCα was purified from insect cells overexpressing the α isoform in the bacculovirus system. Cells were a gift from David Burns and Nancy Rankl of the Sphinx Pharmaceutical Co. The procedure was a modification of that of Stabel et al. (17). Briefly, insect cell pellets were lysed in 2 mM benzamidine, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 40 μg/ml leupeptin,

and 200 μM phenylmethyl-sulfonylfluoride (PMSF). The mixture was homogenized and then centrifuged at 200000 x g for 50 min. The supernatant was applied to a Q-Sepharose column (Pharmacia: 2.5 cm x 20 cm) equilibrated with DEAE buffer (1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5, and 10% (v/v) glycerol). DEAE buffer alone, then DEAE containing 0.05 M, 0.15 M, 0.45 M and 1.0 M KCl were used for elution. PKCα was eluted with the 0.15 M KCl between fractions 20-30. Collected fractions (3.5 ml) were assayed for PKC activity as for the rat brain PKC (16). Fractions with the highest activity were pooled, adjusted to 1.5 M KCl and loaded onto a phenyl-sepharose column (Pharmacia; 1.0 cm x 10 cm) equilibrated with 1.5 M KCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5 and 10% glycerol. PKCα was eluted with a 100 ml salt gradient from 1.5 M to 0 M KCl. Fractions (2 ml) were tested for activity, pooled, and concentrated to 0.4 mg/ml by ultrafiltration using an Amicon YM30 membrane and stored at -70°C in 50% glycerol. The specific activity of PKCα was similar to that of rat brain PKC.

Phospholipid vesicles--Lipid films were made by dissolving phospholipids in 2:1 (v/v) chloroform:methanol, and drying under a stream of nitrogen, followed by desiccation under vacuum for 2 hrs. Films were suspended in either a buffer of 5 mM $MgCl_2$, 20 mM Tris-HCl, 100 mM KCl, or a sucrose buffer of 0.170 M sucrose in 5 mM $MgCl_2$ and 20 mM Tris-HCl pH 7.0. Vesicles were subjected to 5 freeze-thaw cycles and extruded through two 0.1 μm polycarbonate filters in a microextruder. Vesicles containing sucrose were suspended in the $MgCl_2$/Tris/KCl buffer and centrifuged at 100,000 x g for 30 min to dilute out the sucrose.

LPG vesicles--(Incorporation of LPG into the outer monolayer only.) An aqueous solution containing purified LPG or LPG fragments was incubated with LUVs or SLVs (sucrose loaded vesicles) for 20 minutes in a buffer of 100 mM KCl. 5 mM $MgCl_2$. 20 mM Tris/HCl, pH 7.0. The mol % of LPG is always given with respect to the total lipid present, regardless of the sidedness of LPG. Measurement of LPG incorporation into these vesicles using SLVs and LPG which was biosynthesized using ^{3}H-mannose. showed that these conditions resulted in essentially quantitative incorporation of LPG into the vesicles.

(Incorporation of LPG into inner and outer monolayers.) An aqueous solution of LPG was added to dried lipid films before the 5 freeze-thaw cycles. These multilamellar vesicles were then extruded to form LUVs or SLVs.

(Removal of LPG from outer monolayer.) LUVs were prepared with LPG on both inner and outer monolayer, or on the outer monolayer only. LUVs were incubated with a 5 fold excess of sucrose loaded MLVs for 48 hours. During this time. LPG was exchanged from the outer monolayer of the LUVs to the MLVs. The MLVs were sedimented at 12,000 g for 10 min. The supernatant containing the LUVs with no LPG. or LPG on the inner monolayer only were used in the PKC activity assays. The MLVs consisted of POPC only, and had no effect on the assay. The exchange of LPG was monitored by incorporating a small amount of tritium labeled LPG into the vesicles.

PKC binding assays--The sucrose-loaded vesicle (SLV) assay was modified from the procedure of Rebecci et al. (18) as described elsewhere (15). Briefly. PKC was incubated with SLVs and then centrifuged at 100000 x g for 30 min at 25°C to separate

the membrane-bound enzyme. The pellet and supernatant were assayed under identical conditions for enzyme activity toward the R-peptide. The substrate used was one which was not sensitive to the presence or absence of LPG. However, an equal amount of vesicles were added to the supernate fraction prior to the activity assay so that the measured activity in supernate and pellet fractions would be directly comparable. The buffer contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.0, 200 μM $CaCl_2$ and 0.3 mg/ml BSA.

PKC activity assays--The activity assay toward histone, protamine sulfate, or synthetic peptide was performed as previously described (15,19). The buffer was the same as that used for the binding assays. Histone or protamine sulfate was added to a final concentration of 0.2 mg/ml (in 250 μl total volume), acetyl-FKKSFKL-amide was used at a final concentration of 90 μM. Phospholipid was 85 μM in the form of large unilamellar vesicles (LUVs), [γ-^{32}P]ATP (0.2 mCi/ml) was 20 μM, and PKC was 100 ng/ml.

Differential Scanning Calorimetry (DSC)--Bilayer to hexagonal phase transition temperatures were measured as described previously (20). Briefly, lipid films of dielaidoyl phosphatidylethanolamine (DEPE) were suspended in buffer to a final total lipid concentration of 10 mg/ml, and LPG fragments were added. An MC-2 high sensitivity calorimeter was used (Microcal Co., Amherst, MA), with a heating scan rate of 41 K/h.

Determination of Km and Vmax--Activity assays were performed using 100 μM lipid (70:29:1 PS:PC:DG ± 5% LPG) with the MARCKS peptide as a substrate. Other

conditions are the same as described under PKC Activity Assays. Km and Vmax were determined using the program GraFit 3.0 (Erithacus Software, Robin J. Leather-Barrow).

Trypsin cleavage of PKCα--The procedure was a modification of that of Newton and Koshland (21). 300 ng PKCα was incubated with 200 μM Ca^{2+}. 400 μM LUVs of 70:29:1 PS:PC:DG ± 5% LPG at 25°C. 10 μl of 88 units/ml trypsin was added. and the mixture was incubated for 15 min at 30°C. Reaction products were separated by SDS-PAGE on a 7% acrylamide gel which was then silver stained.

Results

LUVs consisting of 50 mol% POPS, 0.5 mol% to 4.0 mol% LPG and the remainder POPC, inhibited PKC-catalyzed phosphorylation of histone relative to LUVs composed of 1:1 POPS/POPC (Fig. 1a). Similar results showing a dose-dependent LPG inhibition of PKC activity using Triton micelles containing PS and DG have previously been reported (9). The largest decrease in activity is seen between 0 and 0.5% LPG.

To ascertain whether the inhibition of PKC activity was caused by LPG interfering with binding of the enzyme to the membrane or by inhibiting the membrane-bound form of PKC, a modified form of the SLV binding assay for PKC was used. The original method (15) used protamine sulfate as a substrate for PKC to determine the distribution of the enzyme between membrane and soluble fractions. However, PKC-catalyzed phosphorylation of protamine sulfate was found to be activated by LPG thus complicating the analysis. Using LUVs of 50 mol% POPS with the remainder POPC. 1-3% LPG increased phosphorylation of protamine sulfate up to 50% (not shown). A

synthetic peptide, the R-peptide, has a sequence corresponding to a segment of protamine. Like protamine sulfate, it is phosphorylated by PKC in a manner largely independent of Ca^{2+} or lipid (22). The rate of phosphorylation of the R-peptide is not affected by the presence of vesicles containing 1-5% LPG (data not shown). This substrate was therefore used instead of protamine sulfate in the PKC binding assay. The results showed that 1 or 2% LPG modestly inhibited the binding of PKCα to membranes but that 5% LPG completely prevented binding (Fig. 1b). The results obtained using rat brain PKC (data not shown) were similar to those shown using PKCα. Therefore, comparisons between experiments performed using the two different PKC preparations are valid, in addition to the fact that both preparations are essentially composed of conventional PKC isoforms.

The percentage of PKC bound to vesicles (Fig. 1b) with a particular LPG content, is higher than the relative activity of PKC towards histone (Fig. 1a) for the same vesicle composition. 1,2-diacylglycerol (DG) is known to increase the binding affinity of PKC for PS-containing membranes (15). Therefore, adding 1% DG and increasing the % PS of the vesicles to 70% resulted in nearly 100% of the PKC binding to LUVs (using the R-peptide as substrate) even in the presence of 5% LPG (Fig. 2a). Since it is possible to achieve ~100% binding in the presence of LPG (at 70 mol% PS), LPG's effects on PKC's binding to membranes is not likely due to a steric hindrance caused by the bulkiness of the molecule, but possibly by an effect on the physical properties of the membrane. PKC phosphorylation of histone is reduced by LPG even at high mol% PS where all of the enzyme is bound to the membrane (Fig. 2b).

It was important to determine whether inhibition of PKC phosphorylation of histone by LPG was due to reduction in the amount of substrate available at the membrane. It has been shown that the peptide substrate acetyl-FKKSFKL-amide (MARCKS peptide) attains its maximal rate of phosphorylation at lower mol fractions of PS than is the case for histone (15). LUVs with 70% POPS and 1% DG should support a maximum rate of PKC catalyzed phosphorylation of this heptapeptide. The fact that LPG caused a similar inhibition of the phosphorylation of this substrate (not shown) as it did for histone indicates that LPG is inhibiting the membrane-bound form of PKC and is not acting by reducing substrate binding to the lipid bilayer.

Since LPG is negatively charged it would be expected to increase the accumulation of cationic substrates at the membrane interface by the Gouy-Chapman electrical double layer effect. Thus, if LPG had any effect on PKC activity through changes in substrate accumulation at the membrane, it would be expected to increase the activity of the enzyme. SLVs were incubated with the MARCKS peptide and centrifuged as in the binding assay to separate free from membrane-bound MARCKS peptide. This showed that there were similar amounts of peptide bound to LUVs composed of 70:29:1 PS:PC:DG either with or without 5% LPG (data not shown). Thus, under the conditions used, one would not anticipate any change in substrate availability to the enzyme.

Km and Vmax were determined for the MARCKS peptide. At a lipid concentration of 100 μM, Vmax was 0.034 ± 0.001 pmol phosphate/min without LPG, and 0.034 ± 0.003 with 5% LPG with vesicles of 70:29:1 PS:PC:DG. Km was 31 ± 4 μM in the absence of LPG, and 105 ± 26 μM with 5% LPG (Fig. 3).

PKCα was subjected to cleavage by trypsin, in the presence of 70:29:1 PS:PC:DG vesicles, with and without added 5% LPG. Binding to membranes results in a conformational change in PKC leading to the exposure of the enzyme's hinge region, which contains a trypsin cleavage site (21). The silver stained SDS PAGE gel (Fig. 4) revealed that PKCα was much less sensitive to cleavage when bound to vesicles containing LPG (lane 2) than when bound to those lacking LPG (lane 1). In the presence of LPG, most of the PKCα remains uncleaved, while in its absence most of the PKCα is cleaved to a 45 kDa catalytic and 35 kDa regulatory domain. Note that the amount of intact PKCα remaining in the presence of LPG (lane 2) is similar to the amount obtained when no vesicles are present (lane 3), although the binding assays reveal that under these conditions, nearly 100% of the PKCα is membrane-bound. Therefore, PKC must be bound in a non-activated conformation, similar to that which occurs in solution and which does not expose the hinge region. It is evident that PKC's regulatory domain interacts differently with an LPG containing membrane, as suggested by the smear in lane 2 instead of the 35 kDa band which appears in lane 1. This is likely, since LPG was found to have no effect on the activity of PKM (the catalytic fragment of PKC) in an *in vitro* assay (9). LPG-containing vesicles were not found to affect the activity of trypsin, in assays using either histone or casein as substrates, relative to vesicles without LPG (data not shown).

To discover which portion of LPG is responsible for the inhibition of the membrane-bound form of PKC, we used LPG fragments consisting of an average of 1.6 or 12 repeating Gal(β1,4)Manα1-PO_4 units in the binding and activity assays in the

presence of 1 mol% DG. It was found that LPG fragments containing an average of 12 and 6 repeating disaccharide units inhibited the membrane-bound form of PKC to a similar extent as the full length molecule of an average of 16 repeating units. However, there was much less of an effect on membrane-bound PKC by LPG fragments containing an average of only 1 disaccharide unit (data not shown). In contrast to previous findings using Triton micelles (8), the 1-O-alkylglycerol portion of LPG (the lipid core containing no carbohydrate) was found to be ineffective in inhibiting the activity of PKC in our LUV assay system (data not shown), or at raising the T_H of DEPE (not shown). Therefore, both enzyme inhibitory effects as well as effects on lipid polymorphism were lost when most of the carbohydrate portion of LPG was removed.

The inhibitory effect of LPG on PKC activity may be a consequence of its bilayer stabilizing effects on membranes. We investigated if the loss of inhibitory activity of the smaller LPG fragment could be correlated with its reduced effectiveness in raising the bilayer to hexagonal phase transition temperature of a synthetic phospholipid. DEPE is a model system often used to measure changes in T_H. The changes in T_H of DEPE reflect the effects of the additive on membrane intrinsic curvature. These shifts in T_H have been correlated with changes in PKC activity (23). Indeed there was a progressive loss in the bilayer stabilizing ability of the LPG fragments as the size of the fragments became smaller (Fig. 5). This is consistent with the suggestion that the bilayer stabilization of membranes by LPG is responsible for its inhibitory action, but steric factors may also play a role.

Strong evidence for the involvement of membrane physical properties is given by the finding that LPG is able to inhibit both the activity and binding of PKCα even from the opposite monolayer to which the enzyme binds. LUVs consisting of 30:69:1 PS:PC:DG + 2.5% LPG on each side of the membrane inhibited PKC catalyzed phosphorylation of histone as well as its binding to vesicles to a greater extent than did the same LUVs containing 2.5% LPG on the outer monolayer only (Fig. 6). LPG is inhibiting the membrane-bound form of PKC , since the percentage bound is larger than the percentage which is active, similar to the effects seen in figure 2 containing LPG on the outer monolayer only. The effects of LPG sidedness on PKCα activity are similar towards both histone and the MARCKS peptide again suggesting that the LPG effects are independent of substrate availability (not shown).

We have also tested the inhibition of PKC by LPG contained on the opposing monolayer. This was done by incorporating LPG on both sides of the membrane followed by extraction of LPG on the outer monolayer with MLVs. As a control, we also extracted LPG from LUVs containing the amphiphile only on the outer monolayer. In this latter case, all of the LPG should be extracted and these LUVs are capable of supporting close to 80% of the total activity (Fig. 7). For LUVs originally containing LPG on both sides of the membrane, extraction with MLVs resulted is vesicles supporting a substantially lower activity, particularly in the case of vesicles originally containing 5% LPG (Fig. 7). This result demonstrates that LPG on the inner monolayer can inhibit PKC bound to the outer monolayer. However, this transbilayer inhibition is

weaker than in cases where LPG is present in the outer monolayer or in both monolayers (no MLV wash, right side of Fig. 7).

Discussion

The inhibition of the PKC of host cells by LPG may have a biological role for the survival of *Leishmania* in macrophages. *Leishmania* is one of only a few microbes that can successfully survive in lysosomes of host macrophages. LPG has been shown to be critical in the infection of a macrophage since *Leishmania* mutants that lack LPG are unable to survive (1). Moreover, the addition of purified LPG to these mutants prolonged their capability of intracellular survival in macrophages. Furthermore, preliminary work in our laboratories has shown that genetic complementation of these mutants restores the expression of cell surface LPG and their ability to infect macrophages, thereby demonstrating the importance of LPG in *Leishmania*-macrophage interactions. The underlying basis for surviving with impunity is accomplished at least in part, by preventing macrophages from becoming activated, a process mediated by PKC.

LPG represents a modulator of PKC activity which is structurally different from those previously studied. A knowledge of its mechanism of inhibition of this enzyme may contribute to a further understanding of the modulation of PKC activity by changes in membrane properties. An interesting finding was that LPG could inhibit both membrane binding and the activity of membrane-bound PKCα from the opposite monolayer. The effects of LPG from the inner monolayer of LUVs on PKCs binding and activity are not quite as strong as its effects from the outer monolayer (compare effects of

5 mol% LPG added to the outer monolayer on binding [Fig. 2a] and activity [Fig. 2b] of PKC at 30 mol% PS with the effects of 2.5 mol% LPG added to both inner and outer monolayers on binding and activity [Fig. 6]). However, there is still a significant amount of inhibition, which supports the hypothesis that the inhibition of PKC by LPG is (at least partially) a consequence of the modulation of membrane biophysical properties. Similar results were obtained when the LPG was removed from the outer monolayer and left only on the inner monolayer. Compare differences in % activity when 5% LPG was originally present on the outer monolayer and then removed with MLVs, with the activity when LPG was originally present on both monolayers (Fig. 7). In the later case 2.5% LPG remains on the inner monolayer and can cause inhibition of PKC activity on the outer monolayer. This provides stronger evidence that LPG can affect PKC activity from the opposite monolayer.

It has been suggested that modulation of PKC activity by additives to the membrane is predictable on the basis of the charge on the additive and its effect on lipid polymorphism (23). In general, cationic substances are inhibitors of PKC, while anionic membrane additives are either activators or cofactors for the enzyme. In addition, uncharged or zwitterionic additives which raise the bilayer to hexagonal phase transition temperature (T_H) of the membrane are inhibitors of PKC[1]. LPG is very potent in raising T_H (20); however, LPG is also anionic. Generally the charge effect predominates for

[1]T_H is the temperature at which model phosphatidylethanolamine bilayers form the inverted hexagonal phase. This phase forms when the bilayer is destabilized by increasing the intrinsic curvature of each monolayer (see ref. 23 for further explanation).

modulation of PKC activity, but not in the case of LPG. LPG may be the only known anionic amphiphile inhibitor of PKC. This may be a consequence of the unusually large effect of LPG in raising T_H which is a property correlated with the inhibition of PKC.

The activity of PKC toward histone is modulated by the calcium concentration at 0.3 nm from the membrane surface (24). Under the conditions of our experiment. however, LPG does not modulate PKC activity solely by altering the amount or distribution of Ca^{2+} at the membrane surface since we find that the effect of LPG on inhibiting the phosphorylation of histone is independent of Ca^{2+} concentration between 0 and 10 mM calcium (results not shown).

Kinetic analysis indicates that LPG affects K_m of the MARCKS peptide but not the V_{max}. In addition, analysis of susceptibility to tryptic cleavage indicates that PKC bound to the membrane in the presence of LPG does not undergo a conformational change associated with activation of the enzyme, as occurs in the absence of LPG. These results indicate that the active site of PKC is less available to substrate when the enzyme is bound to membranes in the presence of LPG. However, the native structure and catalytic mechanism of PKC remain unaltered by LPG as indicated by the invariance of V_{max}. This is also shown by the fact that the PKC-catalyzed phosphorylation of protamine sulfate is not inhibited by LPG.

This is the first example of an amphiphile that inhibits the membrane-bound form of PKC. PKC activity is generally well correlated with membrane binding except for the LPG-containing membranes, which are unique in strongly inhibiting the membrane-bound form of the enzyme. How LPG accomplishes this is still uncertain. It is possible

that LPG may alter the site at which PKC binds to the membrane by binding to the enzyme itself. A number of anionic amphiphiles of varying chemical structure can bind to PKC (14,26). In addition, substances which raise T_H appear to make the rearrangement of proteins in membranes more difficult. For example, this has been suggested as a factor modulating the lifetime of alamethicin channels (27) as well as the bilayer disrupting effects of viral fusion proteins (20), and the inhibition of insulin receptor autophosphorylation and signalling (28).

Thus LPG can function both to prevent binding of PKC to membranes as well as to inhibit the membrane-bound form of this enzyme. This is the first time that an amphiphile has been shown to inhibit the membrane-bound form of PKC in the presence of other cofactors. Our findings also demonstrate the ability of membrane modulation of protein activity to be transmitted by the coupling of opposing monolayers. This mechanism may be of importance to the survival of *Leishmania donovani*, and may therefore be a target in the control of the spread of the parasite.

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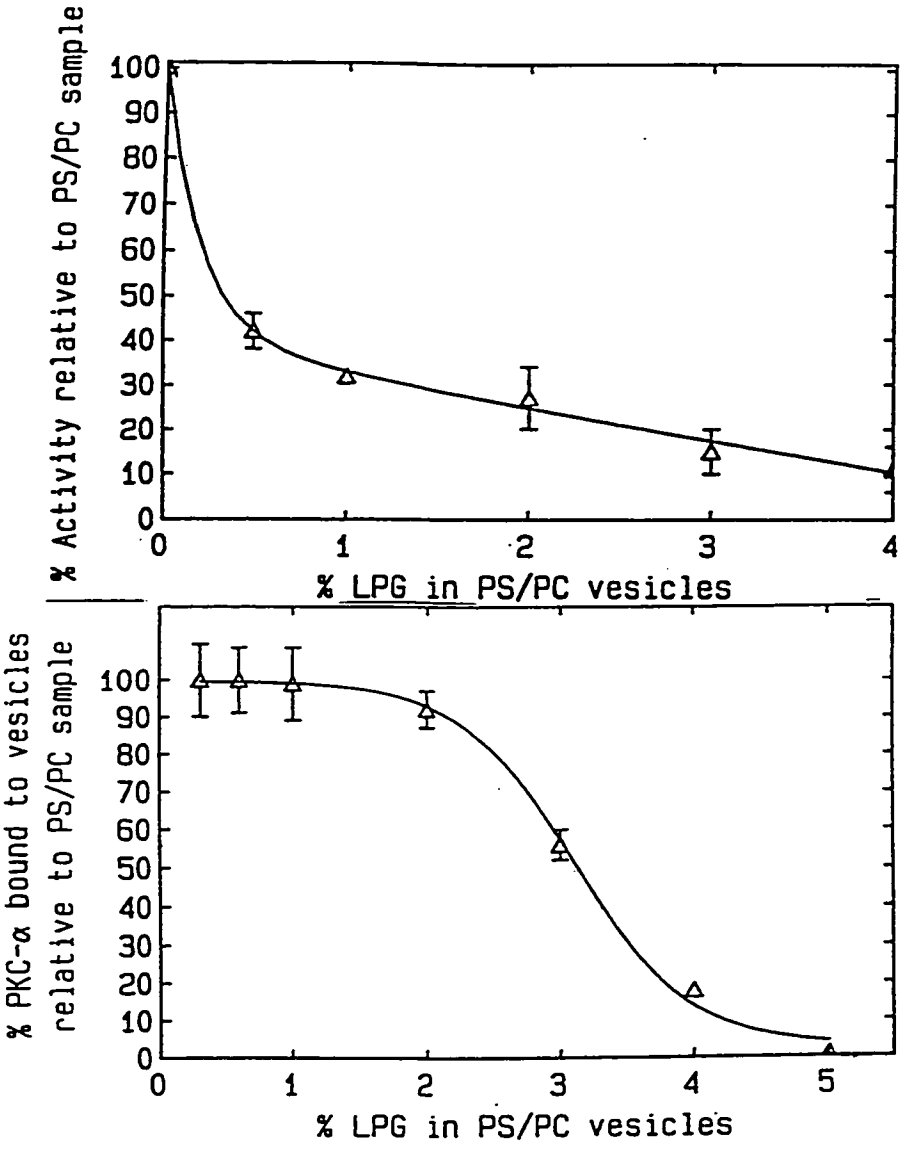

Fig. 1. **LPG Inhibition of PKC-Catalyzed Phosphorylation of Histone and Binding of PKCα to SLVs.** The LUVs were composed of 50 mol% POPS, 0.3%-5.0% LPG, and the remainder POPC. Fig. 1a, Activity: The activity is expressed as a percentage of the activity obtained using LUVs without LPG which was 245 pmol/min/mg. Fig. 1b. Binding: The binding is expressed is a percentage of the binding obtained with vesicles without LPG which was 87% of the added enzyme. Data are expressed as the mean of triplicate determinations ± S.D. at each concentration of LPG.

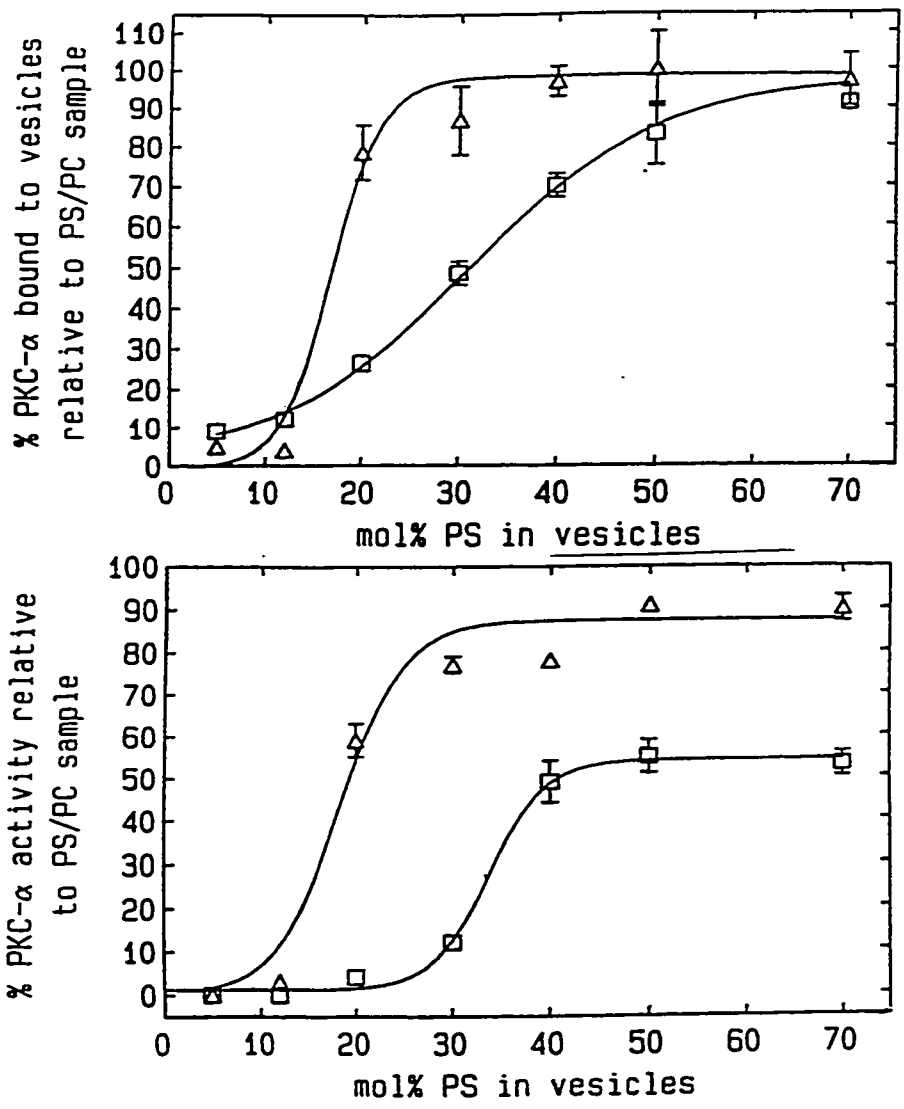

Fig. 2. **Binding of PKCα to SLVs and PKC-Catalyzed Phosphorylation of Histone.** The vesicles were composed of the indicated amount of POPS, 1 mol% DG and either 2% LPG (triangles) or 5% LPG (squares) with the remainder being POPC. Fig. 2a. Binding: The binding is expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to ~100% of the added enzyme bound for vesicles containing 20-70 mol% PS, 84% and 21% for vesicles containing 12 and 5 mol% PS respectively. Fig. 2b, Activity: The activity is expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to a specific activity of 3.8, 4.1, 4.5. 2.6, 2.1, 1.9 and 0.65 nmol/min/mg for vesicles containing 70, 50, 40, 30, 20, 12 and 5 mol% PS respectively. Data is expressed as the mean of triplicate determinations ± S.D. for each concentration of POPS.

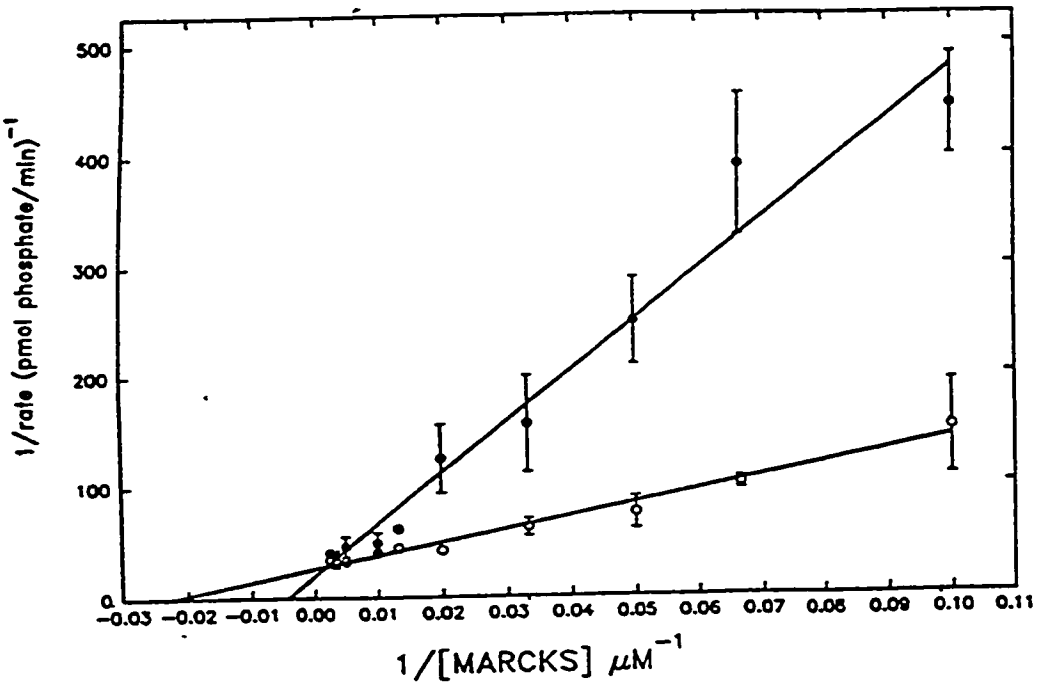

Fig. 3. **Lineweaver-Burk Plot for the Phosphorylation of the MARCKs Peptide by PKCα.** LUVs were composed of 70 mol% POPS, 1 mol% DG, and the remainder POPC, either without (hollow circles) or with 5% LPG (filled circles). Actual Km and Vmax values were calculated using GraFit 3.0. Data are expressed as the mean of triplicate determinations of at least two separate experiments.

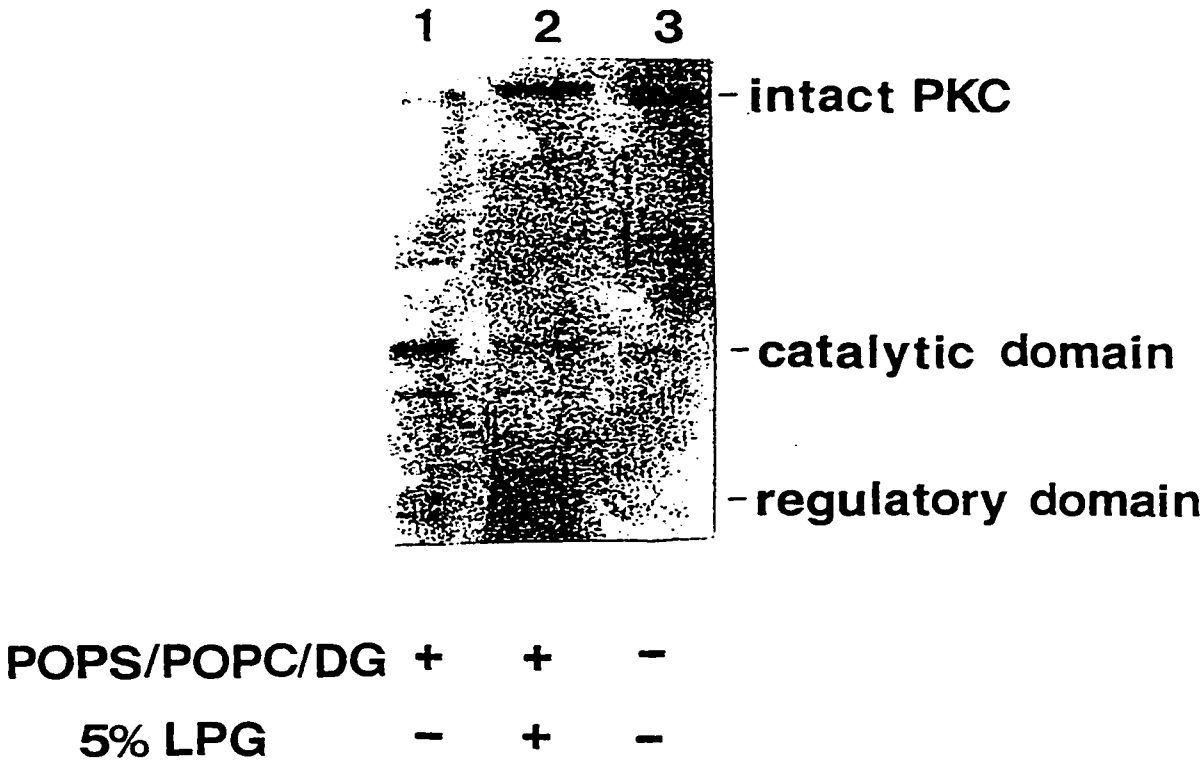

Fig. 4. **Silver Stained Polyacrylamide Gel Showing PKCα's Sensitivity to Tryptic Hydrolysis.** 300 ng PKCα was incubated with LUVs composed of 70 mol% PS, 1 mol% DG, and the remainder POPC, either without (lane 1) or with 5% LPG (lane 2), then treated with trypsin as described in "Methods". Lane 3 was in the absence of LUVs.

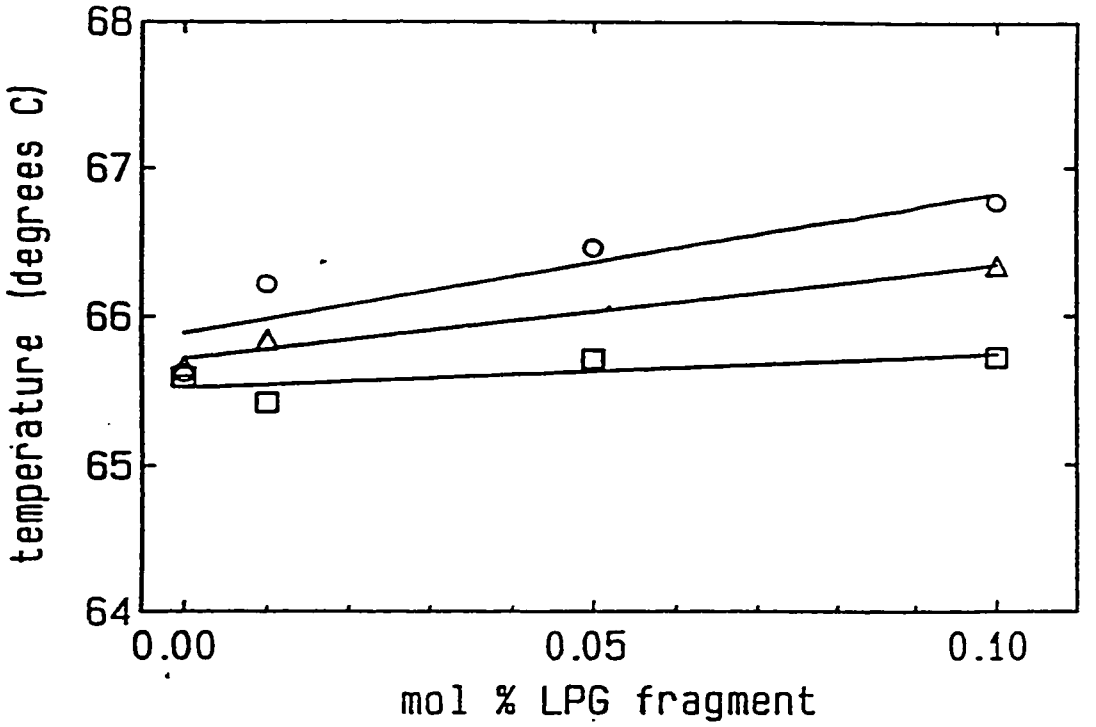

Fig. 5. **Effect of LPG Fragments on the Bilayer to Hexagonal Phase Transition Temperature of DEPE Multilamellar Vesicles.** LPG fragments were of 1 (square), 6 (triangle), or 12 (circle), repeating disaccharide units. T_H is plotted against mol% LPG fragment added to the MLVs.

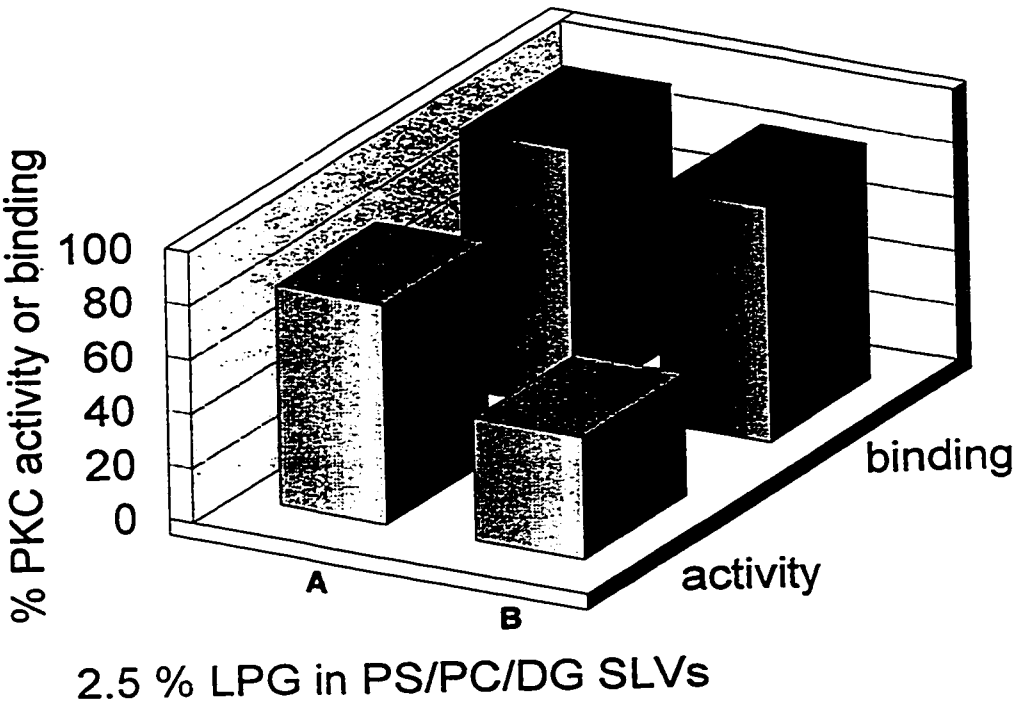

Fig. 6. **Effect of LPG Added to One Versus Both Sides of the Membrane on the Activity of PKCα Towards Histone as Compared to its Binding to SLVs.** Vesicles were composed of 30 mol% POPS, 1 mol% DG, 2.5 mol% LPG (with respect to total lipid) added to the outside (A) or to each side (B) (5% LPG of total lipid) of the membrane, and the remainder POPC. S.D. (not shown) did not exceed ± 7%. Binding and activity are expressed as a percentage of that obtained with vesicles containing no LPG. This corresponds to 100% of the total added enzyme bound, with a specific activity of 3.3 nmol/min/mg. Data is expressed as the mean of triplicate determinations.

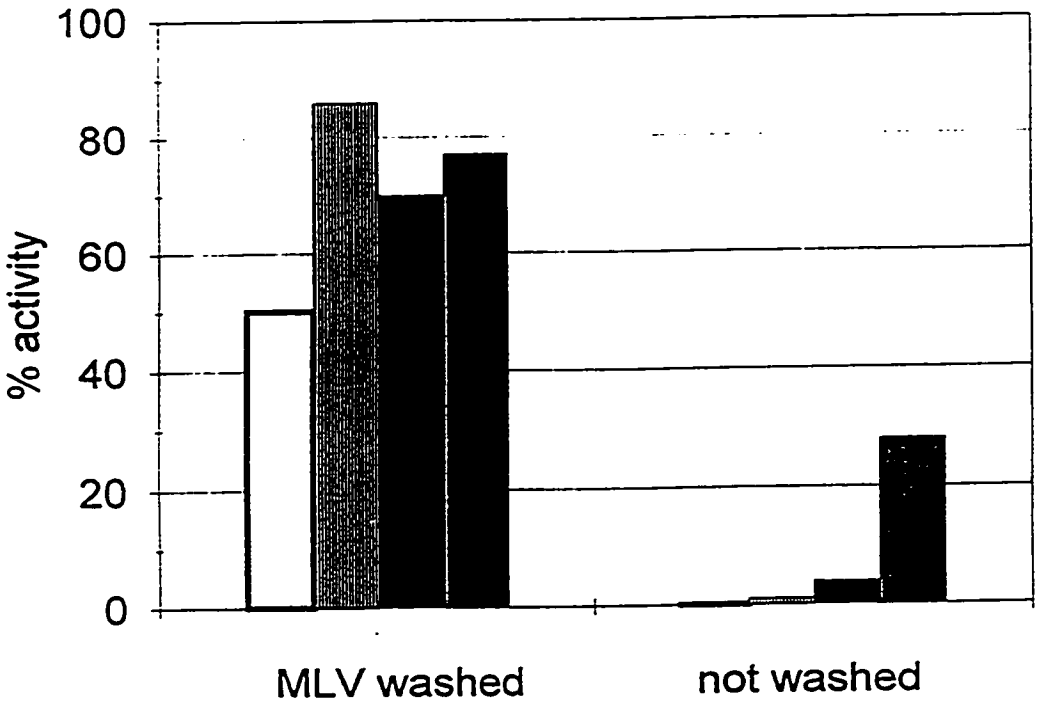

Fig. 7. **LPG on the Inner Monolayer of LUVs Inhibits the Activity of PKC Towards Histone at the Outer Monolayer.** Vesicles were composed of 20 mol% POPS, 1 mol% DG, 2.5 or 5 mol% LPG initially added to both monolayers, and the remainder POPC. Vesicles were incubated with or without sucrose loaded MLVs for 48 hours. MLVs were sedimented, and the remaining supernatant contained LUVs with LPG removed from the outer monolayer. These LUVs were used in PKC activity assays. The left-hand group of bars represent activity using LUVs which had been washed with MLVs and include: LPG originally on both sides of the bilayer at 5% (hollow) or at 2.5% (filled) total lipid and finally containing 2.5% (hollow) or 1.25% (filled) LPG on the inner monolayer only, after the wash. Controls originally containing 5% (stripes) or 2.5% (dots) LPG, originally on the outer monolayer and finally having almost all of the LPG removed by the MLV wash. The right-hand group of bars correspond to the original LUVs described above and have not had any LPG extracted with MLVs. S.D. (not shown) did not exceed ± 8%. Activity was expressed as a percentage of that obtained with vesicles which did not have LPG incorporated. Data is expressed as the mean of triplicate determinations.

Manuscript 3, "The Role of Water in Protein Kinase C Catalysis and its Binding to Membranes"

This study was begun as a continuation to the study of the inhibition of PKC by LPG. Polyethylene-glycol linked phosphatidylethanolamines were used to inhibit the activity of PKC, and were found to inhibit the membrane bound form of the enzyme, suggesting a general mechanism for inhibition of PKC by hydrophilic polymers. Free polyethyleneglycol of different sizes were originally used in activity assays to compare their effects with those of PE-linked PEG. Since free PEG is used as an osmotic stressing agent to control the activity of water around a protein or other macromolecules, this led to an interesting study on the effects of water on PKC catalysis and binding to membranes. Studies of this sort have been carried out with soluble enzymes, but only once has a study used a membrane bound enzyme. It was found that there are at least two distinct regions of exclusion of PKC, one inaccessible to PEGs larger than 1000, the other inaccessible only to PEGs larger than 10000. PKC becomes hydrated upon binding to membranes. The enzyme becomes hydrated with about 2300 water molecules upon binding of histone, and is dehydrated by about 1300 water molecules in going to the transition state. PKC is dehydrated upon binding protamine, and unchanged in going to the transition state with this substrate.

The experiments in this study were carried out by Jennifer Giorgione. The manuscript was written by Jennifer Giorgione, and edited by Richard Epand.

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The Role of Water in Protein Kinase C Catalysis and its Binding to Membranes[†]

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[1]Abbreviations: PKC, protein kinase C; PE, phosphatidylethanolamine; PEG, polyethyleneglycol (if followed by a number, it represents the molecular weight); PE-linked PEG, 1- palmitoyl, 2-oleoyl phosphatidylethanolamine-linked polyethyleneglycol (if followed by a number, it represents the molecular weight of the PEG without PE); POPS, 1-palmitoyl,2-oleoyl phosphatidylserine; POPC, 1-palmitoyl,2-oleoyl phosphatidylcholine; PS, phosphatidylserine; PC, phosphatidylcholine; DAG, 1,2-dioleoyl-sn-glycerol; H_{II}, inverted hexagonal phase; T_H, bilayer to hexagonal phase transition temperature; LUVs, large unilamellar vesicles; SLVs, sucrose loaded large unilamellar vesicles; MARCKS peptide, acetyl-FKKSFKL-amide; pseudosubstrate site peptide, Acetyl-ERMRPRKRQGSVRRRV-amide.

Abstract

The role of hydration in the catalytic activity and membrane binding of rat brain protein kinase C (PKC) was investigated by modulating the activity of water with polyethylene glycols of molecular weights 1,000-20,000, and dextran of molecular weight 20,000. These polymers create an osmotic stress due to their exclusion from hydration shells and crevices on proteins, causing dehydration. Polymers larger than 1,000 Da caused an activation of the PKC catalyzed phosphorylation of histone, while PEG 1,000 had no significant effect. The extent of activation by PEG and dextran 20,000 was larger than that of PEG 6,000 or 8,000 when vesicles were composed of 1:1 POPS/POPC, suggesting the presence of at least 2 distinct regions of exclusion on PKC, one inaccessible to PEGs larger than 1,000, the other inaccessible only to PEGs > 10,000. The extent of activation was dependent on the composition of the vesicles used. If basal activity (without PEG) was low (e.g. with low PS content in membranes), then the extent of activation was similar for all polymers larger than 1,000 Da.

Binding of PKC to membranes containing 50 mol% PS was unaffected by PEG 6,000, but was inhibited by PEG 20,000. At a low PS content of 10%, both PEG 6,000 and 20,000 inhibited binding. This suggests that PKC becomes hydrated upon binding to membranes.

Under conditions in which all of the enzyme is membrane-bound, both K_m and V_{max} for the phosphorylation of histone increased linearly with osmotic stress induced by PEG 6,000. Thus, PKC becomes hydrated with 2,311 ± 476 water molecules upon binding of histone, and is dehydrated by 1,349 ± 882 water molecules in going to the

transition state. K_m and V_{max} for phosphorylation of the MARCKS peptide also increase with osmotic stress induced by PEG 6000. When protamine sulfate was used as a substrate (co-factor independent), V_{max} for the reaction was unaffected, but K_m decreased with osmotic pressure (with PEG 6,000), suggesting that PKC gets dehydrated upon binding protamine. Similar results were found with a peptide substrate derived from the pseudosubstrate site of PKCε.

Since dextran, a polymer unrelated in structure to PEG could cause a similar activation of PKC, the effects seen are likely due to osmotic stress and not to specific binding of PEG to PKC. Also, results obtained with PE-linked PEG were opposite to those with free PEG. PE-linked PEGs of 2,000 and 5,000 Da caused an inhibition of PKC catalyzed phosphorylation of histone when present in membranes. If a specific interaction occurred with PEG, this would be expected to occur even with PE-PEG. The effects observed with free PEG are also independent of ionic strength. Free PEG had no effect on the bilayer to hexagonal phase transition temperature of DEPE membranes, suggesting that the effects on PKC activity are not a consequence of changes in membrane properties at the osmotic pressures used.

Water is known to play a role in the conformational changes that occur between different functional states of macromolecules. Recent attention has been given to the role of hydration in the functioning of ion channels, in membrane phase transitions, in the binding of enzymes and substrates, interactions between DNA molecules, and in the activation of enzymes in solution (reviewed in Parsegian et al., 1995). Poly(ethylene glycols) (PEGs)[1] are routinely used as osmotic stressing agents, to alter the water activity in a solution. These polymers are preferentially excluded from the hydration shells around proteins or other macromolecules. This exclusion creates an osmotic stress, which draws water away from the protein surface or out of a polymer-inaccessible crevice. If an enzyme s environment is osmotically stressed, there will be a change in catalytic activity if the number of bound waters are changed between the enzyme and substrate vs the enzyme-substrate complex (change in K_m) or between the ground state and transition state of the later complex (change in V_{max}). In this study, we have investigated the role of hydration in the functioning of rat brain protein kinase C. While most studies using osmotic stress focus on water soluble enzymes, few discuss membrane-dependent activation (McGee & Teuschler, 1995). The classical isoforms of PKC used in this study are of particular interest because they require lipid and calcium for activation. The activity of PKC is influenced by the physical properties of bilayers (reviewed in Epand, 1992). Osmotic stress is known to have an effect on phase transitions of membranes (Rand & Parsegian, in press), and therefore it is of interest to determine the effects of hydration on PKC's activity and binding to membranes. Also, PKC is an example of an amphitropic enzyme. PEGs of various sizes and dextran were

used as osmotic stressing agents to determine the number of water molecules which are associated with the activation of PKC. The large number of water molecules found to be involved in this process reveals that activation must involve a substantial conformational change in the enzyme and that the extent of enzyme hydration is an important factor in determining its state of activation.

Materials and Methods

Materials: Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Poly(ethylene glycol) 6,000 was from J.T. Baker N.J. PEG 1,000, 8,000, and 20,000 were from Sigma (St. Louis, MO). Histone H1 was from GIBCO/BRL (Grand Island, NY). Protamine sulfate, bovine serum albumin fraction V, and ATP sodium salt were from Sigma. $CaCl_2$ was from Fisher, Tris was from Boehringer Mannheim. [γ-^{32}P] ATP was from ICN, and [9,10-^{3}H] dipalmitoyl phosphatidylcholine was from NEN. The MARCKS peptide, acetyl-FKKSFKL-amide, was purchased from NRC (Ottawa, ON) and the pseudosubstrate site peptide, acetyl-ERMRPRKRQGSVRRRV-amide, was custom synthesized by the Indiana University Biotechnology Facility (Indianapolis, IN).

PKC Purification: Rat brain PKC was purified by a modification of the procedure of Huang et al. (1986) as described elsewhere (Mosior & Epand, 1993). Purified PKC displayed a single band on a silver stained electrophoresis gel.

Phospholipid vesicles: Lipid films were made by dissolving phospholipids in 2/1 (v/v) chloroform/methanol and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in sucrose buffer (0.170 M sucrose/5 mM $MgCl_2$/20 mM Tris-HCl, pH 7.0) for binding assays, or standard buffer (100 mM KCl/5 mM $MgCl_2$/20 mM Tris-HCl, pH 7.0) for activity assays, and subjected to five freeze-thaw cycles. Vesicles were extruded through two 0.1 m pore polycarbonate filters in a microextruder. For binding assays, the sucrose loaded vesicles were suspended in standard buffer and centrifuged at 100,000 x g to dilute out the sucrose.

PKC Binding Assays: The sucrose-loaded vesicle (SLV) assay was modified from the procedure of Rebecci et al. (1992) as described elsewhere (Mosior & Epand, 1993). PKC was incubated with SLVs and then centrifuged at 100,000 x g for 30 min at 25°C to separate the membrane-bound enzyme. Solutions of free PEG in the assay buffer were added prior to centrifugation. For assays using PE-PEG, the additive was incorporated at the stage of making the dry lipid film from chloroform/methanol solution. In assays using PEG 20,000, the vesicles and PKC were centrifuged at (150,000 g) for 2 h to account for the higher viscosity of the solution. The percentage of vesicles sedimented with the highest PEG concentration were typically about 70% of those that sedimented without PEG. This was taken into account when calculating the percentage of enzyme bound. The pellet and supernatant were assayed under identical conditions for activity towards protamine sulfate. PEG was added to the bottom fractions so that the final amount of PEG was equal to that in the top fraction. The buffer contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.0, 200 μM $CaCl_2$ and 0.3 mg/mL BSA.

PKC Activity Assays: The activity of PKC towards histone or protamine sulfate was determined as previously described (Mosior & Epand, 1993; Kaibuchi et al., 1981). Histone or protamine sulfate was added to a final concentration of 0.2 mg/mL (in 150 μL total volume), acetyl-FKKSFKL-amide was used at a final concentration of 90 μM. Phospholipid was 100 μM in the form of large unilamellar vesicles (LUVs). [γ-^{32}P] ATP (0.2 mCi/mL) was 20 μM, and PKC was 750 ng/mL. The reaction temperature was 37° C.

Activity towards peptide substrates was determined in a similar manner, however in this case the reaction was stopped with 5% acetic acid, and a portion of this was spotted onto P-81 Whatman ion exchange paper which was then washed with 0.04% phosphoric acid.

Differential Scanning Calorimetry (DSC): Bilayer to hexagonal phase transition temperatures were measured as described previously (Ling et al., 1995). An MC-2 high sensitivity calorimeter was used (Microcal Co., Amherst, MA) with a scan rate of 41 K/h. Lipid films of dielaidoyl phosphatidylethanolamine (DEPE) were suspended in buffer to a final concentration of 5 mg/mL. For experiments using PE-PEG, the lipid-linked polymer was added directly as a component of the films. For experiments with free PEG, the PEG was added to the hydration buffer.

Kinetics: Activity assays were performed as above, using either histone, protamine sulfate, MARCKS peptide, or the pseudosubstrate site peptide as substrates. K_m and V_{max} were determined from initial rates using the program GraFit 3.0 (Robin J. Leather-Barrow; Erithacus Software).

PEG Purification: A solution of PEG 6,000 (4 g/10 mL H_2O) was dialyzed extensively with Spectra/Por tubing of 1,000 MW cut off. The remaining solution was lyophilized for ~24 h. The PEG was recrystalized according to Lentz et al. (1992). Briefly, PEG was dissolved in chloroform, and an 8-fold excess of diethylether was added to recrystalize it. The PEG was filtered and left to dry.

Results using this purified PEG were similar to those with PEG used as supplied by the manufacturer, indicating that the affects were not caused by the presence of contaminants.

Results:

The effect of polyethyleneglycol-linked phosphatidylethanolamine on the activity and membrane binding of PKC

Polyethyleneglycol-linked PE s, when added to LUV s containing 50 mol% PS. were found to inhibit PKC catalyzed phosphorylation of histone (Fig. 1). PE-PEG 5.000 was a more potent inhibitor than PE-PEG 2,000 on a molar basis and even somewhat more potent on a weight basis.

The inhibition of PKC activity caused by PE-PEG was not due to an inhibition in the amount of PKC which bound to the membrane (Fig. 1). The percentage of PKC bound at each PE-PEG concentration was actually greater in the presence of PE-PEG, especially PE-PEG 5,000. Similar results were found with Lipophosphoglycan (LPG) from *Leishmania donovani*, which inhibited the membrane-bound form of the enzyme (Giorgione et al., 1996). It has been shown that LPG inhibits a conformational change in PKC which is required for the activation of the enzyme. It is possible that this is a general mechanism by which membrane-anchored hydrophilic polymers inhibit the activity of membrane bound PKC. Both PE-PEG and LPG have potent effects in raising the bilayer to hexagonal phase transition temperature at low mol fractions (Fig. 2). It has also been recently shown that DOPE-PEGs stabilize the bilayer phase in mixtures of lipids which normally form hexagonal phases (Holland et al., 1996). Compounds with this property are often found to be inhibitors of PKC (reviewed in Epand, 1992), although LPG is an unusual example of an anionic inhibitor of PKC. LPG is a polymer, with a large negative charge over its entirety, while PE-PEGs contain only one negative charge

localized at the phospholipid headgroup. It is unlikely that PKC is binding directly to PE-PEG, since no binding occurred when Ca^{2+} was removed from the assay (data not shown).

The effects of free PEG on PKC activity and membrane binding.

PEG is a hydrophilic polymer that is impermeable to membranes and because of its large size it may also be excluded from crevices in proteins as well sites at which proteins bind to membranes. A consequence of the exclusion of PEG from certain compartments will be to create an osmotic gradient which will cause dehydration of the regions to which PEG is not accessible. PEG can then affect the activity of membrane-bound enzymes by shifting the position of equilibrium between different states based on their extent of hydration.

This analysis is dependent on the assumption that there is no direct interaction between PEG and the enzyme or membrane (Rand et al., 1993; Parsegian et al., 1995). This is indicated by the fact that the effects of PE-PEG on PKC activity are opposite to those of free PEG. The free polymer (PEG 6,000) activates the PKC-catalyzed phosphorylation of histone when it is present in solution at low concentrations (Fig. 3a). This suggests that the effects are not a consequence of the specific structure of PE-PEG interacting with PKC, but rather an effect on membrane properties. At higher concentrations (>15 mM) PEG 6,000 shows less activation and even causes a slight inhibition at 20 mM and above. This inhibition may be due to the polymer's ability to aggregate or precipitate protein, or to the increased viscosity produced at higher PEG

concentrations. This latter explanation is likely a factor. When glycerol was used in a similar assay to increase the viscosity without creating an osmotic stress. the only effect was an inhibition at higher concentrations which correlated with the inhibition seen at higher PEG concentrations. Glycerol increases viscosity without being excluded from crevices or precipitating proteins. A similar trend to that which is seen in Fig 3a with PEG 6,000 was observed when the peptide acetyl-FKKSFKL-amide was used as the substrate (not shown), which represents a phosphorylation site of the MARCKS protein. a physiological substrate of PKC. When activity is plotted against the log of osmotic pressure, the effects of PEG of different sizes on the phosphorylation of histone can be compared. Activation of PKC was seen for both PEG 6,000 and PEG 8,000. while PEG 1,000 had no significant effect on PKC activity (Fig. 3a). This suggests that PEG 1.000 is not excluded from the compartment that is dehydrated by the larger PEG species. There is no biphasic effect seen with PEG 20,000, even at the highest osmotic pressure used. The activation by either PEG 6,000 or PEG 20,000 reaches almost 25 fold if the concentration of PS in the vesicles is decreased from 50% to 10% so that basal PKC activity is lowest (not shown).

Activation of PKC phosphorylation of histone is seen with PEG 6.000 (Fig. 4a) and PEG 20,000 (Fig. 4b) when the PS concentration is 10 or 20%, even when diacylglycerol is present in the membrane, which normally would increase the amount of PKC that binds. However, at 50 mol% PS with DG, the maximal activation by PEG 20,000 is only 1.4 fold (not shown). The activation seen at any particular vesicle composition is usually higher with PEG 20,000 than with PEG 6,000 or 8,000. (unless the

vesicles already support maximal PKC activity, in which case PEG of any size has no additional effect, or if basal activity is minimal, then PEGs of both sizes show maximal activation). The differing effects on the activation of PKC which were obtained with PEGs of various sizes indicates the presence of at least two aqueous compartments around PKC. One is inaccessible to PEGs, of around 6,000 Da, while the other is inaccessible only to those of 20,000 Da. It is likely that PEG 6,000 is also excluded from a hydration shell around PKC, but this shell is of smaller volume than that excluded to PEG 20,000. There is no significant activation by PEG 1,000, even though basal activity is at a minimum (not shown). This supports the conclusion that PEG 1,000 is not excluded from a crevice on PKC.

The activation of PKC by free PEG was specific for histone phosphorylation, while having little effect on the phosphorylation of protamine (not shown). The phosphorylation of histone requires the presence of Ca^{2+}, and PS for a structural rearrangement, while the phosphorylation of protamine is independent of these factors (Bazzi & Nelsestuen, 1987). Thus PEG affects the membrane-dependent activation process of PKC. In the case of Arg-rich substrates, the activation of PKC can be accomplished by the substrate itself (Bruins & Epand, 1995). Activation of PKC occurs whether the PEG is present on the outside or both sides of the LUVs (not shown).

However, despite this activation, PEG 20,000 inhibits the binding of PKC to the membrane (Fig. 3b). There is essentially no activity of PKC in the absence of lipid. Therefore, the surprisingly small fraction of PKC that is actually bound must have an even higher fold activation. This suggests that a hydration step may be involved when

the enzyme binds to the membrane. Binding is also inhibited by PEG 6.000 if the concentration of PS in the vesicles is lowered to 10% (Fig. 3b), while PEG 20.000 inhibits binding even in the presence of 50% PS and 1% DG (not shown). No effect is observed with PEG 1.000 because it likely has access to all compartments.

Although PEG 6,000 inhibits binding, this is not observed if there is sufficient PS (50 mol%) in the membrane (not shown). There is slight inhibition of binding at higher concentrations of PEG, where the inhibition in activity is also seen, and is most likely due to precipitation of protein or solution viscosity inhibiting the attainment of equilibrium at these higher concentrations. The lack of effect on membrane binding with high PS content due to PEG 6,000 suggests that there is no change in hydration of PKC upon binding to SLVs, and that the effect of PEG 6,000 is only on catalysis.

Both K_m and V_{max} were determined for phosphorylation of histone by PKC at various osmotic pressures, using PEG 6,000 with high PS where all of the PKC is bound (Fig. 5). Both constants increased with increasing concentrations of PEG 6,000. The change in the number of bound water molecules upon activation of PKC was determined (equations in appendix). Measuring the change in ln V_{max} with change in osmotic pressure (Fig. 6) revealed that $1{,}349 \pm 882$ water molecules are removed per PKC molecule in going to the transition state. The dehydration step involved in the activation of PKC must be in the membrane dependent conformational change, since there is an increase in V_{max} with increasing osmotic pressure. The number of water molecules associated with PKC s rearrangement to the catalytically active form is reasonable when

compared to the volume of 95,700 $Å^3$ for PKC itself as calculated from its specific volume and molecular mass, which is equivalent to 3,190 water molecules. Similarly, from K_m data, 2,311 ± 476 water molecules are "added" in binding of histone to the active site (Fig. 6).

K_m and V_{max} were also found to increase when 8 mM PEG 20,000 was used to increase osmotic pressure. {K_m 3.8 ± 1.9 M to 5.1 ± 2.7 M, V_{max} 0.0050 ± 0.0014 to 0.0078 ± 0.0026 pmol phosphate/min}. A similar trend was seen when the MARKS peptide (which is lysine rich, like histone) was used as a substrate, although the % PS of LUVs used was lower. When protamine or the pseudosubstrate peptide (another arginine-rich substrate) are the substrates, K_m decreases while V_{max} is unchanged with PEG 6,000. For the pseudosubstrate peptide, V_{max} = 0.0139 ± 0.0016 pmol phosphate/min and K_m = 34.6 ± 9.8 μM without PEG, and V_{max} = 0.0137 ± 0.0007 pmol phosphate/min, K_m = 2.2 ± 1.3 μM with 10.2 mM PEG 6,000.

Dextran of molecular weight 20,000 was also found to activate PKC phosphorylation of histone (Fig. 7). However, binding couldn't be tested due to sedimentation of the dextran upon centrifugation.

PEG 6,000 and PEG 20,000 had no effect on the bilayer to hexagonal phase transition temperature of DEPE, even when present in the highest concentrations used for PKC assays (not shown).

We also verified that the effects observed with PEG were not a consequence of increased ionic strength. PKC activity towards histone was measured with increasing

NaCl concentrations in the presence and absence of 12.4 mM PEG 6,000 (not shown). Increasing the ionic strength decreased the activity of PKC both with and without PEG. The extent of inhibition was the same in both cases. If the effects of PEG were due to an increase in ionic strength, one would expect the combined effects to be additive and in the opposite direction. Therefore, this variable can be ruled out.

Discussion:

PE-linked polyethylene glycols are used extensively in liposome mediated drug delivery. Since they enter the bloodstream, it is useful to understand their effects on cellular enzymes. These compounds were found to inhibit the membrane-bound form of PKC. The most significant finding was that these PE-linked PEGs have opposite effects on PKC as does free PEG, suggesting that there is no direct interaction between the polymer and the enzyme which causes a change in activity. This is an important prerequisite for the analysis of the effects of osmotic stress on a protein, enzyme or channel (Parsegian et al., 1995). In addition, PEG has no effect on lipid polymorphism and the observed effects are independent of ionic strength demonstrating that the effects are specific for PKC. Further support that the observed effects result from changes in hydration comes from the findings obtained with dextran as the stressing agent. Similar levels of activation of PKC catalyzed phosphorylation of histone were obtained with this polymer, which is unrelated in structure to polyethylene glycol. If there were a specific interaction between the enzyme and PEG, then similar effects should not be seen with dextran.

Large variations have been found in the numbers of water molecules associated with conformational changes of enzymes and proteins. Studies with hemoglobin found that 65-72 water molecules were associated with the binding of four oxygen molecules (Colombo & Bonilla-Rodriguez, 1996; Colombo et al., 1992), and binding of glucose to hexokinase resulted in the release of 65 water molecules (Rand et al., 1993). Different results have been found for the role of water in the generation of coagulation factor Xa in the aqueous phase vs generation of the intermediates on phospholipid membranes (McGee & Teuschler, 1995). Without membranes present, over 5,000 water molecules were found to be removed from the protein surface during the reaction. When the reactions occurred on phospholipid membranes, about 800 water molecules were found to be added to the surface. Therefore, differences in hydration can exist depending on whether the proteins are membrane bound or not. This may be the case for the K_m values found with protamine vs histone, which change oppositely in the presence of osmotic stress, a possible consequence of the fact that histone binds to the active form of PKC, while protamine binds to the inactive form. Thus, effects of PEG on the binding of histone to membrane-bound PKC indicates that hydration is increased when substrate binds to an activated form of PKC. Protamine binding itself also likely requires hydration but this process is counter-balanced by a dehydration of the enzyme resulting from a conformational change that exposes hydrophobic groups (Bruins & Epand, 1995). Thus the overall effect of PEG on the K_m of protamine is to lower it, indicating a dehydration upon forming the enzyme-substrate complex. V_{max} is increased by PEG in the case of histone but is unchanged for protamine. Since the conversion of the enzyme-

substrate complex to the transition state is likely to be similar for both substrates. the greater effect of PEG on the V_{max} of histone is likely caused by a dehydration resulting from a greater insertion of the complex into the membrane upon formation of the transition state with histone. This conclusion is strengthened by the finding that a small lysine-rich peptide substrate shows similar trends as histone does. while an arg-rich peptide behaves similarly to protamine. These may represent general effects of osmotic stress on the kinetics of the two types of substrates.

Although the equations used to calculate the number of water molecules associated with PKC activation involve the equilibrium constant K, our analysis assumes that K_m and V_{max} are a close approximation if the reaction follows Michaelis-Menten kinetics. K_m is a ratio of $(k_2 + k_3)/k_1$ and will be close to a dissociation constant if $k_2 > k_3$ according to the scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

Additional complexities occur with interfacial catalysis because of the exchange rates of enzyme, substrate and product between the membrane and aqueous phase. For example. it has been shown recently that the apparent K_m for the prothrombinase reaction depends on whether the substrate comes to the active site from the membrane rather than the solution (Lu & Nelsestuen, 1996). However, in the present study there is evidence to suggest that the measured K_m approximates an equilibrium dissociation constant for the

enzyme-substrate complex. For peptide substrates (e.g., the MARKS peptide), Lineweaver-Burke plots are linear over a wide range of concentrations (Giorgione et al., 1996). Also, different substrates show the same effects with PEG 6.000. The activation is of a similar extent, as well as the inhibition at higher concentrations of PEG. Protamine and histone have similar K_m values, even though one involves interfacial catalysis and the other does not. The difference in the direction of the change in K_m with PEG is a consequence of protamine binding to active PKC and histone binding to inactive PKC.

PEG inhibits the binding of PKC to membranes (Fig. 3b). This indicates that the binding process is accompanied by increased hydration. There are two kinds of morphological features that can account for this increase in water binding. One is the creation of new or deeper crevices in the membrane-bound form of PKC. The crevices would be inaccessible to PEG and therefore osmotic stress would inhibit their formation and reduce the binding of PKC to membranes. In addition, the aqueous volume between PKC and the membrane, close to the point of membrane attachment of PKC, may also be inaccessible to PEG. However in this case, unlike the conventional explanation for osmotic stress effects, removal of water from this region will increase PKC binding to the membrane. A similar phenomenon occurs for PEG-induced vesicle aggregation. We thus favour PEG-induced osmotic stress on the hydration of membrane-bound PKC, to explain the inhibition of binding.

The differences observed in the binding of PKC to membranes of 50 mol% PS in the presence of PEG 6,000 vs. PEG 20,000 may be a consequence of PKC not having

access to the membrane in the presence of PEG 20,000. It has been shown that PEG 20,000 absorbs to the bilayer surface and prevents the close approach of two bilayers because of its size. It does not have the same ability to draw water out from between the bilayers as PEGs of less than 10,000 do, which stabilize interactions between membranes (Kuhl et al., 1996). If PEG 20,000 is not allowing PKC access to the membrane, this would inhibit binding.

The general conclusion about changes in hydration can be summarized as follows (where E = PKC, L = lipid vesicle, P = protamine, H = histone, * = transition state):

$$E + L + H_2O \rightleftharpoons EL$$

$$E + P \rightleftharpoons EP + H_2O$$

$$E + H + H_2O \rightleftharpoons EH$$

$$EP \rightleftharpoons EP^*$$

$$EH \rightleftharpoons EH^* + H_2O$$

These findings are important for the understanding of the molecular mechanisms of PKC-dependent processes. It is the first example of a study of hydration changes in a membrane-bound allosteric enzyme.

The physiological relevance of these findings is uncertain. However, PKC has been shown to play a role in osmotically induced cellular signalling *in vivo*. Activation of PKC has been found to inhibit collecting duct osmotic water permeability (Han et al., 1994; Ando et al., 1992). In GH_4C_1 cells, PKC was found to be involved in Ca^{2+} influx and prolactin secretion caused by cell swelling (Sato et al., 1992). *Saccharomyces cervisiae* mutants lacking the PKC1 gene product were found to be viable when grown on

osmotically supported medium, but lysed at restrictive temperatures on hypotonic medium (Paravicini et al., 1992; Levin & Bartlett-Heubusch, 1992). PKC has been shown to phosphorylate and inhibit the activity of the Na/K ATPase in intact renal epithelium (Middleton et al., 1993). Since this pump is involved in transport of solutes across epithelial cells, it may regulate the osmotic balance as well (since water would be transported in response to solutes). It remains to be proven if some of these relationships are a consequence of the sensitivity of PKC activity on hydration pressure.

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Appendix

Determination of # of H_2O molecules associated with PKC's active site and transition state. Studies on hexokinase used changes in K_{diss} and K_m with osmotic pressure to determine # of H_20 molecules associated with binding of glucose to hexokinase (Rand et al., 1993).

$\Delta G = -RT \ln K_d + nRT \ln W$

where W = water activity

$nRT \ln W = \Pi \Delta V_w$

where ΔV_w = the volume of water removed

$\Delta G = -RT \ln K_d + \Pi \Delta V_w$

so

$\Delta V_w = RT \, \Delta \ln K_d / \Delta \Pi = RT \ln (K_d^{\circ}/K_d^{\Pi})/\Delta \Pi$

You can also substitute K_m for K_d. We determined K_m and V_{max} using histone as the substrate, at different osmotic pressures. The plot of $\ln (K_m^{\circ}/ K_m^{\Pi})$ vs Π should yield a straight line with slope = $\Delta V_w/RT$.

From

$\ln(V_{max}^{\Pi}/V_{max}^{\circ})$ vs Π (Fig. 6a)

slope = 0.945 ± 0.618

slope = $0.945 = \Delta V_w/RT$ RT = 2,577 J/mol

The units for slope are $cm^2 \times 10^{-6}$ / dynes. Since 10 dynes/cm^2 = 1 J/m^3, then slope = 9.45 $m^3 \times 10^{-6}$/ J.

9.45 $m^3 \times 10^{-6}$/ J = ΔV_w/ 2.577 J/mol

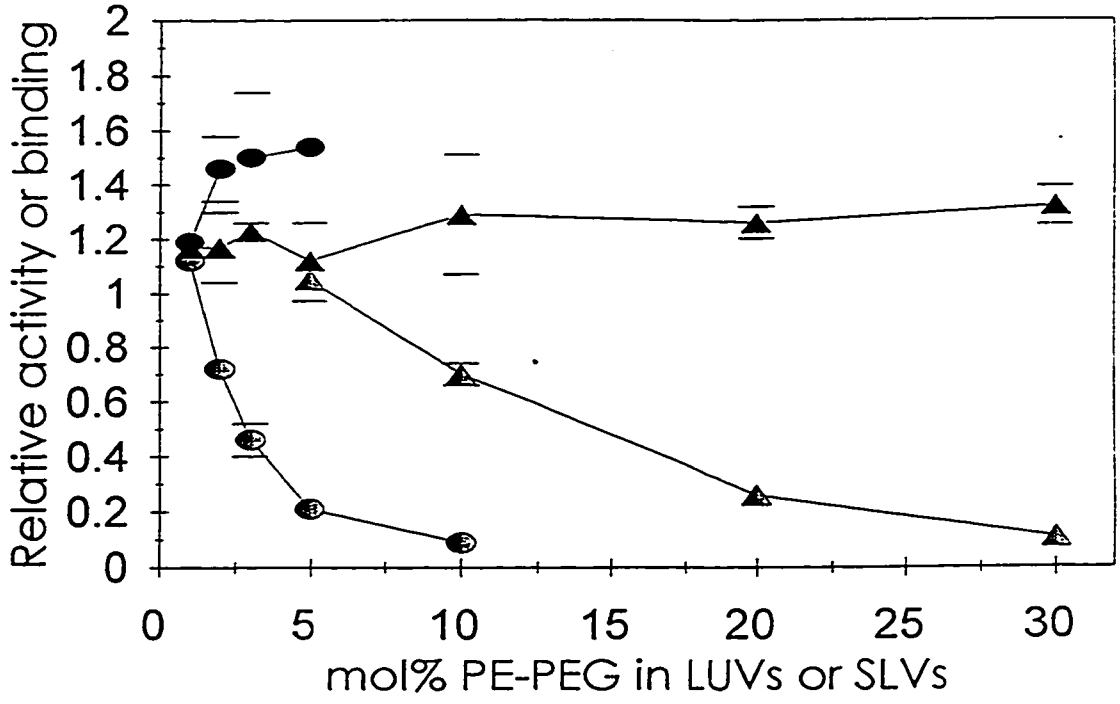
0.0243 m^3/mol = ΔV_w

= 4.047 x 10^{-26} m^3/molecule

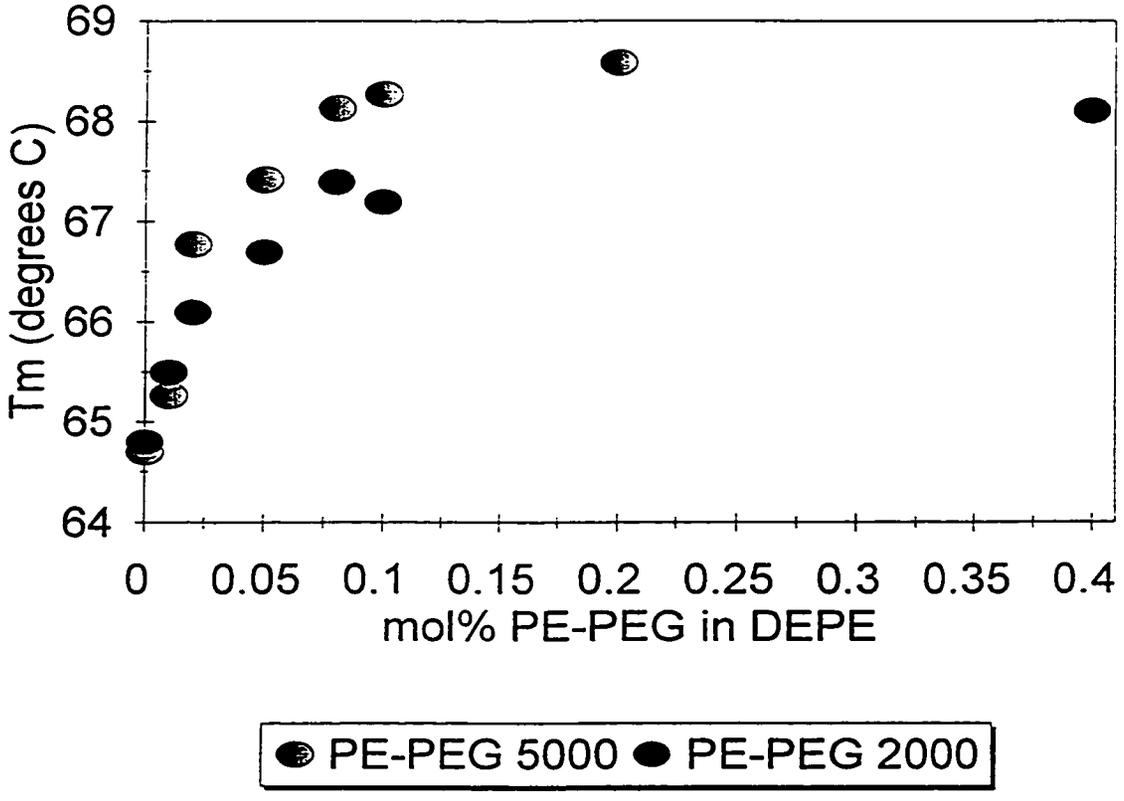
= 40,470 $Å^3$/molecule

1 H_2O = 30 $Å^3$

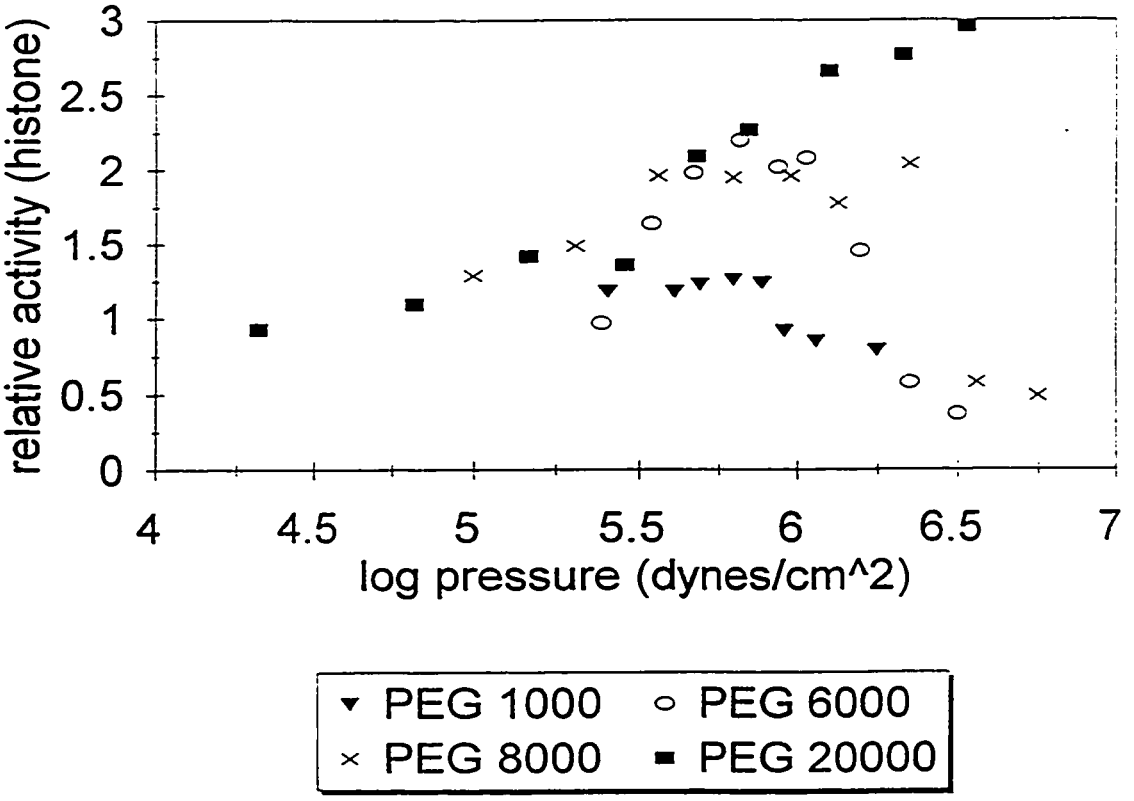
Therefore, 1,349 ± 882 water molecules removed per PKC histone complex in going to the transition state similarly, from K_m data, 2,311 ± 476 water molecules "added" in binding of histone to the active site of PKC.



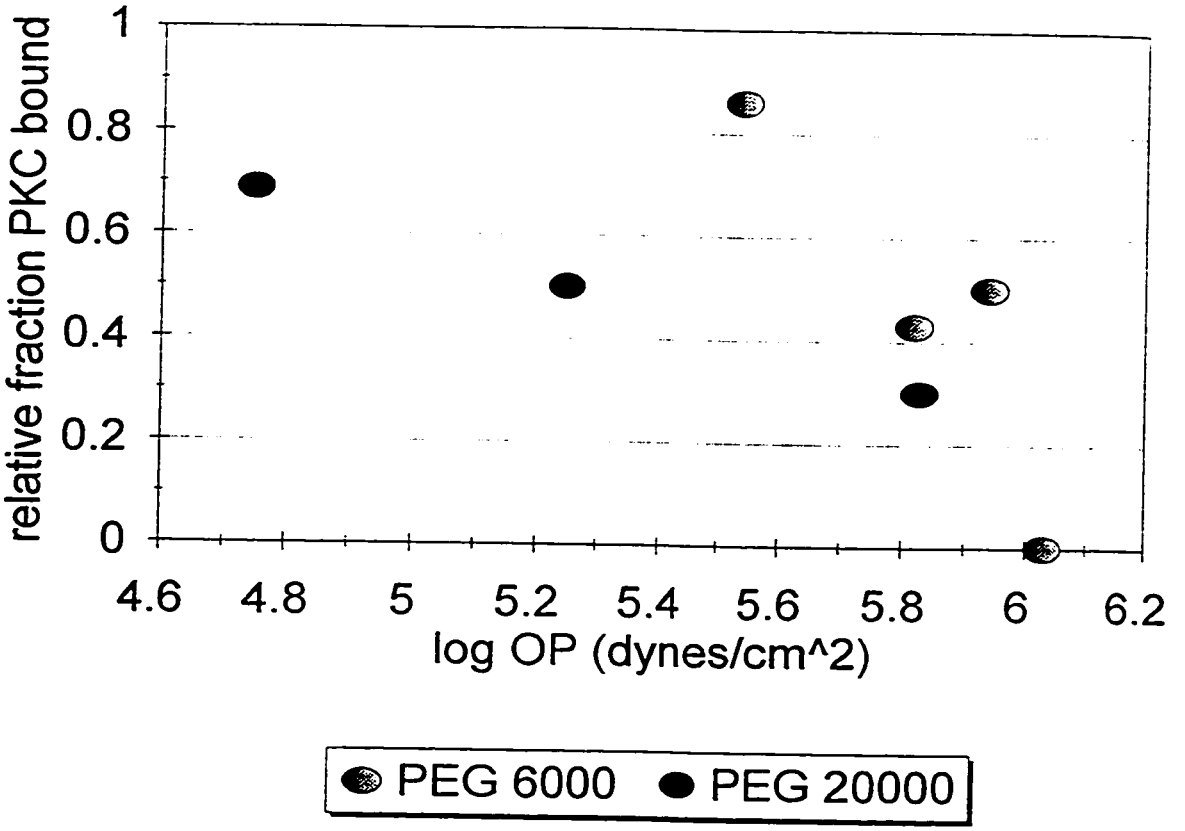
1. PE-PEGs 2,000 and 5,000 inhibit the PKC-Catalyzed Phosphorylation of Histone and its Binding to SLVs. Activity: The LUVs were composed of 50 mol% POPS, 0-30 mol% PE-PEG 2000 or 5000, and the remainder POPC. The activity is expressed relative to the activity obtained using LUVs without PE-PEG. Shaded circles (PE-PEG 5,000), shaded triangles (PE-PEG 2,000). Binding: The sucrose loaded vesicles were composed similarly to LUVs. Binding is expressed as a percentage of the binding obtained with vesicles without PE-PEG. Solid circles (PE-PEG 5,000), solid triangles (PE-PEG 2,000). Data are expressed as the mean of triplicate determinations ± S.D. (some error bars omitted for clarity).



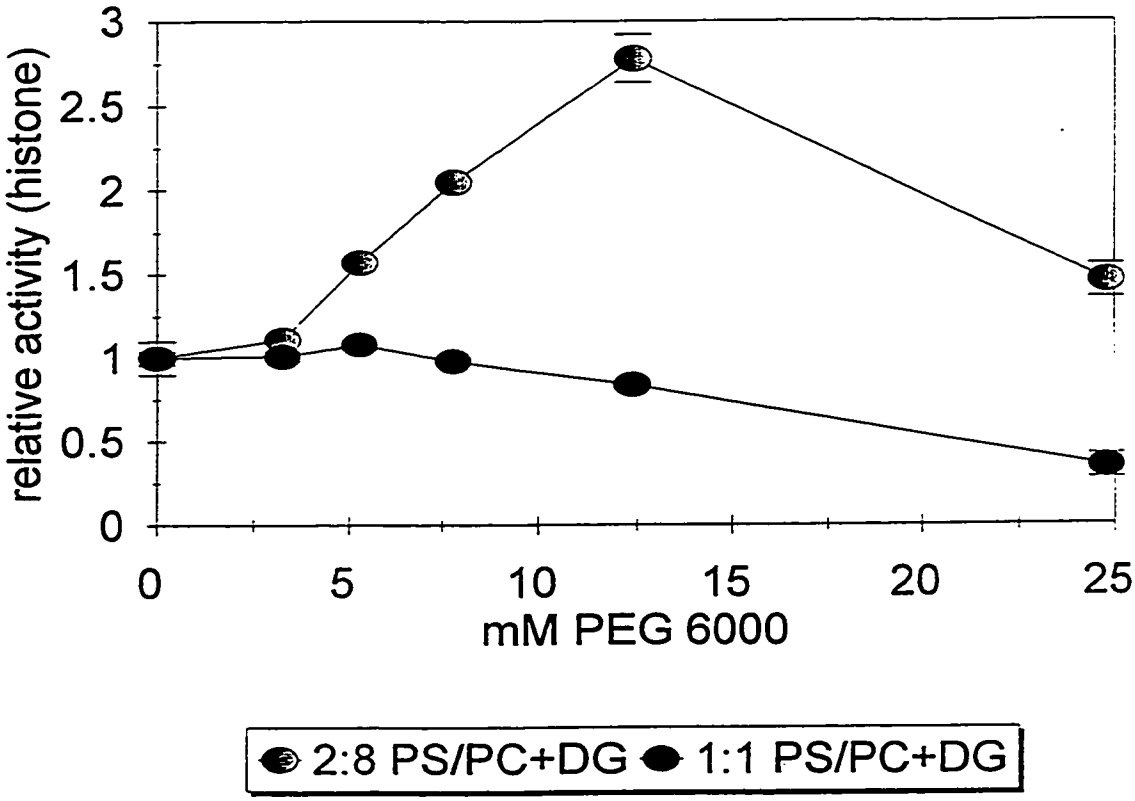
2. PE-PEGs 2000 and 5000 raise the Bilayer to Hexagonal Phase Transition Temperature of DEPE Multilamellar Vesicles. T_H is plotted against mol% PE-PEG in MLVs.



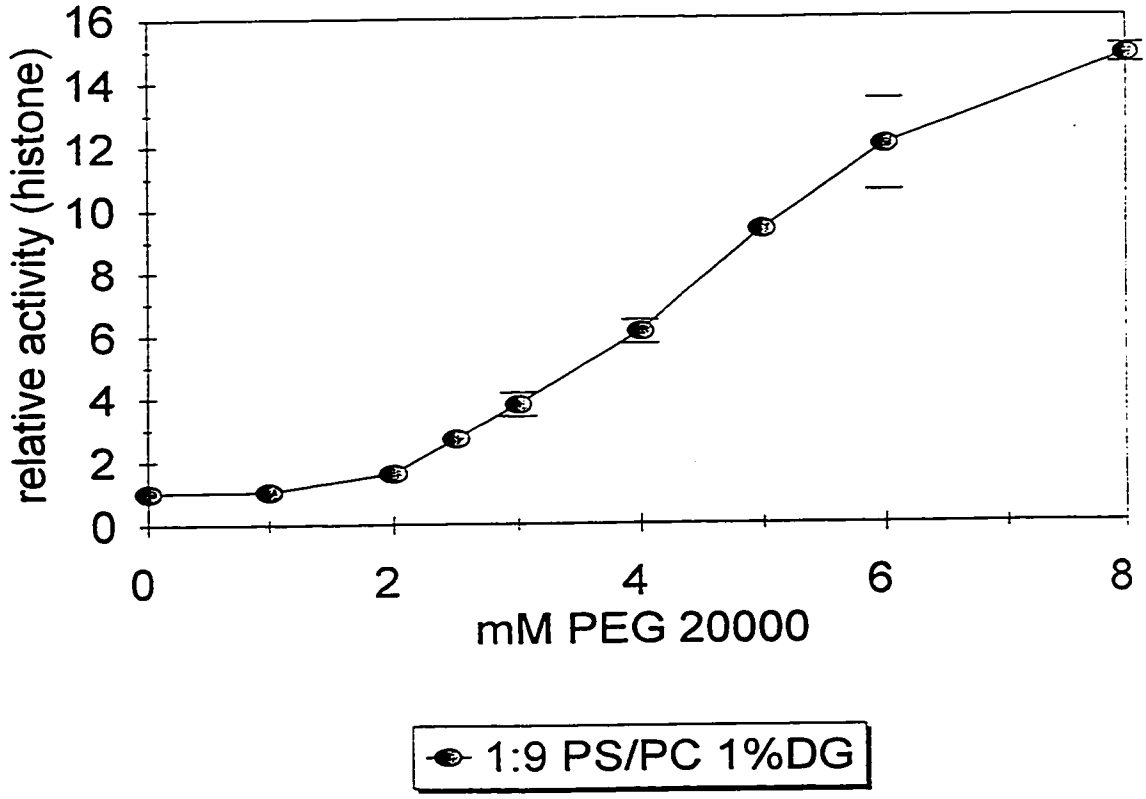
3a. The Effects of Osmotic Pressure created by PEGs of various sizes on the PKC Catalyzed Phosphorylation of Histone. The LUVs were composed of 50 mol% POPS and 50 mol% POPC. PEGs were added to the reaction buffer as in "methods". The activity is expressed as a fold increase in the activity obtained without PEG. and plotted as a function of osmotic pressure. S.D (not shown) did not exceed ± 9% in all but one case.



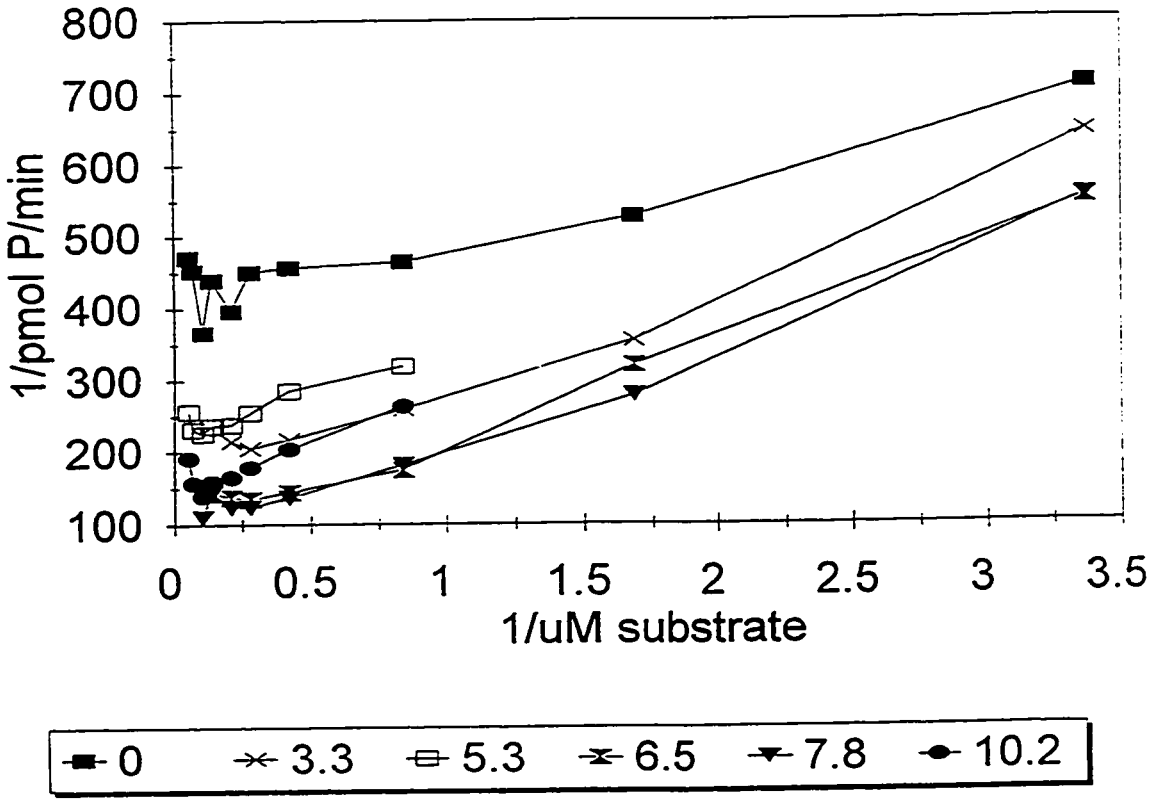
3b. PEGs 6,000 and 20,000 Inhibit the Binding of PKC to SLVs Containing 10 mol% POPS. The SLVs were composed of 10 mol% POPS and 90 mol% POPC. PEGs were added to the reaction buffer as in "methods". The binding is expressed as a fraction of that obtained in the absence of PEG, and plotted as a function of osmotic pressure. Data are expressed as the mean of triplicate determinations ± S.D.



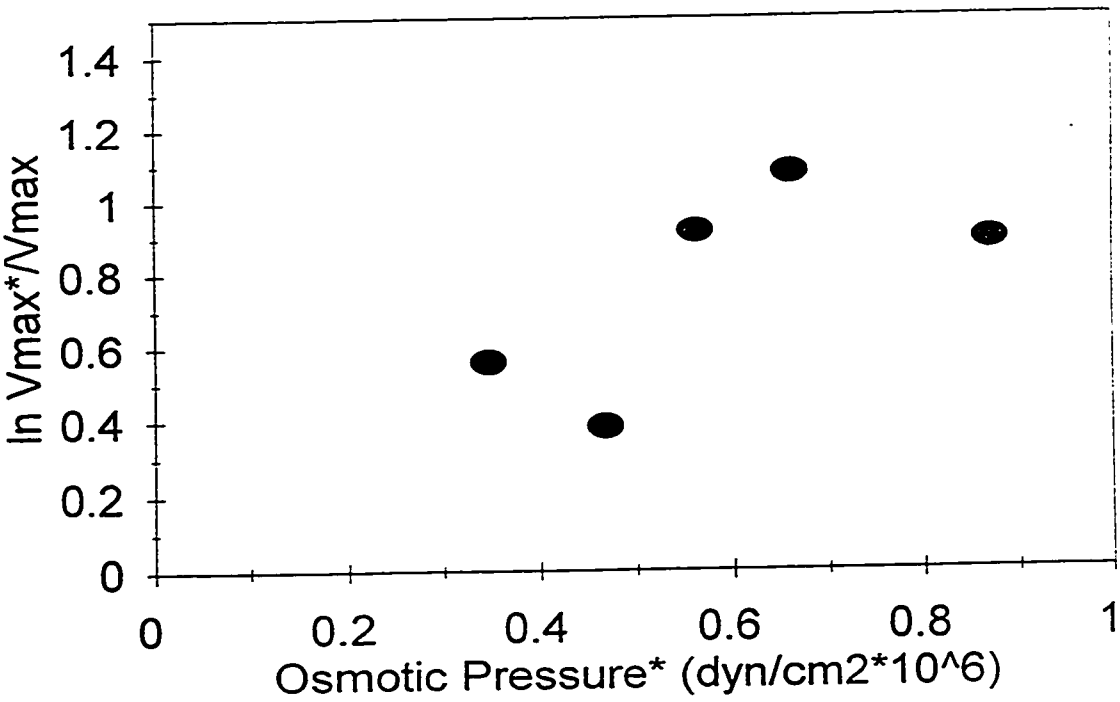
4. Effect of PEG 6,000 and 20,000 on PKC-Catalyzed Phosphorylation of Histone. **4a)** PEG 6,000: The LUVs were composed of either 20 mol% POPS, 1 mol% DAG with the remainder POPC, or 50 mol% POPS, 1 mol% DAG with the remainder POPC. The activity is expressed relative to that obtained in the absence of PEG. Data are expressed as the mean of triplicate determinations ± S.D.



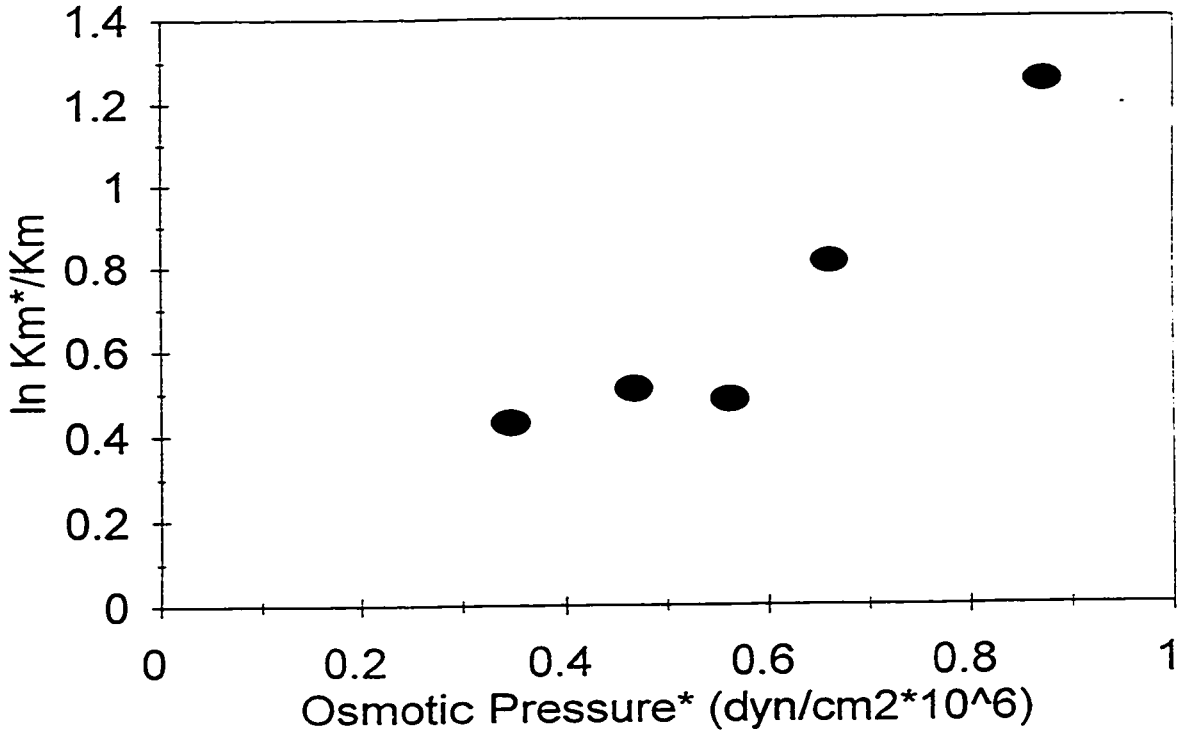
4b. PEG 20,000: The LUVs were composed of 10 mol% POPS, 1 mol% DAG, and the remainder POPC. PEG was added to the reaction buffer. The activity is expressed relative to the activity obtained without PEG. Data are expressed as the mean of triplicate determinations ± S.D.



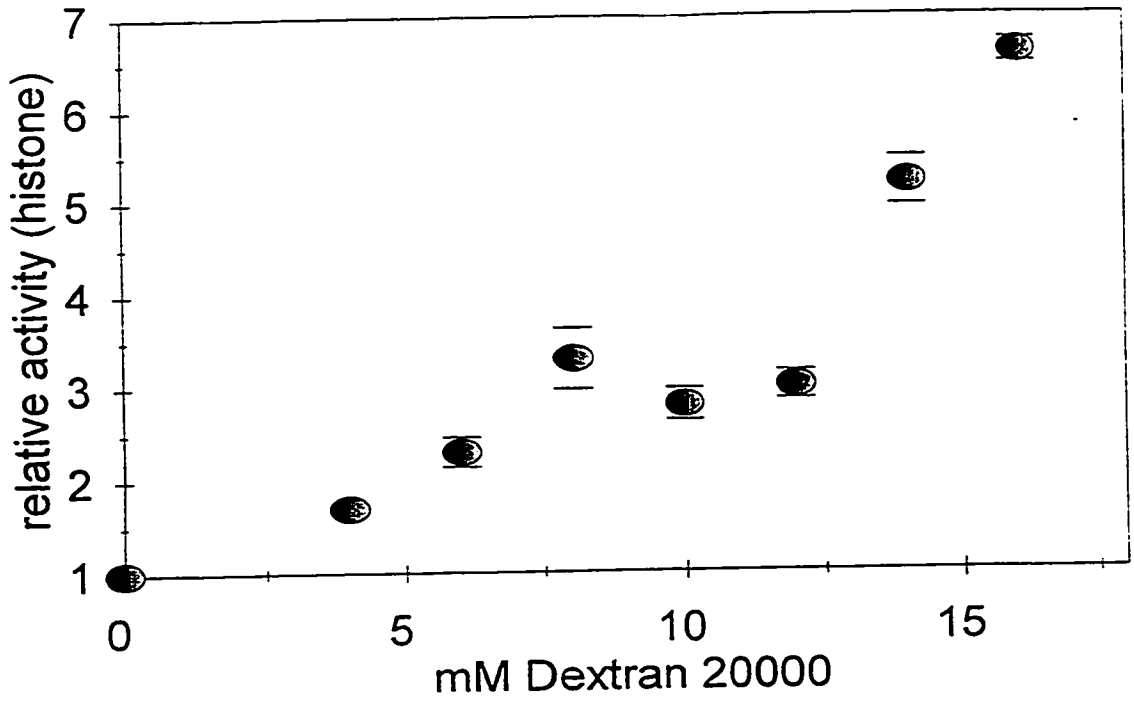
5. Lineweaver-Burk Plots for the Phosphorylation of Histone by PKC in the presence of PEG 6,000. LUVs were composed of 50 mol% POPS and 50 mol% POPC. PEG was added to the reaction buffer. K_m and V_{max} values were calculated using GraFit 3.0. Data are expressed as the mean of triplicate determinations of at least two separate experiments. The legend under the graph gives the concentration of PEG in mM units.



6a. Change in (ln V_{max}) with Increase in Osmotic Pressure with PEG 6,000. V_{max} values were those obtained in figure 5. The change in V_{max} was measured relative to that obtained without PEG. Linear regression and the equations in the appendix were used to calculate the number of water molecules removed from PKC in going to the transition state as 1,349 ± 882.



6b. Change in (ln K_m) with increase in Osmotic Pressure with PEG 6,000. K_m values were those obtained in figure 5. Calculations were as in figure 6a. The number of water molecules added to PKC upon histone binding was found to be 2,311 ± 476.



7. Dextran 20,000 Activates the PKC-Catalyzed Phosphorylation of Histone. LUVs were composed of 1:1 POPS/POPC. Dextran was added to the reaction buffer. Activity is expressed as the fold increase in activity relative to that obtained without dextran. Data are expressed as the mean of triplicate determinations ± S.D.

Manuscript 4, "Increased Activation of Protein Kinase C with Cubic Phase Lipid Compared with Liposomes"

This study was undertaken to investigate the possibility that PKC can become activated in the presence of lipid which is not in the lamellar phase. It has been suggested that there is an optimal curvature strain associated with maximally activated PKC (Slater *et al.* 1994). Cubic phases possess are lower curvature strain than lamellar phases, so it was interesting to determine how the activation of PKC would compare to that seen in the lamellar phase. It was shown that the specific activity of PKC bound to the cubic phase was much greater than that of PKC bound to the lamellar phase. Therefore, curvature strain is not the only factor causing activation of PKC in this system. Other factors, such as a curved morphology and interfacial polarity must also play a role.

The experiments in this manuscript were carried out by Jennifer Giorgione, with the exception of the X-Ray diffraction (Table 1), which was carried out by Richard Epand, the NMR measurements (Figures 1 and 6) which were carried out by Don Hughes. However, the samples for X-Ray diffraction and NMR were prepared by Jennifer Giorgione. Also, Zhi Huang carried out the microscopy (referred to in results section but not shown) and also showed Jennifer Giorgione how to prepare cubic phase lipid. The manuscript was written by Jennifer Giorgione, with some input from Zhi Huang due to his experience with cubic phases, then edited by Richard Epand.

Manuscript 4, as published in Biochemistry 37, 2384-2392, 1998.

INCREASED ACTIVATION OF PROTEIN KINASE C WITH CUBIC PHASE LIPID COMPARED WITH LIPOSOMES[†]

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Running Title: PKC and non-lamellar phases.

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ABSTRACT

Protein kinase C (PKC) activation is measured using liposomes containing phosphatidylserine. Certain lipids display a wide range of polymorphism, depending on conditions. They can give rise to non-lamellar phases, such as hexagonal or cubic phases as well as to lamellar phases. In this paper, we studied the activity and membrane binding of PKC in lipid bicontinuous cubic phases and hexagonal phases. The cubic phase lipid systems were: 1) mono-olein with 1-palmitoyl-2-oleoyl-3-phosphatidylserine (MO/PS) and 2) dielaidoylphosphatidylethanolamine/alamethicin (DEPE/ alamethicin). Under fully hydrated conditions, both of the above lipid mixtures are bicontinuous cubic phases with a space group of Pn3m within certain concentration ratios and temperature ranges. Dioleoylphosphatidylethanolamine (DOPE) with up to 10 mol% PS exists in the hexagonal phase at room temperature. These cubic and hexagonal phases were able to support the PKC catalyzed phosphorylation of histone. The amount of PKC bound to the MO/PS cubic phase showed little increase between 5 and 10 mol% PS. For both of the cubic phase systems studied, only a minor fraction of the PKC was bound to the membrane. This indicates that the specific activity of the enzyme bound to cubic phase membranes is much greater than that bound to phospholipid in the lamellar phase. Addition of up to 50 mol% MO to lipid in the lamellar phase had relatively small effects on the activity of PKC. The increase in PKC activity correlated well with an increase in PKC binding, resulting in little change in the specific activity of the membrane-bound form. These findings may be physiologically relevant due to the apparent presence of the cubic phase in certain biological structures. Also, these phases have little or no

curvature strain, a property which has been shown to correlate with activation of PKC. Therefore, other factors, such as a curved morphology and/or interfacial polarity, must be responsible for the activation of PKC in these lipid systems.

Protein kinase C (PKC), which is widely distributed in tissues and organs, is a family of enzymes important in signal transduction (1). The mechanism of activation of PKC and its interaction with membranes have attracted much attention (2-4). PKC consists of several isoforms which are classified into subfamilies. The classical isoforms used in this study are regulated by Ca^{2+} and phospholipid, as well as by diacylglycerol. It is known that PKC activity is influenced by the presence of non-lamellar forming lipids (5-8). Furthermore, Stubbs & Slater (9) suggested that there is an optimal membrane curvature leading to maximally activated PKC.

Lipid systems used in PKC studies are in the form of vesicles, in which the lipids are in a lamellar phase. A great deal of experimental work has been done on the structure and phase behaviour of phospholipid bilayers (10). The lamellar liquid crystalline phase is an appropriate model for biological membranes. However, many studies have shown that the lamellar phase is only one of a large variety of phases presented by phospholipids. Many non-lamellar phases have also been found with purified phospholipids which are major components of biological membranes (11,12). These non-lamellar phases or more likely, the propensity toward their formation, can greatly affect the function and behaviour of membranes (13-16).

Among non-lamellar phases, the cubic phase is an important family. As its name states, this phase has a three-dimensional periodic molecular arrangement with cubic

symmetry. Recently, cubic phases formed by phospholipid systems have received a great deal of attention. Structurally, cubic phases have two distinct families: bicontinuous cubic phases, which are based on underlying periodic minimal surfaces. and discontinuous cubic phases, consisting of complex packing of discrete micellar or inverse micellar aggregates (15,17-20).

The bicontinuous cubic phase is of great interest and importance in biological systems, since it has been observed by microscopy in many biological specimens. including the infoldings of the plasma membrane, the smooth endoplasmic reticulum. nuclear, and mitochondrial membranes (20). The bicontinuous cubic phase can organize space within the cell, while at the same time allowing aqueous solutes to diffuse to any location within the aqueous compartments of the structure. In the present work, we studied the activation of PKC in two different lipid cubic phase systems. One cubic phase used was mono-olein which presents a cubic phase with a space group of Ia3d in low water content and another cubic phase of the space group Pn3m in excess water (21,22). Up to 10 mol% PS was added to MO while maintaining an isotropic phase as determined by ^{31}P NMR and a Pn3m space group by x-ray diffraction. Another cubic lipid system was a dielaidoylphosphatidyl-ethanolamine (DEPE)/alamethicin mixture. which was reported to present a Pn3m bicontinuous cubic phase within a composition range of 1 to 10 wt% alamethicin over a large temperature range in excess water (23). Both these cubic phases have phospholipids with negative curvature lining aqueous channels, but less curvature strain than the corresponding lamellar phases of the same

lipid (24). Bicontinuous cubic phases have zero mean curvature at the midplanes of bilayers, but locally have a continuous gradation of curvature; therefore PKC can potentially select regions with optimal lipid packing for binding and activation. The mean curvature at the water-membrane interface is negative.

Hexagonal phases are not known to exist in biological membranes although they have been suggested to be an intermediate structure facilitating the movement of lipid to the air/water interface in the lung (25). The hexagonal phase system used in this work was dioleoylphosphatidyl-ethanolamine/1-palmitoyl-2-oleoyl-phosphatidylserine (DOPE/PS). Bilayers containing PE with unsaturated acyl chains have negative curvature strain, but no curved morphology. In contrast, PE in the inverted hexagonal phase has almost no curvature strain, but does have a curved morphology. It was of interest to determine the relative importance of the curvature strain, or the curved morphology in regulating PKC function.

MATERIALS AND METHODS

Materials. 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphospha-tidylcholine (POPC), 1,2-dielaidoylphosphatidylethanolamine (DEPE), 1,2-dioleoylphosphatidyl-ethanolamine (DOPE), and 1,2-dioleoylglycerol (DAG) were purchased from Avanti Polar Lipids (Alabaster, AL). Mono-olein (MO) was from Nu Chek. Alamethicin (a synthetic polypeptide), bovine serum albumin (BSA) fraction V and protamine sulfate were obtained from Sigma (St. Louis, MO). Histone H1 was from GIBCO/BRL (Grand Island, NY). The MARCKS peptide (acetyl-FKKSFKL-amide)

was purchased from the Institute for Biological Science, National Research Council (Ottawa, Ontario). [γ-^{32}P] ATP was from ICN, and [9,10-^{3}H] dipalmitoylphosphatidylcholine was from NEN. Other chemicals were from either Fisher or Sigma.

PKC purification. Rat brain PKC was purified by a modified procedure of Huang et al. (26) as described previously (27). The purified protein displayed a single band on a silver-stained electrophoresis gel.

Lipid. Lipid films were made by dissolving phospholipids in 2:1 (v:v) chloroform/methanol and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in standard buffer (100 mM KCl/5 mM $MgCl_2$/20 mM Tris-HCl, at pH 7.0), and subjected to 5 freeze-thaw cycles. Lamellar phase lipids were extruded through two 0.1 μm pore polycarbonate filters in a microextruder to form LUVs. Cubic and hexagonal phase lipids were not extruded.

PKC Binding Assays. Binding assays for the vesicular system were a modification of the sucrose-loaded vesicle assay of Rebecci et al. (28) as described elsewhere (27). For the cubic phase, lipids were suspended in standard buffer rather than sucrose buffer, and separated from the bulk solvent by centrifugation at 100,000 x g for 45 min. at 25°C in 1.03 M sucrose. The lipid floated to the sides of the centrifuge tubes, and could be removed from the sucrose buffer. Lipid was resuspended in sucrose buffer, and centrifuged again under the same conditions, with PKC, Ca^{2+}, and BSA, to separate membrane-bound enzyme. The sedimented lipid/PKC and supernatant were assayed under identical conditions for activity toward protamine sulfate as previously described

(27). For the hexagonal phase, it was sufficient to sediment the lipid in a bench top Eppendorf centrifuge (12,000 x g) for 10 min. No sucrose was necessary.

PKC Activity Assays. The activity of PKC toward histone was determined as previously described (27). Histone was added to a final concentration of 0.2 mg/mL. Lipid was either 125 μM, or 1.25 mM as indicated in the figure legends. PKC was 575 ng/ml. [γ-^{32}P] ATP (0.2 mCi/mL) was 200 μM and Ca^{++} was 200 μM. The activity was assayed at 25°C for MO/PS, 40 C for DEPE/alamethicin, and 25 C for DOPE/PS. For activity assays using the MARCKS peptide, the reaction was stopped using 75 μl of 5% acetic acid. 100 μl was spotted on Whatman P-81 filter paper (3 cm^2) and washed for 20 min with 0.04% phosphoric acid.

Western Blotting. The binding assay was performed with 10% PS/90% MO, as described above for the cubic phase. However, activity of the top and bottom fractions was not determined. The total volume sedimented was 375 μl. This was removed after centrifugation, and the lipid was resuspended in 200 μl sucrose buffer. These were loaded on a 7.5% polyacrylamide gel which was run overnight at 50V. Protein was transferred to nitrocellulose (Hybond ECL nitrocellulose, Amersham). The gel and membrane were placed between 2 pieces of 3 MM Whatman paper, and placed in an electroblotting cassette (Bio-Rad TransBlot) in 15.6 mM Tris, 120 mM glycine: 20% methanol. Transfer was for 3 hrs at 70V. The nitrocellulose was incubated in blocking buffer which contained 3% BSA in 10 mM Tris, 150 mM NaCl (TBS) for 2 hrs, washed with TBS 3 times, then incubated overnight with primary monoclonal antibodies to either PKC , PKC , or PKC (Bio/Can Scientific, Ontario). The nitrocellulose was washed 3

times with TBS, and was then incubated with a polyclonal secondary antibody (peroxidase-linked goat anti mouse IgG from Bio/Can Scientific, Ontario) for 3 hrs. The nitrocellulose was washed for 45 min in TBS with 3 changes. The ECL-chemi-luminescent detection system (Amersham) was used to visualize the bands. These were exposed to Reflection NEF-496 Autoradiography Film (Mandel Scientific) for 1-10 seconds.

Microscopy. As an initial indication of the presence of the cubic phase, polarized microscopy was used. As an isotropic phase, a cubic phase would be dark when observed with a microscope under crossed polarizers. Whereas for the lamellar and hexagonal phases, "birefringent" patterns would be observed. A polarizing microscope with two crossed polarisers equipped with a heat stage was used.

^{31}P-NMR. ^{31}P-NMR was used to determine the presence of lamellar, hexagonal and isotropic phases. Lipid films were prepared as described above, and hydrated with standard buffer containing BSA and Ca^{2+}, (and histone in one case as noted in Results). 25 mg of phospholipid was used in each case (total lipid varied accordingly, see figure legends), and was hydrated with 0.5 ml of buffer. The samples were freeze-thawed 5 times, and transferred to NMR tubes. A Bruker DRX 500 spectrometer operating at 202.45 MHz was used with a radio frequency pulse length of 9 μs with broad band proton decoupling. The temperature was maintained within 0.1°C with a Bruker B-VT 1000 variable temperature unit. Exponential line broadening of 100 Hz was applied prior to Fourier transformation.

X-ray Diffraction Experiments. Nickel-filtered CuK (λ = 1.54 Å) X-rays were obtained from a Rigaku-Denki rotating anode. X-rays were focused using a Frank's type camera and recorded using a position-sensitive proportional counter (TEC model 205) (29). Unoriented lipid dispersions were prepared by hydrating dried lipid films with a buffer similar to that used for PKC activity assays and containing 200 μM $CaCl_2$ and BSA but not histone or PKC. The lipid suspension was not extruded but was packed into 1.5 mm glass capillaries using a metal wire. Some of the samples were also centrifuged in the capillary. Measurements were made at 25 ± 0.2°C as used in the PKC assays. The x-ray exposure times were typically 10-15 minutes. Typical absolute lattice spacing. calibrated against freshly crystallized nonadecane (d=26.2 A), are accurate to ±0.5 for lattices up to 80 A and to ±1-2 A for larger lattices.

RESULTS

The Pn3m Bicontinuous Cubic Phase. In excess water, MO was reported to present a Pn3m cubic phase with a lattice parameter of 105 A at 22°C (21). With the polarizing microscope, MO in excess Tris buffer was confirmed to exist in the cubic phase over a wide range of temperatures. To activate PKC, PS was incorporated into the MO cubic phase system. It is known that PS is a bilayer stabilizer with respect to inverted phases (30). It was important to determine the cubic to lamellar phase boundary of the MO/POPS system in the reaction buffer. Using a polarizing microscope, mixtures containing low amounts of PS at 25°C revealed a dark view field, indicating the presence of the cubic phase. When the POPS content was increased to about 15 mol%, two co-existing phases were observed, and only a lamellar phase was seen with about 18 mol% or more POPS. Similar results were obtained using ^{31}P-NMR. At 25°C, mixtures containing either 5 or 12 mol% POPS were isotropic, while those containing 20 mol% had the characteristic bilayer shape powder patterns (Fig. 1). We also studied this system using X-ray diffraction. Five reflections could be observed from samples of MO containing 0, 2, 5 or 10 mol % POPS. The relative spacings of the diffraction lines were: $\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$ and $\sqrt{8}$ for the five orders, corresponding to a Pn3m cubic phase. The first two orders were much stronger than the remaining three and the third order was weakest, but observable. There was no evidence of additional peaks corresponding to coexisting ordered lamellar or hexagonal phases. The lattice spacings calculated from an average of the five reflexions is given in Table I. Very similar average values, but with higher precision, were calculated from the first two stronger orders (not shown).

This cubic phase was able to support the PKC-catalyzed phosphorylation of histone (Fig. 2). The activity was determined as a function of PS concentration. The activity was rather insensitive to PS mol fraction between 2 and 12%. Mixtures containing over 12 mol% PS were present in the bilayer phase in this assay, as extruded large unilamellar vesicles. The activity seen in the MO/PS cubic phase is higher at lower lipid concentrations (data not shown). When the lipid concentration is increased 5 fold from 125 μM to 625 μM; the level of activity drops about 2 fold. This is possibly a result of larger cubic aggregates forming, so that there is less surface area exposed (see Discussion) for PKC binding. Also, the enzyme may get trapped among the aggregates.

One mol% diacylglycerol could be added to MO mixtures containing up to 10 mol% PS, and still remain as an isotropic phase, as shown by ^{31}P-NMR (data not shown). DAG is known to increase the affinity of PKC for membranes, and cause maximal activation. In the bilayer phase, addition of 1 mol% DAG to 20 mol% PS containing membranes increased the activity by 1.9 fold (Fig. 3). Addition of DAG to the cubic phase showed between 1.4 and 1.9 fold activation, with larger effects at greater PS concentrations.

Binding of PKC to cubic phase membranes was studied using a modified binding assay (see Methods). It was found that no more than 10% of the PKC was bound to MO/PS mixtures containing up to 10 mol% PS (Fig. 4), despite the relatively high activity seen with these mixtures. The amount of enzyme bound to the lamellar phase (20-50 mol% PS + MO) increases greatly in comparison. For cubic phase lipid, both activity (Fig. 2) and binding (Fig. 4) are insensitive to the amount of PS present between

about 2 and 10 mol%. This indicates that some PS is required for PKC activity and binding with cubic phases of MO, but that a maximal level of activity is reached at low mol fractions of PS. As a control, we performed binding and activity assays using DOPC/POPS/MO mixtures, which exist in the lamellar phase (31). PKC activity and % PKC bound to these vesicles at each composition are shown in Table 2. As the concentration of MO increases at a constant PS concentration, the level of PKC activity increases, as well as the percentage of PKC bound. However, the amount of PKC bound to this lamellar phase is much higher than the amount bound to MO cubic phases at the same PS level (compare Fig. 4 and Table 2).

Addition of 0.5-10 mol% alamethicin to dielaidoylphosphatidylethanolamine (DEPE) has been found to promote formation of the Pn3m cubic phase in excess water, at temperatures from 38 to 75°C (23). This cubic phase was also shown to exist when the phospholipid/peptide mixture was in excess buffer (data not shown). Without alamethicin, DEPE forms a lamellar phase up to 65°C. Addition of 4 mol% alamethicin to DEPE at 40°C caused a 2.8 fold activation of the measured PKC catalyzed phosphorylation of histone, relative to DEPE alone (Fig. 5). The binding of PKC to this cubic phase was less than 1%. (Binding was very weak, and values varied slightly. Values were always below 0.7% and went as low as 0) (not shown). Since the measured activity with DEPE and alamethicin was 0.0024 pmol P/min with less than 1% PKC bound, the specific activity is at least twice that observed with 10% PS/MO which supported activity levels of 0.0063 pmol P/min with a minimum of 6% PKC bound. Alamethicin itself was not the cause for this activation, since 4 mol% alamethicin added

to MO/PS mixtures in the cubic phase slightly decreased the PKC catalyzed phosphorylation of histone, in comparison with that obtained with MO/PS itself. Addition of alamethicin to MO/PS mixtures did not change the phase, as determined by ^{31}P-NMR (data not shown).

We compared the activity of PKC measured with MO/PS in the cubic phase using the small MARCKS peptide, instead of histone as the substrate for phosphorylation. The activity measured with MO containing 10% PS was similar for both the MARCKS peptide and histone (not shown).

We compare the specific activity of the membrane-bound form of PKC in cubic and lamellar phases (Table III). Although in set one the amount of lipid used for activity and binding was different, the results are valid for comparative purposes. The specific activity in the cubic phase is many fold higher than in the lamellar phase. Only at very high PS does the specific activity of the enzyme bound to lamellar phase liposomes approach one of the points of the cubic phase system. The measured activity in the cubic phase is not related to the amount of lipid present. Thus set two having the activity measured at lower lipid concentration gives a relative specific activity comparable to the cubic phase lipid used in set one. Furthermore, lamellar phase lipid with 20% PS (set 3) has a comparable specific activity to the 20% PS mixture reported in set one and much lower than the specific activities of PKC in cubic phase lipids. These results confirm our conclusion that PKC bound to cubic phase lipid is more active than the enzyme bound to a liposome of comparable lipid composition.

Since only about 10% of the PKC binds to the cubic phase, we were interested in determining whether this was due to binding of one specific isoform. Western blotting with antibodies to each of the three PKC isoforms found in rat brain (α, β, γ), on top and bottom fractions after incubation of PKC with cubic phase lipid (10% PS/90% MO). showed that each isoform was bound in about equal amounts to the cubic phase (data not shown).

The Inverted Hexagonal Phase. Dioleoylphosphatidylethanolamine (DOPE) forms an inverted hexagonal phase at room temperature (32,33). Addition of up to 10 mol% PS to this phospholipid does not change the phase as determined by ^{31}P-NMR (Fig. 6), even in the presence of histone, BSA, Ca^{2+}, or low concentrations of ATP (data not shown). The presence of an H_{II} phase was confirmed by x-ray diffraction which showed three orders indexing in the ratio 1: $\sqrt{3}$: $\sqrt{4}$. The H_{II} lattice spacing was rather insensitive to the presence of PS, increasing only slightly above the experimental error at 10% PS (Table I). This hexagonal phase was able to support the PKC catalyzed phosphorylation of histone (Fig. 7). The activity was not due to the PE itself, since similar DEPE/PS mixtures which exist in the L_β gel phase at 25°C did not show any increase in activity with increasing PS concentration (not shown). Binding of PKC to the hexagonal phase correlates with activity, with the percentage of enzyme bound, increasing with the concentration of PS (Fig. 7).

DISCUSSION

It has long been known that membrane phospholipids or other additives which are hexagonal phase promoters activate PKC when added to bilayer phases (34). However, the physical property responsible for this correlation is not known. It has been suggested that the activity of membrane-bound enzymes may be modulated by curvature strain, i.e., an instability of the bilayer caused by the intrinsic tendency of the constituent monolayers to form curved structures (35). This formulation was modified in terms of the dependence of lateral pressure on the position within the membrane (36). This mechanism of the lipid modulation of protein properties has been proposed for integral membrane proteins (e.g., see 37). It may be less applicable for an amphitropic protein, such as PKC, which readily partitions between aqueous and membrane environments. Furthermore, PKC can be activated by Triton micelles containing PS (38). Such micelles would be devoid of negative curvature strain. It was therefore suggested that changes in the interfacial properties of amphiphile-water systems was the direct cause for the modulation of the activity of this protein kinase (5), although there certainly could be other differences in the nature of the interaction between PKC and a detergent micelle *vs* a bilayer.

One can test the relative importance of curvature strain/lateral pressure profiles of the phospholipid monolayers in modulating PKC activity with the use of non-lamellar phases. The curvature strain would be progressively reduced in going from the lamellar to the bicontinuous cubic and finally to the hexagonal phase (24). If this property were responsible for the modulation of PKC activity, the activity should progressively decrease

in going through these three phases. The complication is that under a particular set of conditions the lipid will take up only one of the three phases. We therefore chose systems in which a minor change in the composition of the lipid resulted in a phase change, as well as performing appropriate controls.

We have studied two lipid systems that form bicontinuous cubic phases with a Pn3m space group. One of them is MO (21,22). This lipid alone does not support the activity of PKC (Fig. 2). However, at % PS between 2 and 10, there is measurable activation of PKC. At these low mol fractions of PS only a small fraction of the PKC is bound to these cubic phase lipids (Fig. 4). Thus, the specific activity of the PKC bound to cubic phase membranes is much higher than that obtained with lamellar phase membranes of similar composition (Table 3). There is most likely an optimal monolayer curvature in the bilayer phase for PKC binding and activation (9). The phospholipid in the cubic phase likely contains this optimal curvature, since the aqueous channels are lined with headgroups arranged in such a way that they would have a continuous variation of curvature. In addition, at a composition of about 12% PS there is a conversion from cubic to lamellar phase with increasing PS content. This results in an increased binding of PKC to the membrane but there is no abrupt increase in PKC activity over the range of PS concentrations, i.e. 12-13%, at which the lipid undergoes a conversion of phase.

It is well known that PS is required for the activity of PKC and its binding to membranes (1,27). The amount of PS that can be mixed with MO, while maintaining the lipid in the cubic phase is limited. However, we can assess the effect of MO addition to

PS-containing vesicles in a bilayer phase (Table 2). MO increases the maximal activity observed at high concentrations of PS and it shifts the dependence of the activity on PS to lower PS concentrations (full curves not shown). This is typical for the addition of a membrane component, like MO, which lowers the bilayer to hexagonal transition temperature (39). However, the activity of PKC, in all cases, correlates well with the fraction of PKC bound to a membrane. Thus, MO does not greatly reduce the requirement for PS, and it only slightly increases the activity of membrane-bound PKC.

The Pn3m cubic phase consists of a bicontinuous array of phospholipid, with the headgroups forming the lining of interconnected aqueous channels. These aqueous channels connect spherically shaped compartments of water which have a slightly larger diameter than the channels (connecting tubes). Despite this complication of non-uniform aqueous pore size, we can estimate the approximate average diameter of the aqueous channels of the cubic phase. The diameter of the water channel is (sin 45°)(lattice spacing) - (bilayer thickness). The thickness of an MO monolayer has been estimated at about 17Å (40). From our measured unit cell dimensions for MO (Table I), we estimate a water tube diameter of 39 Å . This is in good agreement with the estimate of Longley and McIntosh (21) of 40 Å . This is smaller than the diameter estimated for PKC as a globular protein of molecular mass 82 kDa and $v \equiv 0.72$, corresponding to a particle of diameter 57 Å . The cubic phase lattice parameter increases markedly with PS as does the diameter of the water cylinders (Table I). At 2% PS, the calculated aqueous tube diameter, 43 Å, is still too small to accommodate PKC. However, at 5% PS, the tube

diameter is 51.5 Å, which is not too much smaller than the 57 Å calculated for PKC as a globular structure. If PKC could become elongated or enter the polar region of MO or locally distort the lattice structure of MO, it could enter the cubic phase matrix. At 10% PS, the lattice spacing is large enough for PKC to enter. The fact that PKC binding does not increase between 5% and 10% PS (Fig. 4), suggests that there is no change in lipid accessibility between these two mol fractions of PS. However, between 2 and 5% PS there is a large percent increase in PKC binding which likely reflects the greater accessibility of the lipid. However, the absolute amount of PKC bound to the cubic phase remains low because of the relatively low mol % PS required to maintain a cubic phase and the lack of affinity of PKC for MO. In contrast to binding, 2% PS does support some activity (Fig. 2). This activity likely arises from PKC bound to the exterior of the cubic phase aggregates, and to the regions at the entrance to the water channels on the surface. Also, the observation that there was not a 2 fold increase in PKC activity when the amount of lipid was doubled, can be explained by larger cubic aggregates forming, which do not expose twice as much outer surface area, even though there would be twice the headgroup area exposed inside the channels. This is true also for the 5 and the 10% PS mixtures, suggesting that surface-bound PKC contributes significantly to the activity observed even in these cases in which the enzyme can enter the cubic phase matrix. Because of the low binding of PKC to MO with 2% PS, the specific activity of the bound enzyme must be very high. Thus, this membrane surface is very effective in activating PKC. This is also shown by the fact that in going from the cubic (10% PS) to the lamellar (15% PS) phase, the binding (Fig. 4) increases much more than activity (Fig.

2). Thus, the specific activity of the membrane-bound form of PKC is higher in the cubic phase. The binding to lamellar phase lipid is higher than to that of the cubic phase, despite the fact that half of the lipid in the lamellar phase is on the internal monolayer of the vesicle and not accessible to PKC. The greater binding to the lamellar phase lipid could have several causes. The dependence of binding on PS concentration is highly sigmoidal (27), hence a small increase in PS can cause a large increase in binding. In addition, there may not be complete access to all the membrane-water interface in the cubic phase and there may also be a difference in the binding affinity of PKC for cubic *vs* lamellar phase lipid.

An increase of PS from 2% to 5 or 10% increases the accessibility of the lipid surface to PKC, as a result of an increase in size of the aqueous tubes. Hence there is an increase in binding, but the activity is not greatly changed. It would appear that increased PS above 2%, allows more PKC to bind but that the specific activity of the bound enzyme is lower. The factors responsible for interfacial catalysis are complex and involve several binding and dissociation rates, in addition to the kinetics of the reaction itself. In particular the rate of binding of histone (21 kDa) to the membrane-bound form of PKC and the diffusion of enzyme and substrates in and out of the cubic phase matrix, may limit the rate of catalysis by PKC bound in the interior of the cubic phase matrix. Thus, even with 5 and 10% PS in MO, a large fraction of the activity of PKC may come from the enzyme bound to the surface of the cubic phase aggregate. This is not because there is a high concentration of the substrate, histone, at the surface of the cubic phase. Substitution of histone by a smaller substrate, the MARCKS peptide, had little affect on

the observed rates. The MARCKS peptide has much lower binding affinity for anionic membranes than histone, but is an equally effective substrate. It is also not likely that substrate accessibility limits the rate of PKC phosphorylation since the MARCKS peptide would have facile access to the aqueous pores of the cubic phase.

PKC activity in the cubic phase is not only a property of MO/PS systems, but was also found with DEPE/alamethicin (Fig. 5). Overall, a 2.8 fold increase in activity was found with the cubic phase compared to the lamellar phase, although the activity was not very high due to the lack of PS. Although PS is required to maximally activate PKC, it has been shown that PE can give rise to a lower level of activity (7, 41). Formation of the cubic phase in DEPE is triggered by the addition of only a small mol fraction of alamethicin. The alamethicin itself shows some inhibitory activity against PKC with MO/PS cubic phases. Therefore the enhanced activity of the alamethicin-DEPE cubic phase, compared with DEPE in the lamellar phase, shows that the cubic phase can support a higher level of activity than the lamellar phase. This is especially true when one considers the activity of PKC bound to the membrane. In the case of DEPE/alamethicin, very little PKC is bound to lipid, despite the increased measured activity of PKC. This again indicates that the specific activity of PKC bound to cubic phase membranes is very high. The lattice constant for this system was measured to be 160-180 A (23), larger than the MO/10% PS mixture and sufficient to allow PKC to enter the structure.

An increase in monolayer curvature concomitant with a decrease in monolayer curvature *strain* occurs in the hexagonal phase. In the presence of small mole fractions of PS, this phase supports the activity of PKC (Fig. 7). However, unlike the cubic phase, substantial amounts of PKC are bound to the hexagonal phase in the presence of PS, and this amount increases markedly with increased PS (Fig. 7). Unlike the cubic phase systems, in which a relatively minor perturbation can convert the lamellar to the cubic

phase, there is no other phase of comparable chemical composition to compare the hexagonal phase to. Nevertheless, it appears that the hexagonal phase can support some activation of PKC. From the hexagonal phase lattice spacings (Table I), we calculate the diameter of an H_{II} cylinder to be 75.2 Å for DOPE. This is in reasonable agreement with previous measurements (32,33,42,43). The length of the hydrocarbon chain was determined to be 20 Å for this lipid (43), although it has also been suggested that the hydrocarbon chains are not completely segregated away from the polar headgroups (44). The internal core of an H_{II} cylinder has a diameter of 35.2 Å, much too small for PKC to enter. Curiously, the presence of PS did not make the cylinder diameters of the H_{II} phase substantially larger (Table I). It is thus likely that, as in the case of the cubic phase, PKC binds to the entrances of the aqueous cores of the H_{II} phase. However, there is uncertainty about the nature of the ends of the cylinders. If they were blunt ends, they would expose the hydrophobic portions of the acyl chains, unless they were covered by a monolayer of micelle-type lipid or if they closed upon themselves to form a toroid, as has been shown in freeze fractures of the disk membranes of frog retinal rod outer segments, observed by electron microscopy (45), as well as in bovine retinal rod outer segments (46,47). It is also not known how the hydrophobic exterior of an aggregate of hexagonal phase cylinders is protected from contact with water. It is possible that, as suggested with the ends of the cylinders, there is a covering of a lipid monolayer with positive curvature.

We can thus conclude that lipids in inverted phase structures can support the activity of PKC. This is particularly well demonstrated for cubic phase lipid in which we

can show that lipid mixtures of essentially the same chemical composition in the cubic phase support considerably greater activity of membrane-bound PKC than in the lamellar phase. This may have relevance to biological systems since biological membranes have been shown to contain cubic phases (20).

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TABLE I

LATTICE SPACINGS OBSERVED BY X-RAY DIFFRACTION

	Pn3m phase of MO		H_{II} phase of DOPE
% PS Added	Lattice spacing (A)	Calculated water tube diameter (A)	Lattice spacing (A)
0	103 ± 2	39	65.1 ± 0.2
2	109.5 ± 1	43	65.0 ± 0.1
5	121 ± 1	51.5	65.2 ± 0.2
10	136 ± 2	62	65.6 ± 0.2

TABLE II

PKC ACTIVITY AND BINDING TO LAMELLAR DOPC/POPS/MO VESICLES

Vesicle Composition	PKC Activity (pmol P/min x 10^{-3})	% PKC Bound to SLV
100% PC	1.98 ± 0.30	19 ± 2
90% PC/10% PS	2.01 ± 0.08	15 ± 1
80% PC/20% PS	5.66 ± 0.23	60 ± 5
80% PC/20% MO	1.36 ± 0.05	16 ± 1
70% PC/10% PS/20% MO	2.86 ± 0.14	27 ± 2
60% PC/20% PS/ 20% MO	6.37 ± 0.19	64 ± 5
50% PC/50% MO	0.92 ± 0.05	2 ± 0
40% PC/10% PS/50% MO	4.90 ± 0.05	47 ± 5
30% PC/20% PS/50% MO	7.90 ± 0.40	80 ± 8

Selected points from curve of PKC vs % PS at 0, 10 and 20% PS which approximately corresponds to PS-independent activity, midpoint of activity curve and maximal activity at high PS. Conditions of assay are the same as in experimental section. The substrate is histone. % PKC bound is defined as the percentage of total enzyme added that is bound to the membrane fraction as measured by the SLV binding assay.

TABLE III

Comparative Specific Activity of Membrane-Bound PKC

Specific activity is expressed as $\frac{\text{pmol P/min}}{\text{\% PKC bound}} \times 10^{-4}$

Set one used activity data from Fig.2 at 1.25 mM lipid and binding data from Fig. 4 at 125 μM lipid; set two uses activity and binding data from Fig. 3 (no DAG) and 4, respectively at 125 μM lipid; set three uses activity and binding data from Table 2 at 125 μM lipid.

SET ONE	% PS	Specific activity
cubic phase	2	39.1 ± 13.7
	5	3.77 ± 0.90
	10	8.19 ± 2.01
lamellar phase	15	1.60 ± 0.27
	20	1.08 ± 0.06
	30	2.83 ± 0.17
	50	3.72 ± 0.26
SET TWO		
cubic phase	2	16.0 ± 6.3
	5	5.19 ± 1.09
	10	8.45 ± 0.23
SET THREE		
lamellar phase	20% PS/30% PC/50% MO	1.00 ± 0.15
	20% PS/60% PC/20% MO	1.00 ± 0.11
	20% PS/80% PC	0.95 ± 0.12

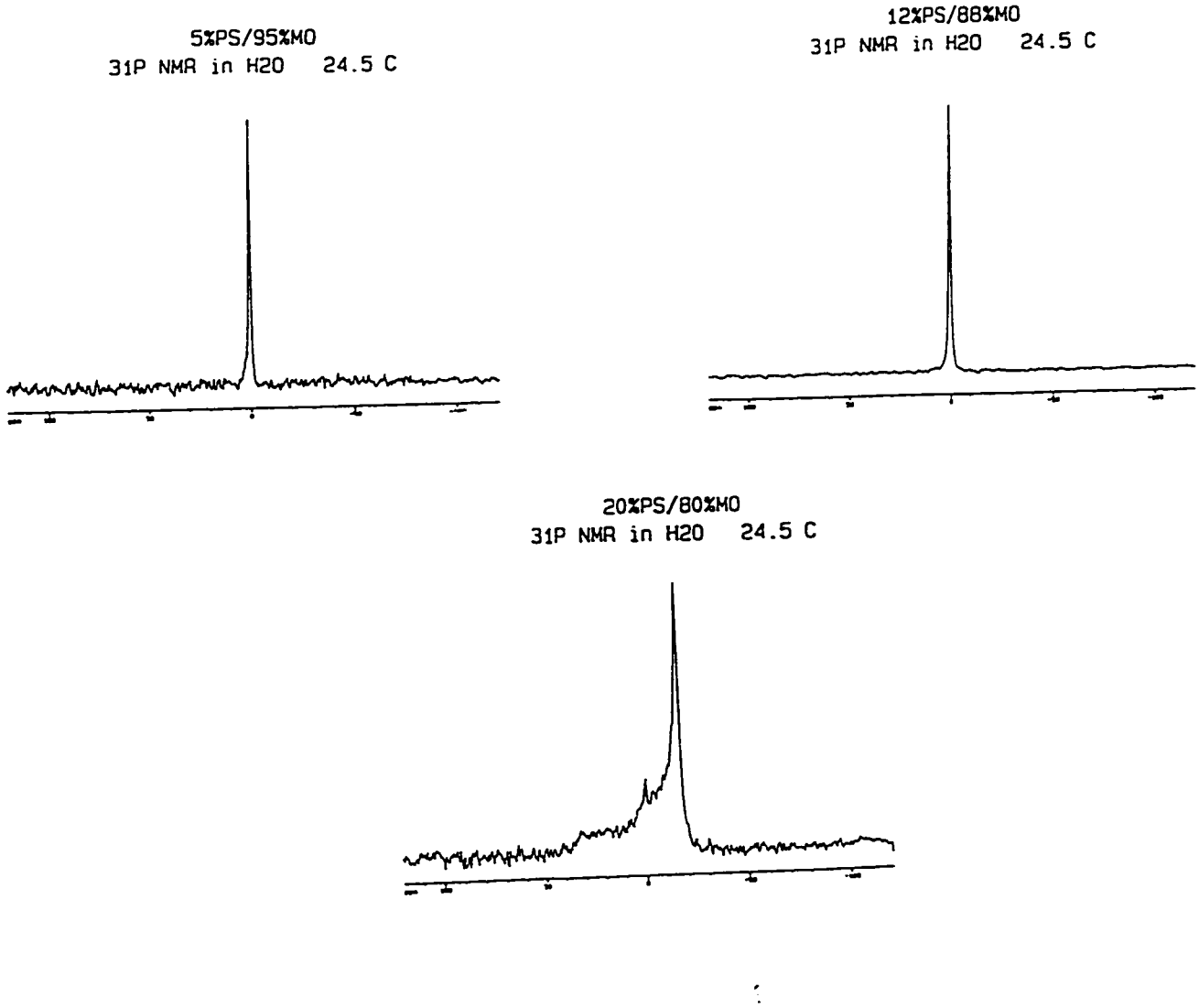

Fig. 1. **^{32}P-NMR spectra of MO/PS mixtures.** 25 mg of phosphatidylserine was used for each measurement, with an amount of MO to make up the desired composition. Scans were done at 25°C.

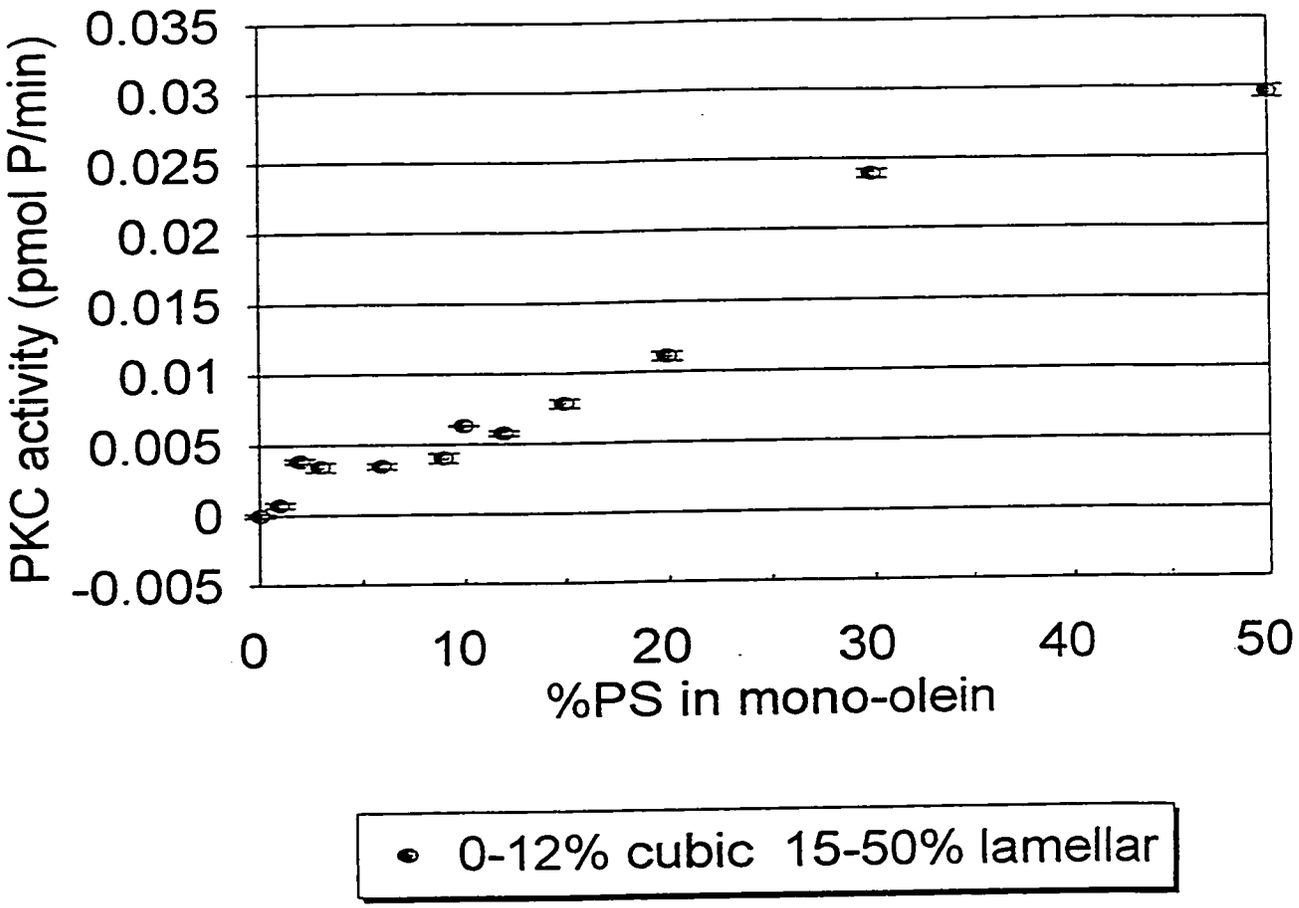

Fig. 2. **The PKC-catalyzed phosphorylation of histone increases as a function of PS concentration in a MO cubic phase.** Lipid was composed of MO plus the indicated concentration of PS and used at a final concentration of 1.25 mM. Temperature, 25 C. Rate of phosphorylation is expressed as pmol phosphate incorporated per minute. Data are expressed as the mean of triplicate determinations + standard deviation.

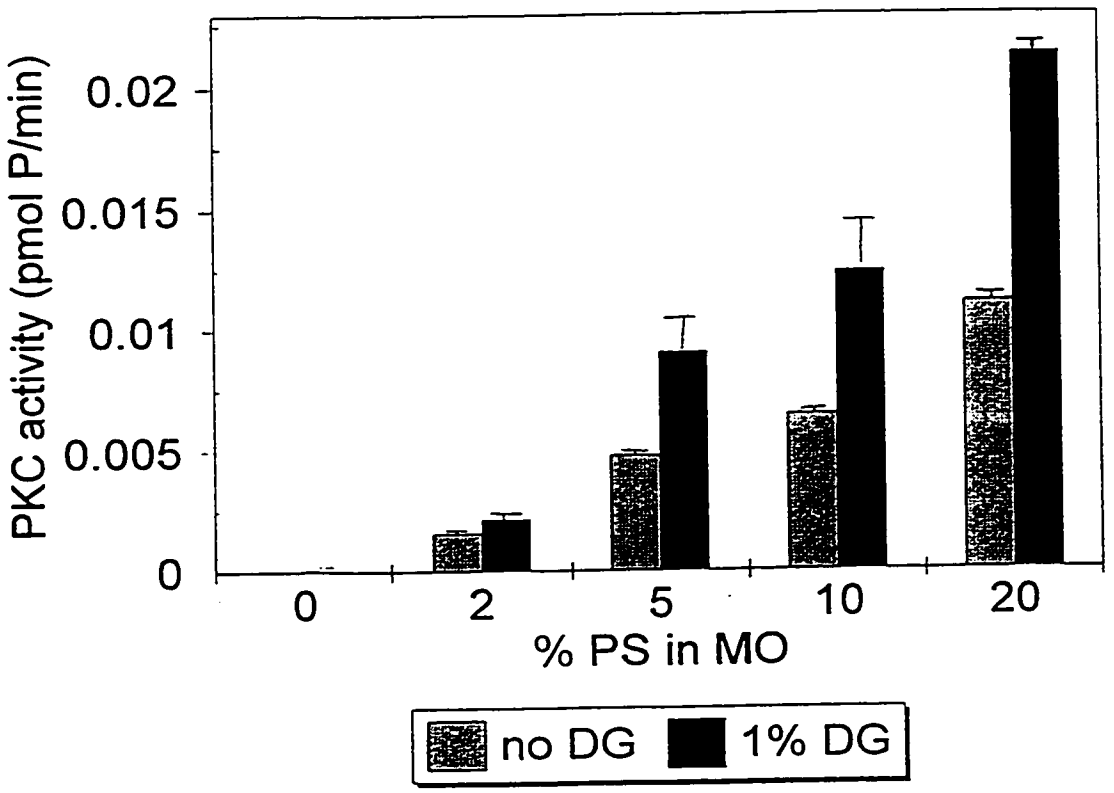

Fig. 3. **DAG increases the rate of PKC-catalyzed phosphorylation of histone when added to the PS/MO cubic phase.** Lipid was composed of MO plus the indicated concentration of PS, with or without 1 mol% DAG, and used at a final concentration of 125 μM. Results from vesicles containing 20% PS in the lamellar phase, with and without DAG are shown for comparison. Rate of phosphorylation is expressed as in Fig. 2.

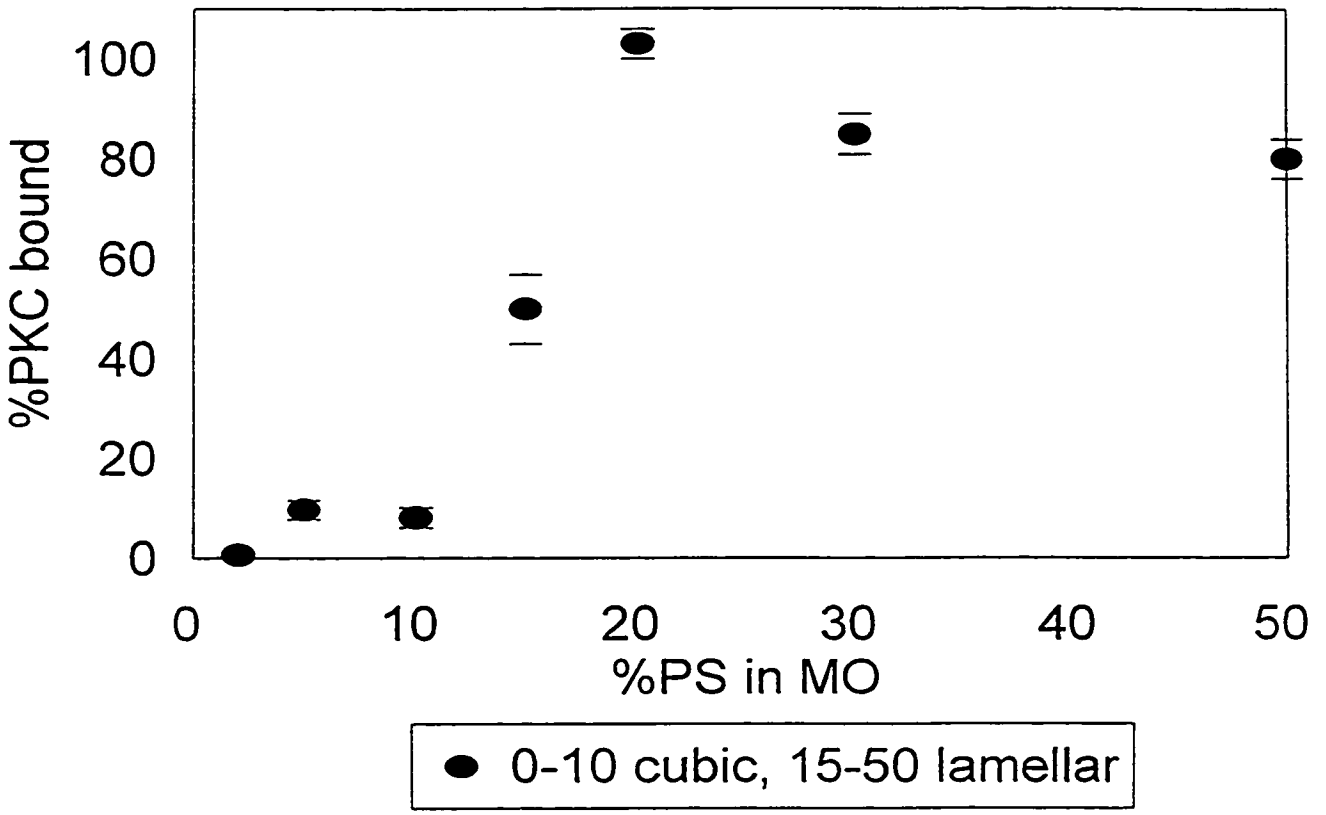

Fig. 4. **Binding of PKC to the PS/MO cubic phase.** Lipid was composed of MO plus the indicated concentration of PS and used at a final concentration of 125 μM. Binding is expressed as a percentage of the total enzyme added.

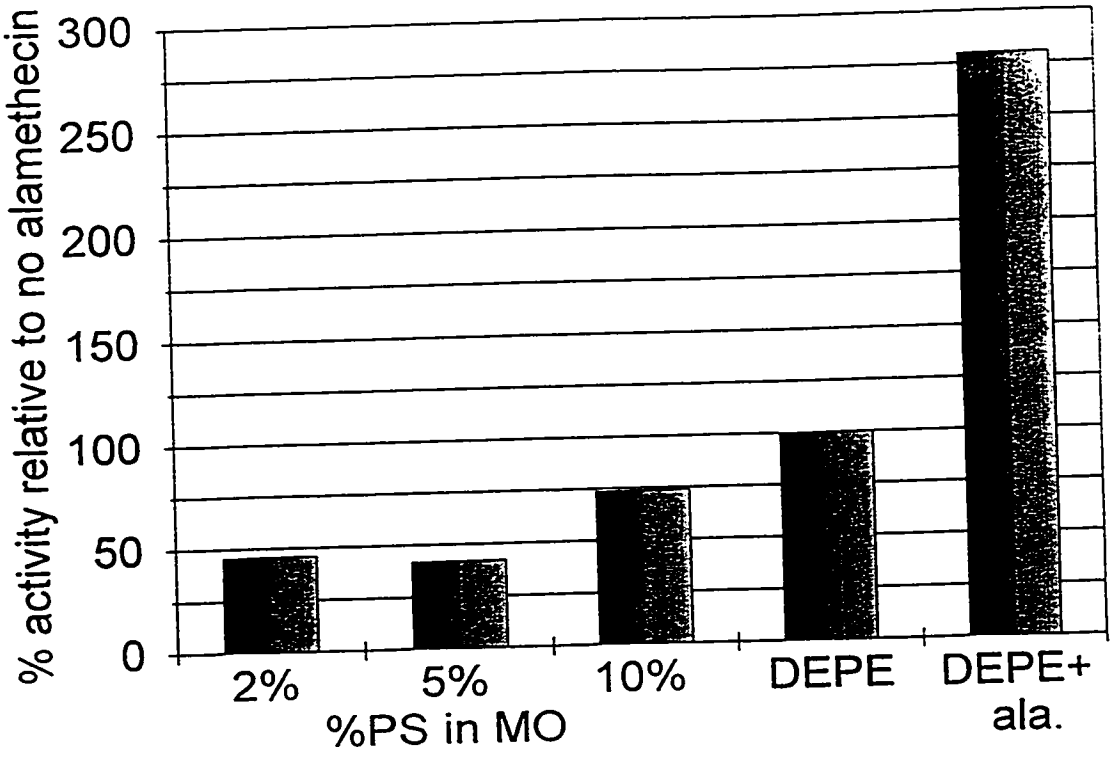

Fig. 5. **Increase in PKC activity in the DEPE/alamethicin cubic phase.** For bars 4 and 5 (far right), lipid was composed of DEPE with or without 4 mol% alamethicin and used at a final concentration of 1.25 mM. Temperature 40° C. DEPE alone is lamellar, and DEPE with alamethicin is cubic. Standard deviation was less than 1%. For bars 1-3 (left), lipid was composed of MO/PS with 4 mol% alamethicin at 25°C. All 3 were in the cubic phase. Standard deviation was 20, 13 and 5% respectively. Activity is expressed as a percentage of that obtained (with MO and the indicated concentration of PS) in the absence of alamethicin for bars 1-3, and with DEPE alone for bars 4 and 5.

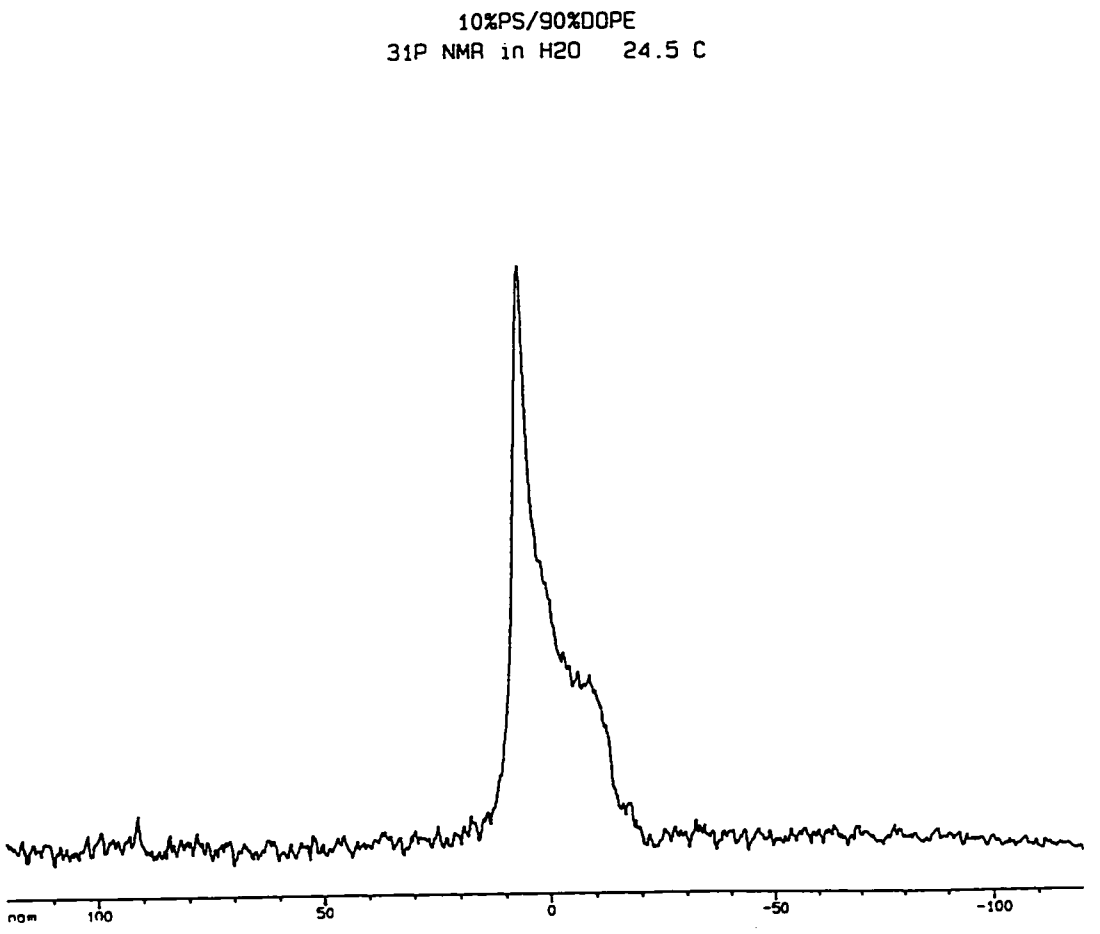

Fig. 6. **^{31}P NMR spectra of DOPE/PS mixtures.** Total phospholipid was 25 mg for each measurement. Temperature, 25°C. Similar powder patterns were obtained at 15°C.

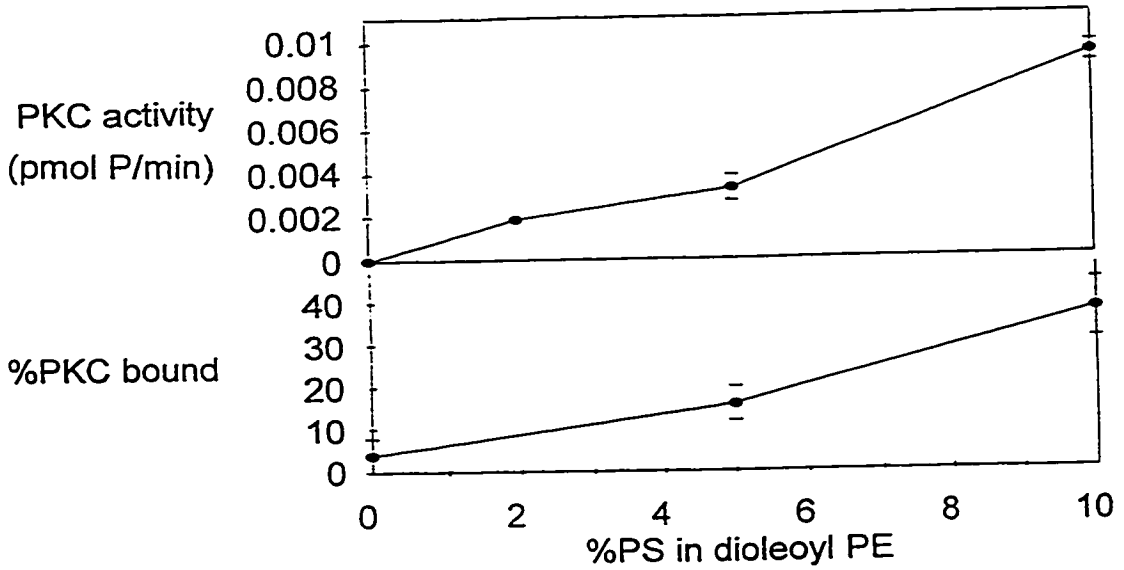

Fig. 7. **Hexagonal phases support PKC-catalyzed phosphorylation of histone and PKC binding.** Phospholipid was composed of DOPE plus the indicated concentration of PS. Phospholipid was used at a final concentration of 125 μM. Temperature was 25°C. Data are expressed as the mean of triplicate determinations + standard deviation. Binding (bottom) is expressed as a percentage of the total enzyme added. Activity (top) is expressed as pmol P incorporated per minute.

Manuscript 5, "Interfacial Membrane Properties Modulate Protein Kinase C Activation: Role of the Position of Acyl Chain Unsaturation"

This study was undertaken to better understand the relationship between hexagonal phase propensity and PKC activation. The physical properties of three phosphatidylethanolamines which vary only in the position of their double bond have been well characterized (Epand *et al.* 1996). It was interesting to determine whether the relative effects of these phospholipids on PKC activation and binding correlated with any of the physical properties previously determined. It was found that PKC activation did not correlate with either intrinsic radius of curvature or monolayer bending modulus of the three PEs, but instead correlated well with the interfacial polarity of membranes containing the three PEs. These findings tie in well with those in Manuscript 4, showing that curvature strain is not the only factor causing activation of PKC.

All experiments were performed by Jennifer Giorgione, who also wrote the manuscript, which was edited by Richard Epand. The probe DTMAC was synthesized in collaboration with Ruud Kraayenhof.

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Interfacial Membrane Properties Modulate Protein Kinase C Activation: Role of the Position of Acyl Chain Unsaturation

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Running Title: PKC and non-lamellar phases.

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Abbreviations used:

PKC	protein kinase C
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PS	phosphatidylserine
DPLPE	Dipetroselinoyl-PE (6)
DOPE	Dioleoyl-PE (9)
DVPE	Divaccenoyl-PE (11)
DTMAC	4-(n-dodecylthiomethyl)-7-(N,N-dimethyl-amino) coumarin
5-doxyl-PC	1-Palmitoyl-2-Stearoyl(5-Doxyl)-phosphatidylcholine
T_H	bilayer to hexagonal phase transition temperature
LUVs	Large unilamellar vesicles
SLVs	Sucrose-loaded vesicles
POPS	1-palmitoyl-2-oleoylphosphatidylserine
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
R_o	intrinsic radius of curvature
K_c	elastic bending modulus
DAG	Dioleoylglycerol

Abstract

We studied the effects of the addition of a series of 1,2 dioctadecenoyl-*sn*-glycerol-3-phospho-ethanolamines to vesicles composed of 1-palmitoyl-2-oleoyl phosphatidylserine and 1-palmitoyl-2-oleoyl phosphatidylcholine, on the activity and membrane binding of protein kinase C (PKC). The three phosphatidylethanolamines (PE) were dipetroselinoyl-PE, dioleoyl-PE, and divaccenoyl PE, which have double bonds in positions 6, 9 and 11, respectively. These lipids represent a group of structurally homologous compounds whose physical properties have been compared. We also used a fluorescent probe, 4-(n-dodecylthiomethyl)-7-(N,N-dimethyl-amino)coumarin to measure the relative interfacial polarities of LUVs containing each of the three PE's. We find dipetroselinoyl-PE to allow the least access of the fluorescent probe into the membrane. This is also the lipid that shows the lowest activation of PKC. The activity of PKC was found to correlate best with the interfacial properties of the three PE's rather than the curvature energy of the membrane. The results show the sensitivity of the activity of PKC to small changes in lipid structure.

Protein kinase C (PKC) is a family of Ser/Thr protein kinases (1) which play an important role in signal transduction (2). The principle isoforms of rat brain PKC are the Ca^{2+}-dependent, c-PKC isoforms (3). Aspects of the structural and regulatory properties of PKC have recently been reviewed (4-7).

Protein kinase C can be activated by binding to membranes. The enzyme activity is modulated, in part, by the physical properties of the membrane to which it binds (8, 9). It has been shown that uncharged or zwitterionic additives which lower the bilayer-to-hexagonal phase transition temperature of model PE bilayers are activators of PKC (10). In addition, replacement of phosphatidylcholine (PC), a lipid that forms stable bilayers, with phosphatidylethanolamine (PE), a lipid which is prone to form inverted phases, has been shown to increase the activation of PKC (11-14). It has been suggested that the relative activation seen with different PEs correlates with their bilayer to hexagonal phase transition temperature (T_H), with the strongest activators having the lowest T_H (11,12). There are two aspects by which agents that lower T_H increase the activity of PKC. One aspect is a lowering of the mol fraction of PS required for activation and the other is the maximal activity observed at high PS (12,15). The exact physical property responsible for this correlation is not known.

To further investigate this phenomenon, a series of 1,2 dioctadecenoyl-sn-glycerol-3-phosphoethanolamines (di-18:1 PEs), differing only in the position of the double bond, was used. Double bonds were at position 6, 9, or 11. This series of structurally homologous PEs has been well characterized with regards to T_H, intrinsic radius of curvature and monolayer bending modulus (16). Determining the effects of

these homologous phospholipids on PKC binding and activation can lead to a better understanding of the physical properties responsible for the observed correlation between "hexagonal phase propensity" and enzyme activity (8).

EXPERIMENTAL PROCEDURES

Materials. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Histone H1 was from GIBCO/BRL (Grand Island, NY). Bovine serum albumin fraction V, protamine sulfate, ATP sodium salt, and EGTA were from Sigma (St. Louis, MO). [γ-^{32}P] ATP was from ICN, and [9, 10-^{3}H] dipalmitoyl phosphatidylcholine was from NEN. $CaCl_2$ was from Fisher, and Tris was from Boehringer Mannheim. The synthesis of 4-(n-dodecylthiomethyl)-7-(N,N-dimethyl-amino) coumarin (DTMAC) has been described previously (17).

PKC Purification. Rat brain PKC was purified by a modified procedure of Huang et al. (18) described previously (15).

Lipid vesicles. Lipid films were made by dissolving phospholipids, with or without DTMAC in 2:1 (v:v) chloroform/methanol and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in standard buffer (100 mM KCl/5 mM $MgCl_2$/20 mM Tris, at pH 7.0), and subjected to 5 freeze-thaw cycles. These were extruded through two 0.1 μm pore polycarbonate filters in a microextruder (Avanti Mini Extruder, Avanti Polar Lipids, Alabaster, AL) to form LUVs. Sucrose loaded vesicles (SLVs) were prepared by hydrating in a buffer of 0.170 M sucrose/5 mM $MgCl_2$/20 mM Tris at pH 7.0. The vesicles were sedimented in standard buffer for 30 min at 100,000g to dilute out the excess sucrose.

PKC activity assays. The activity of PKC towards histone was determined as previously described (15). Histone was added to a final concentration of 0.2 mg/mL, in a 150 μL total volume. Phospholipid was 100 μM in the form of either LUVs or sucrose-

loaded vesicles (SLVs) (as noted in the figure legends). [γ-^{32}P] ATP (0.2 mCi/mL) was 20 μM, and PKC was 575 ng/mL. Ca^{2+} was 200 μM unless otherwise stated. The reaction temperature was 25°C.

PKC Binding Assays. The sucrose-loaded vesicle (SLV) assay was modified from the procedure of Rebecci et al. (19) as described previously (15). PKC was incubated with SLVs and then centrifuged for 30 min. at 100,000 g at 25°C to separate the membrane-bound enzyme. The pellet and supernatant were assayed under identical conditions for activity towards protamine sulfate.

Fluorescence measurements. LUVs, 100 nm in diameter contained 20 mol% POPS, 50 mol% one of the di 18:1 PE, 0.5 mol% DTMAC, and either 15 mol% 1-palmitoyl-2-stearoyl-(5-doxyl)-PC (5-doxyl PC) and 14.5 mol% POPC, or 29.5 mol% POPC. The final concentration of LUVs was 3.5 mM in standard buffer. The synthesis and initial characterization of DTMAC were reported in (17). Fluorescence was measured in glass cuvettes at 25°C with an SLM Aminco Series II spectrofluorimeter. Temperature was regulated with a constant temperature bath which circulated fluid through the cell holder. A filter which blocked wavelengths below 420 nm was used between the sample and photomultiplier. The excitation wavelength for all LUVs containing DTMAC was 397 nm. An emission scan was taken to determine the wavelength of maximum emission specific for LUVs containing each of the three PEs. There was little difference in the emission maxima in the presence of each of the three PEs, with the maximum being 475 nm ± 1 nm.

RESULTS

We have compared PKC activity at various Ca^{2+} concentrations, in the presence of LUVs composed of 30% POPS with or without the addition of 20% of one of the three PEs, the remaining lipid being POPC (Fig. 1). It is clear that LUVs containing 20 mol% PE, significantly increased the PKC-catalyzed phosphorylation of histone relative to LUVs containing only 30 mol% PS and 70 mol% PC. DOPE supported the highest level of PKC activity under differing Ca^{2+} concentrations. DPLPE and DVPE generally supported similar levels of activity, which were lower than that of the DOPE. A similar order for the three PEs was seen when the mol% of PE was varied from 25 to 75 mol% at a constant 20 mol% POPS and 200 μM Ca^{2+} (Fig. 2).

Binding was determined as a function of PS, at a constant 50 mol% of each PE (not shown). The binding shows sigmoidal behavior, typical for PKC (15). However, there is more error in the binding curves than is usually obtained. It may be that these vesicles, with their particular lipid compositions, are not stable to centrifugation and resuspension. Therefore, two PS concentrations were chosen for a more detailed study with a higher number of replicates (see below).

Activity curves revealed half-maximal activity at 16±2, 14±2 and 11±2 mol% PS, for DPLPE, DOPE, and DVPE, respectively (Fig. 3). Similar results were obtained when LUVs were used instead of SLVs (data not shown). Because the differences in activity were small for the curves for the three different PEs, to further test whether there was a difference among these PEs, we did multiple assays at a fixed PS concentration, either

near half maximal activity (20% PS, Fig. 4) or at maximal activation (40% PS, Fig. 5). At 40% PS, the activity is greatest in the presence of DOPE, while the activity is similar for both DOPE and DVPE at 20% PS. In both cases, activity is least with DPLPE. This is also the case in the presence of 1% DAG (Fig. 6). Binding assays under these conditions revealed that there was significantly more PKC bound to DPLPE-containing membranes than either DOPE or DVPE. Therefore, the specific activity of membrane-bound PKC is much lower with DPLPE.

DTMAC is a fluorescent probe whose depth of burial in the membrane has been found to be sensitive to the nature of the lipid headgroup (17, 20). DTMAC fluorescence can be quenched by the 5-doxyl-PC quencher, which has a nitroxide group at the 5 position of the sn-2 acyl chain. The position of the quencher is more or less fixed by the phospholipid headgroup. DTMAC is more free to move along the bilayer normal. Therefore, a greater amount of quenching of DTMAC fluorescence is expected when the interfacial fluorescence probe is more deeply inserted into the membrane, since the 5-doxyl group will be embedded at a fixed position in the membrane. The fluorescence intensity ratio was calculated by measuring the fluorescence of 0.5 mol% DTMAC, at the wavelength of maximum emission, in the presence *vs.* the absence of 15 mol% 5-doxyl-PC (Fig. 7). Since the intensity ratio is significantly higher for DPLPE than for the other two PEs, there is less quenching of DTMAC in DPLPE containing membranes, and therefore, the depth of insertion of DTMAC is greater in LUVs containing 50 mol% DOPE or DVPE than those containing the same amount of DPLPE. This suggests a difference in the interfacial polarity of these membranes, since DTMAC has been

suggested to partition into a membrane with its fluorophore group in a region with a particular dielectric constant (20).

DISCUSSION

It has been shown that phospholipids or hydrophobic substances that favor the formation of the hexagonal phase are generally activators of PKC. Some studies have shown that the level of activation correlates with the bilayer to hexagonal phase transition temperature (T_H) of the phospholipid (11, 12). The results from the present study are in agreement with this generalization. DOPE (Δ9) exhibits the greatest activation, although in some cases the activity in the presence of DVPE (Δ11) is comparable. DOPE and DVPE have the lowest values of T_H, of 8 and 28°C, respectively. DPLPE (Δ6) almost always has the lowest activity of the three PEs and it has the highest T_H of 37°C. The relative activities also correlate with the L_β to L_α phase transition temperature, -1, -8 and 16° C for DVPE, DOPE and DPLPE, respectively. However, there is no other evidence for a correlation between this transition temperature and PKC activation. In fact, Senisterra and Epand (12) have shown that there is little change in activity in going from the L_β to L_α phase.

However, the specific physical property which is responsible for the correlation between T_H and the activation of PKC is not known. When hexagonal phase promoting lipids are added, there is an increase in headgroup spacing which may make the acyl chains more accessible and allow greater interaction of PKC with the membrane bilayer (21, 22). It should be noted however, that even under conditions which promote strong interaction of PKC with membranes, the bnding of the enzyme is reversible, indicating that the protein does not penetrate deep into the hydrohobic core of the membrane (23).

There are several physical parameters that determine the relative stability of lamellar and hexagonal phases. The differences among phospholipids with regard to lipid polymorphism result from differences in their intrinsic radius of curvature, R_o, and from their elastic bending modulus, K_c. The intrinsic radius of curvature was found to be similar for DOPE and for DPLPE over a range of temperatures (see Fig. 5 in reference 16). Since these two PEs show the highest and the lowest extent of activation of PKC, it is not likely that R_o determines the extent of activation of PKC. The bending modulus, K_c correlates better with activation in that DOPE, the best activator, has the highest K_c. However, DPLPE and DVPE have similar K_c yet DVPE is a better activator. We can also assess the curvature strain energy of a monolayer of each of the PEs, constrained to be a flat bilayer. This energy is given by, $0.5\ K_c/R_o^2$ (24). Using the values of K_c and R_o at 25°C (16), we calculate the curvature energy to be 3.08, 3.91 and 2.04 ergs/cm^2 for DPLPE (Δ6), DOPE (Δ9) and DVPE (Δ11), respectively. The lipid that supports the least activity is DPLPE but the curvature energy for this lipid falls intermediate between those of the other two. The addition of 1 mol% DAG to LUVs containing 20% PS and 50% PE did not alter the relative activity levels (compare Fig. 6 with Fig. 4). Activation was similar for DOPE and DVPE and lowest for DPLPE. Therefore none of the parameters directly associated with membrane curvature is well correlated with PKC activation. This is in agreement with recent evidence showing that cubic phase membranes with lowered curvature strain can support greater activity of membrane-bound PKC than lamellar lipid of similar composition (25).

The quenching of the fluorescence of DTMAC by the nitroxide-labeled PC is indicative of the facility with which substances can enter the membrane bilayer. The results show the sensitivity of the extent of quenching on lipid structure (Fig. 6). There is also an excellent correlation between the extent of quenching in the presence of the three lipids and the activation of PKC. It should be noted that the PKC assays were performed at several different calcium concentrations. Calcium would also affect the interfacial properties of membranes rich in PS, although in the present case there is only 20% PS in the membrane. The fluorescence measurements were done to compare the properties of membranes containing each of the three PEs at a single fixed condition. Calcium was omitted from these experiments in order to avoid complication due to differing effects of this ion on the fluorescent probe. In most activity assays, the lipid that shows the least activation of PKC is DPLPE which stands out as the lipid allowing less entry into the membrane (Fig. 7). This lipid has the double bond position closest to the headgroup. Because of the rigidity of the double bond and the fact that it will cause disordering at positions further into the bilayer center, the accessibility of DTMAC to DPLPE is smallest. DOPE and DVPE generally show similar extents of activation and they have similar behavior in this quenching assay. Thus this quenching assay appears to be an even better predictor of PKC activation than T_H, since the activities and fluorescence quenching of DVPE and DOPE are similar but the T_H value of DVPE is intermediate between those of the other two PEs. The penetration of DTMAC into the bilayer may be related to the ability of PKC to enter the membrane. This property has previously been suggested to be of importance for PKC activation (26).

The differences in activity of PKC in membranes composed of each of these three similar lipids is not very great. However, in the assays done with many replicates, it is clear that DPLPE shows the lowest extent of activation. The fact that the activity shows a sigmoidal dependence on PS means that at a particular PS concentration that is found *in vivo*, the activity of PKC may be quite different depending on subtle factors of lipid structure, such as the position of a double bond in the acyl chain in a mixed lipid system.

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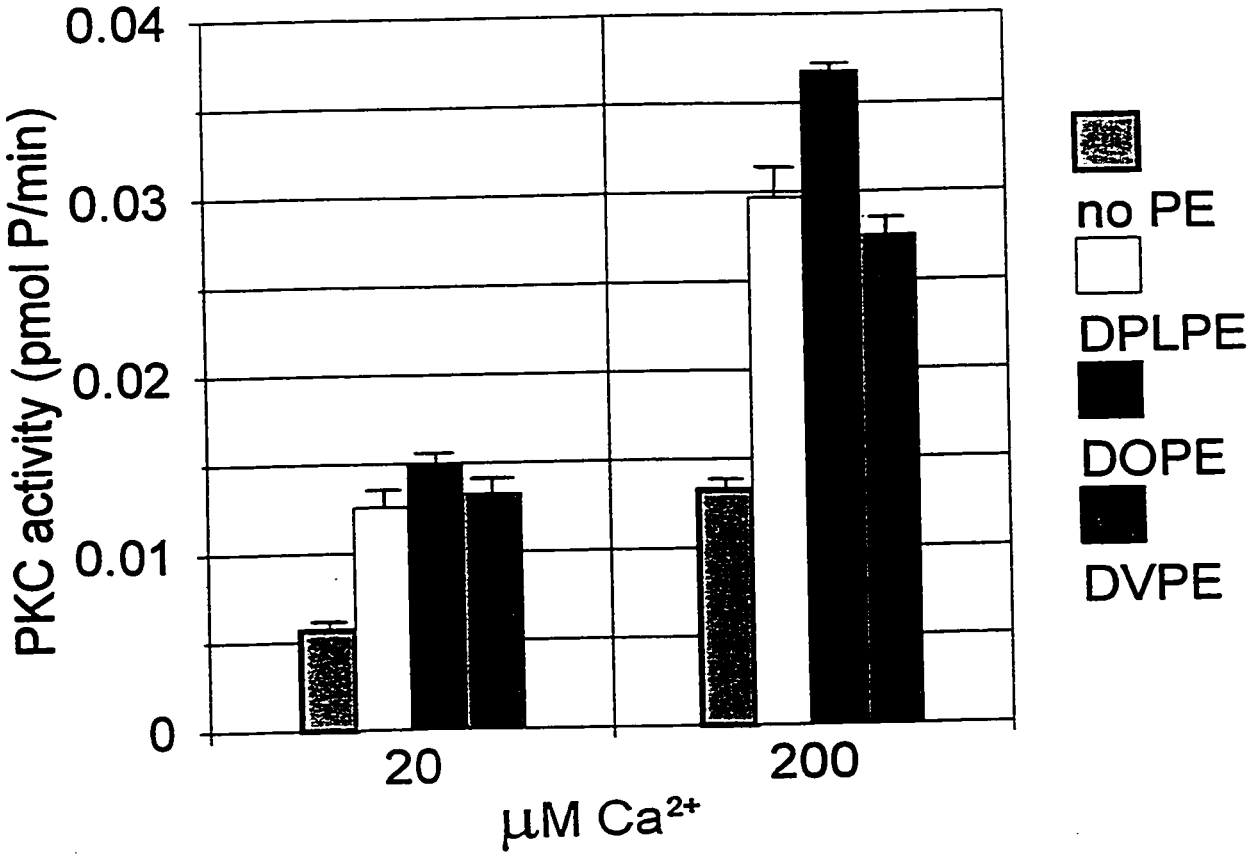

Figure 1. **The effect of DPLPE, DOPE, or DVPE on the PKC-catalyzed phosphorylation of histone with various Ca^{2+} concentrations.** LUVs were composed of 30 mol% POPS, and 70 mol% POPC, with 20 mol% PE when present, replacing POPC. Activity is expressed in pmol phosphate incorporated per minute. Data is expressed as the mean of triplicate determinations +/- SE. Assay temperature was 25 °C.

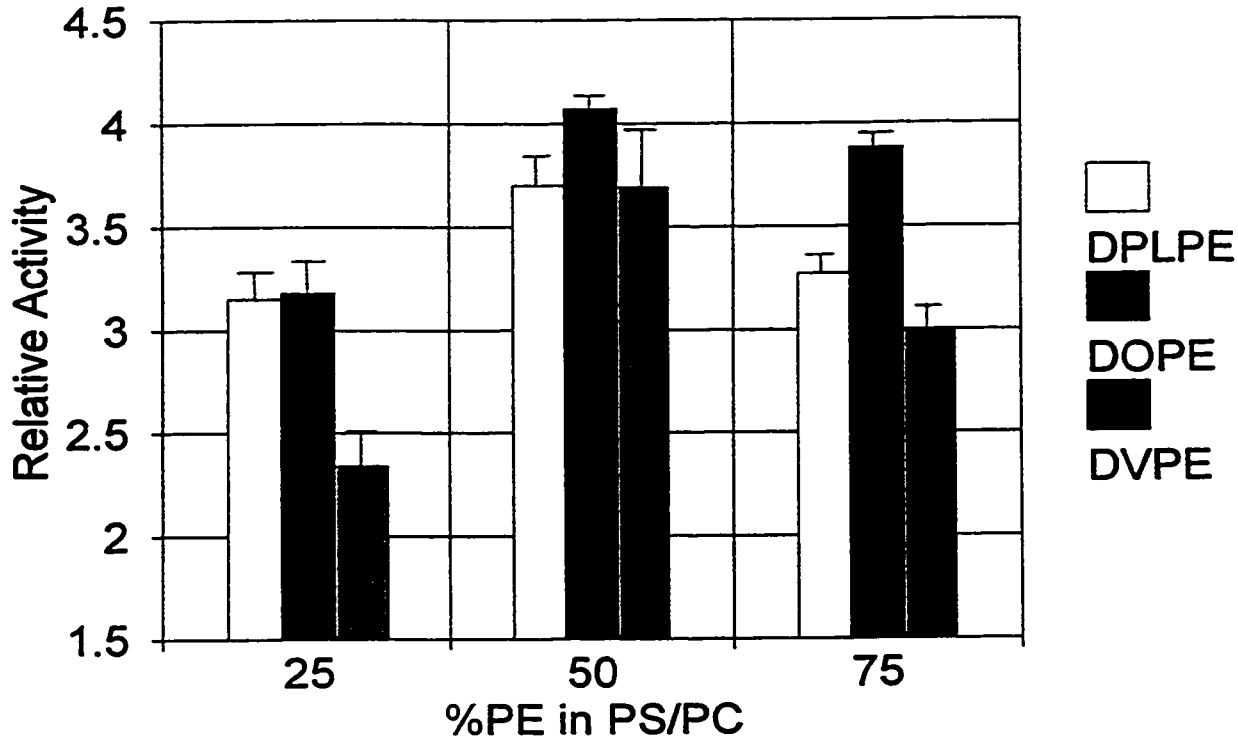

Figure 2. **PKC-catalyzed phosphorylation of histone in the presence of various mol percentages of DPLPE, DOPE, or DVPE.** SLVs were composed of 20 mol% POPS. with the indicated percentage of PE, and the remainder POPC. Activity is expressed in pmol phosphate incorporated per minute. Data is expressed as the mean of triplicate determinations +/- SE. Ca^{2+} was 200 μM, assay temperature was 25°C.

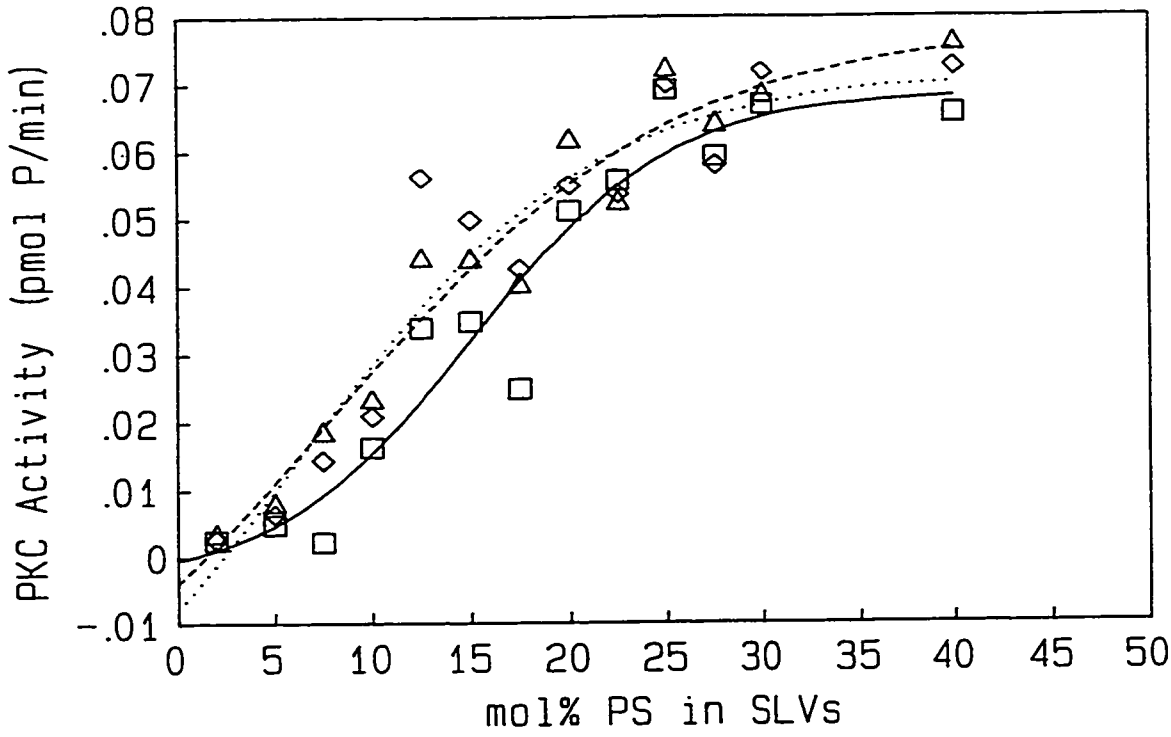

Figure 3. **PKC-catalyzed phosphorylation of histone in the presence of PE-containing SLVs as a function of mol% POPS.** SLVs contained 50 mol% PE, and the indicated POPS, with the remainder POPC. Activity is expressed as pmol histone phosphorylated per minute, and is the mean of triplicate determinations. Curves were fitted to the non-linear Hill equation. DPLPE (squares, solid curve), DOPE (triangles, dashed curve), DVPE (diamonds, dotted curve).

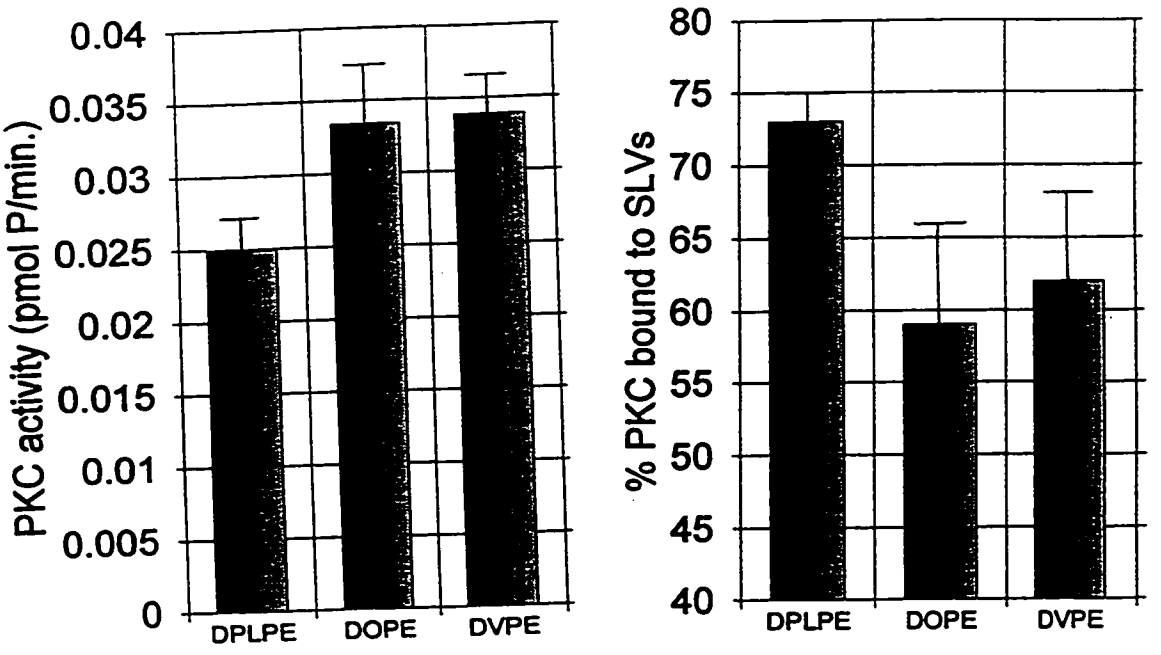

Figure 4. **Comparisons of PKC-catalyzed phosphorylation of histone, and %PKC bound to LUVs or SLVs containing 50 mol% PE.** Vesicles were composed of 20 mol% POPS, 50 mol% PE, and 30 mol% POPC. Six samples of each vesicle composition were made up and extruded separately. Activity or binding was then assayed in triplicate for each sample. Activity is expressed as pmol phosphate incorporated per minute, and binding is expressed as a percentage of the total enzyme added. Data is expressed as the mean of 18 determinations +/- SE. Ca^{2+} and temperature were as above. The differences between DPLPE & DOPE, as well as DPLPE & DVPE are significant ($p<0.01$) as determined by the 2-tailed students T-test using Quatro Pro.

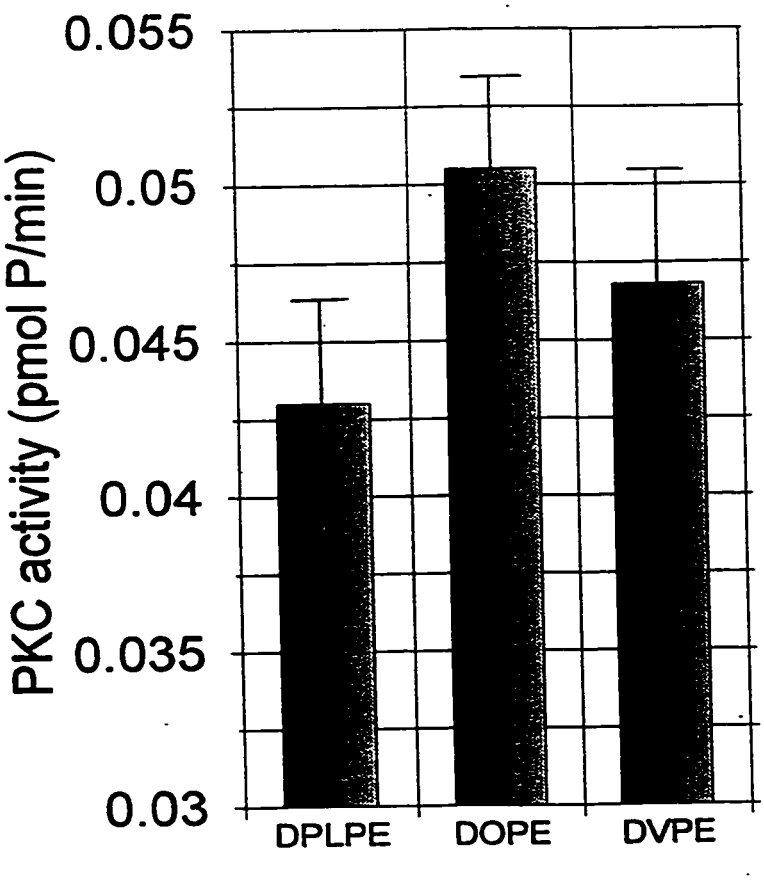

Figure 5. **Comparisons of PKC-catalyzed phosphorylation of histone with LUVs containing 50 mol% PE.** Vesicles were composed of 40 mol% POPS, 50 mol% PE, and 10 mol% POPC. Six samples of each vesicle composition were made up and extruded separately. Activity was then assayed in triplicate for each sample. Activity is expressed as pmol phosphate incorporated per minute. Data is expressed as the mean of 18 determinations +/- SE. Ca^{2+} and temperature were as above. Differences between all 3 values are significant. For DPLPE and DOPE, $p<0.01$. For DPLPE & DVPE, as well as DOPE & DVPE, $0.01 < p < 0.05$. Calculations were as in Fig. 4.

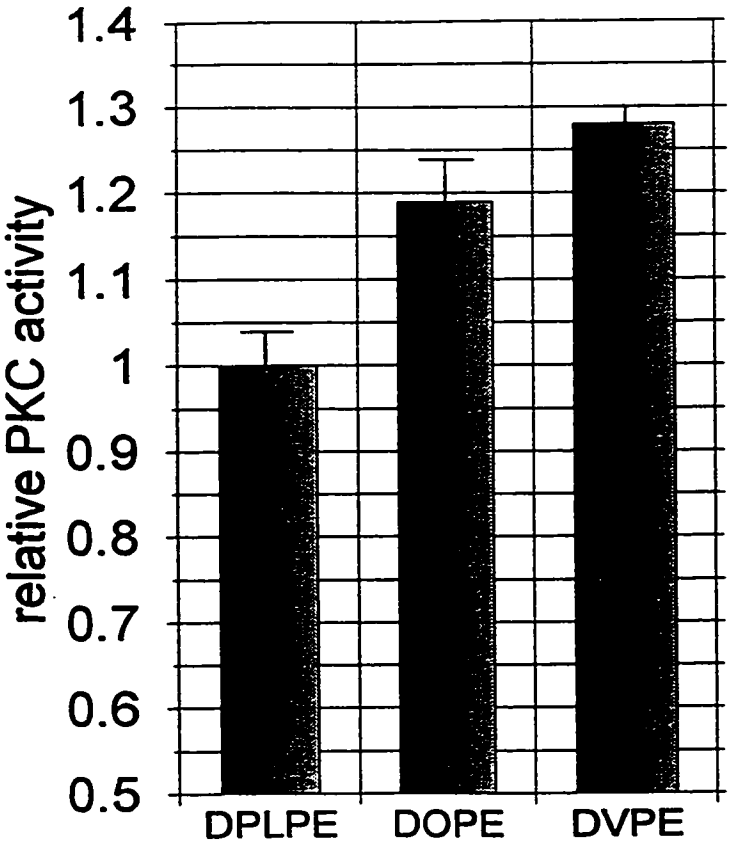

Figure 6. **Comparisons of relative PKC-catalyzed phosphorylation of histone with LUVs containing 50 mol% PE and 1 mol % DAG.** Vesicles were composed of 20 mol% POPS, 50 mol% PE, 1 mol % DAG and 29 mol% POPC. Activity was assayed in triplicate for each sample. Activity is plotted relative to that obtained in the presence of DPLPE. Activity is shown as relative activity since a new batch of PKC was used and comparison of absolute activity levels is not possible. Differences between DPLPE and DOPE, as well as DPLPE and DVPE are significant. Calculations were as in Fig. 4.

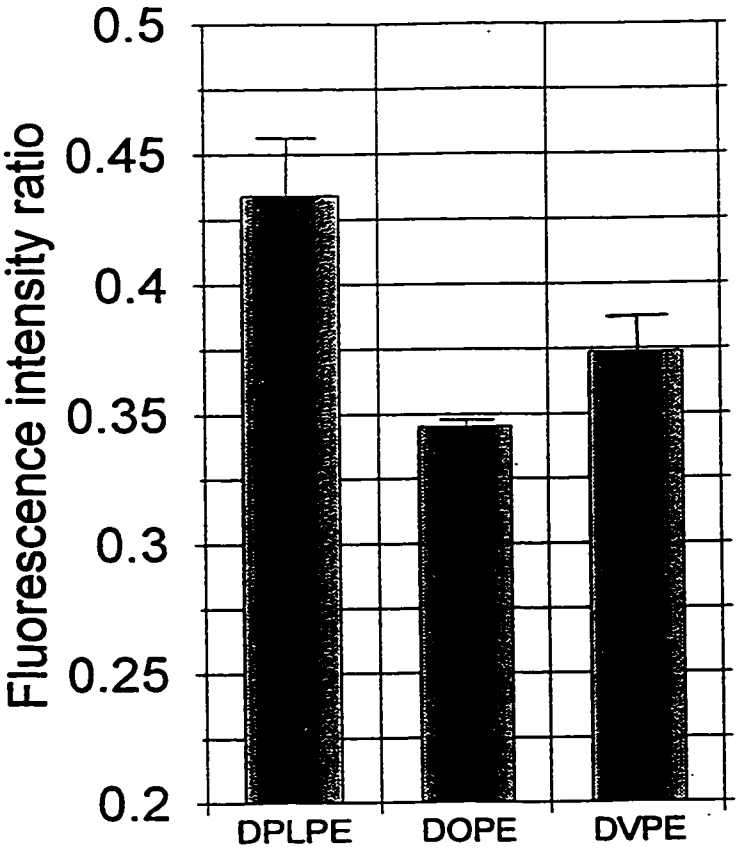

Figure 7. **Fluorescence quenching of DTMAC by 5-doxyl PC in PE-containing LUVs.** The fluorescence intensity of 0.5 mol% DTMAC in LUVs composed of 20 mol% POPS, with 50 mol% PE and the remainder POPC was compared to that of LUVs containing 15 mol% 5-doxyl PC as a quencher. The ratio of fluorescence intensity with the quencher vs. without the quencher is shown. The smaller the ratio, the more quenching. Data are the mean of three separate experiments +/- SE. Differences between all three measurements were significant, and determined as for Fig. 4. For DPLPE & DOPE, $p < 0.01$. For DPCPE & DVPE, as well as DOPE & DVPE. $0.01 < p < 0.05$.

Chapter 5 Discussion

PKC activation and binding have previously been shown to be affected by the biophysical properties of the membrane. The studies outlined in this thesis were designed to study the role of different activators and inhibitors of PKC, and the mechanisms by which they function, in order to better understand the role of membrane structure in PKC regulation.

Lipophosphoglycan from *Leishmania* is a novel PKC inhibitor. Usually, negatively charged substances are activators of PKC. However, the strong effects of LPG on raising T_H predominate in this case, so that LPG acts to inhibit PKC. (Although steric effects may play a role as well). It is also interesting that it is able to specifically inhibit the membrane bound form of the enzyme. Although all of the PKC added to the assay is able to bind to the membrane, only about 50% of it is active (figure 2, manuscript 2). The finding that it is an apparent competitive inhibitor with respect to the MARCKS peptide (figure 3, manuscript 2) reveals that the active site of PKC is much less accessible in the presence of the inhibitor, suggesting that the conformational change required for activation has not occurred, and that the pseudosubstrate site may still be sitting in the active site. The trypsin cleavage assay (figure 4, manuscript 2) supports this theory as well. The hinge region separating the catalytic from the regulatory domain is much less accessible to proteolysis in the presence of LPG. It was also expected that the hinge region separating the pseudosubstrate domain from the C1 domain would be accessible in

the absence of LPG under those assay conditions since all of the enzyme is membrane bound, and DAG is present. However, nicked PKC was not visible as a separate band from full length PKC. One possibility is that there is no full length PKC in lane 1, but that it is in fact all nicked, while that in lane 2 is full length. Regardless of this, it is evident that there is much more cleavage by trypsin in the absence of LPG, signifying a difference in conformation of the enzyme.

Other inhibitors have been shown to inhibit the membrane-bound form of PKC. However, one of these, cholesterylphosphoryldimethylethanolamine (CPD) is zwitterionic and interacts with the catalytic domain (Epand *et al.*, 1989), unlike LPG. Another inhibitor, cholesteryl-3β-carboxyamidoethylene-trimethylammonium iodide, is a bilayer stabilizer, but it also reduces the substrate availability at the membrane, due to its positive charge (Bruins and Epand 1995a).

LPG is a unique inhibitor, because it can transfer its properties from one monolayer of a bilayer to the other. This is the first example of a PKC inhibitor that is able to accomplish this. Although it has been shown previously that LPG can inhibit PKC (McNeely and Turco, 1987, Descoteaux *et al.*, 1992), both LPG and PKC were present on the same side of the bilayer in the *in vitro* assays. The finding that this inhibitor can function from the opposite monolayer has several interesting implications. First, the effects of LPG must be due at least in part, purely to effects on properties of the membrane itself. Therefore, it can function without actually interacting with the enzyme or coming into any physical contact with it. It rules out the possibility that LPG is sterically hindering the conformational change associated with PKC activation by

blocking access to the substrate binding site, or by not allowing substrate to come into contact with PKC. Second, the stabilization of the membrane cause by LPG on one monolayer can be sensed by the opposing monolayer of a bilayer. Since the only difference between the control vs. inhibited condition is the presence of LPG on the inner monolayer of LUVs, the effect must be due to the LPG and not some other protein in these assays. It is possible that the stabilization of the bilayer caused by LPG has an effect on PKC, since there may be less free energy (bilayer may have less energy stored since it is more stable and further from a transition to the hexagonal phase) that can be transferred to PKC for activation. This finding is novel in a way, since it has been shown previously in viral fusion assays that an additive that inhibits viral fusion on one monolayer, activates it if on the opposite monolayer (reviewed in Chernomordik *et al.*, 1995). However, viral fusion is a complex process that requires changes in lipid organization that are opposite on opposite monolayers. (The outer monolayer needs to change in a way that may accommodate formation of hexagonal phase intermediates, while the inner monolayer must do the opposite). Interestingly, it has previously been shown that LPG is a strong inhibitor of viral fusion, while LPG was on the outer monolayer of LUVs or red blood cell ghosts (Miao *et al.*, 1995). An interesting study looked at the effects of LPG on viral fusion when LPG was added to the inner monolayer of the vesicles (Martin *et al.* 1998). It was found that LPG could transfer its properties to the outer monolayer, so that it inhibited viral fusion even from the inner monolayer, unlike previously studied inhibitors. Since LPG has this property, it will likely be a PKC

inhibitor even if added to the outer monolayer of SUVs, which have a positive monolayer curvature.

In vivo, it is known that PKC and LPG bind to opposite sides of the bilayer. when *Leishmania* invades a macrophage cell. It is believed that the parasite is able to survive in the macrophage cell by overcoming the defense mechanisms of the host. One of these is an oxidative burst that produces toxic oxygen radicals. PKC plays a role in this process (Wilson *et al.*, 1986). It is possible that one of the physiological roles of LPG is to protect *Leishmania* from the oxidative burst by inhibiting PKC. As mentioned, the inhibition of PKC by LPG has been shown *in vivo*. However, it requires that LPG inhibit the enzyme without actually coming into contact with it, since LPG has been shown to coat the surface of macrophage cells (Tolson *et al.*, 1990). The findings of this study (manuscript 2) reveal that this is in fact possible. However, it cannot be ruled out at this time that LPG elicits some signalling mechanism involving PKC and other proteins *in vivo*. Knowing the mechanism by which this inhibition takes place may lead to ways of interfering with it in hopes of controlling the spread of Leishmaniasis.

One problem encountered during these studies was that it was difficult to create liposomes with LPG only on the inside monolayer. Several methods were employed, but did not lead to the desired outcome. BSA was originally used to extract LPG from the outer monolayer. While this was effective at removing the LPG, the BSA itself was found to interfere with the PKC activity assay if present in some samples and absent from others. LUVs were then used to extract the LPG from the outer monolayer of SLVs. However, the problem was that the LUVs couldn't be completely separated from the

SLVs afterwards. Therefore, LUVs were constructed with LPG on both sides, and MLVs were used to extract the LPG. This yielded the desired LUVs with LPG on the inner monolayer, which could be easily separated from MLVs in a eppendorf centrifuge.

Polyethyleneglycol-linked phosphatidylethanolamines have been the focus of many studies, due to their use in drug delivery systems (Kilbanov *et al.*, 1990). Due to their overall similar structure to LPG (polymers with a large hydrophilic head group region and a negative charge), it was interesting to compare the effects of LPG on PKC with those of PEG-linked PEs. Not surprisingly, they were found to be strong inhibitors of PKC, as predicted by their effects on T_H. Like LPG, these compounds were able to inhibit the membrane bound form of the enzyme. Strong inhibition was observed while all of the enzyme added to the assay was bound to the membrane. This inhibition of membrane bound PKC may be a general property of hydrophilic negatively charged polymers. It is possible that the negative charge induces binding of PKC to the membrane through electrostatic interactions (at least in the case of the inhibition that occurs due to LPG on the outer monolayer of LUVs). The C1 domain may not contribute much to this binding if DAG or phorbol esters are not included, so that most of the binding seen is due to the C2 domain. There may be partial removal of the pseudosubstrate domain from the active site, resulting in much less activity than would be expected if binding was through both domains. In the presence of DAG (assays with LPG were done in the presence as well as the absence of DAG), full binding may occur, but due to the stabilization effects of the inhibitor on the membrane, the necessary conformational change may not occur. However, in the case of PEG-linked PE's, it

cannot be ruled out that part of the inhibition of PKC activation seen is due to steric interference from the large PEG polymer, which has been shown to adopt a "brush" conformation at higher concentrations, which may partially cover the surface of the membrane (Kenworthy *et al.*, 1995). However, the fact that all of the enzyme binds in the presence of these inhibitors, suggests that the main mechanism of action is the stabilization of the bilayer.

Some activators of PKC have the opposite effects on the bilayer, and serve as destabilizers. Of the four phospholipids used in the study described in manuscript #1, 1-oleoyl, 2-docosahexaenoyl phosphatidylethanolamine (18:1/22:6 PE) was found to be the strongest activator. The order of activation of the three PEs correlated with their effects on T_H, so that the strongest activator had the largest effect on lowering T_H. However, the effects were not entirely correlated in magnitude, in that there was a difference in the effects of 18:1/18:1 PE with those of 18:0/22:6 PE on T_H, but their effects on PKC activation were not significantly different. Therefore, the general trend that hexagonal phase promoters activate PKC is supported, but the actual magnitude of the activation does not always correlate with the magnitude of the effects of a phospholipid or other compound on T_H. Interestingly, incorporation of a 22:6 chain into the sn-2 position of DAGs was found to activate PKC to a greater extent than incorporation of 20:4 or 20:5 (Marignani *et al.*, 1996). Note that although it is stated in manuscript 1 that the addition of 18:1/22:6 PE had no effect on the PKC, it should be clarified that this is true in the sense that it had no effect in comparison to the POPC which it replaced in the membrane.

However, the effect of a phospholipid on T_H of a membrane is better correlated with its' effect on PKC than other effects, such as acyl chain order or defect formation. One effect of the addition of bilayer destabilizers (H_{II} phase promoting phospholipids) to a bilayer is the increase in order of the acyl chains (Lafleur *et al.*, 1996, also reviewed in Gawrisch and Holte, 1996). This can be caused by the replacement of PC with PE headgroups in a bilayer, which has been shown to increase acyl chain order and favour tighter packing. The opposite effect on acyl chain order is observed when the length of the chains increases, they become unsaturated, or the temperature is increased. The PKC activation observed in manuscript 1 is not correlated with acyl chain order, or fluidity of the membrane, since exchange of PC headgroups for PE which increases acyl chain order activated PKC, while replacing acyl chains with less saturated ones (18:0/22:6 to 18:1/22:6) which decreases order, also activates PKC (manuscript 1). Similar results have been shown previously. For example, cholesterol activates PKC (Slater *et al.*, 1994, Bolen and Sando, 1992) but increases acyl chain order (Yeagle, 1985), while alkanes are also activators, but do not have significant effects on chain order (Pope and Dubro, 1986, Gawrisch and Janz, 1991). Activation of PKC has also been shown not to be a result of simple defect formation, since vesicles with phase boundaries don't activate PKC (Senisterra and Epand, 1993).

One of the physiological consequences of the alteration in phospholipid unsaturation with changes in temperature of the environment is to support the activity of membrane bound enzymes which would normally decrease at lower temperatures. Certain species of bacteria such as *Acholeplasma laidlawii* are known to alter the composition of their

membranes in response to changes in growth conditions such as temperature, and availability of phospholipids from their surroundings. (reviewed in Rilfors *et al.*, 1993). The molar ratio of lamellar to non-lamellar forming lipids is raised in the presence of factors that lower T_H (Weislander *et al.*, 1995). The Na^+/Mg^{2+}-ATPase from this organism is stimulated by H_{II} forming lipids (George *et al.*, 1989). Also, membrane composition is altered so that the hexagonal phase transition temperature of its lipids is kept 20 degrees above the growth temperature in *E. coli* (Rietveld *et al.*, 1993). It is easy to see how such an evolutionary adaptation would provide an advantage to organisms that need to survive in varied environments. It is possible that the rate of PKC (or other enzyme) catalysis is lower overall in the carp living in colder waters. Changing the composition of PE species may compensate for the lower rate. An interesting experiment to test this hypothesis would have been to carry out the PKC assay with the added PE at the lower temperature, to see if the rate of catalysis increases to a similar level as what is observed without the additional PE at 37°C.

Addition of hexagonal phase promoters to a bilayer results in the bilayer becoming stressed, since each monolayer of the bilayer has a tendency to curve due to the cone shaped lipids, but is restrained by the opposing monolayer and the other lipids of cylindrical shape. The bilayer is higher in free energy than one made up entirely of lamellar forming lipids. This is one factor that may contribute to the activation of PKC. It is possible that the extra energy stored in this bilayer can be transferred to PKC upon binding. This can be used by the enzyme to undergo the necessary conformational change for activation (also suggested in Stubbs and Slater, 1996).

It was previously found that there is an optimum curvature strain associated with activation of PKC (Slater *et al.*, 1994). If curvature strain was the only requirement for activation, one would expect an increase in activation in going from the cubic phase to the lamellar phase, since the lamellar phase is higher in curvature strain. However, manuscript 4 showed the opposite. These results fit in well with those of Slater *et al.* (1994).

The composition of the cubic phase membranes was very similar to that of the lamellar phase LUVs used. Only a small change in percentage of PS was used to go from cubic to lamellar phases, so that the lamellar phases at the boundaries between lamellar and cubic compositions would be expected to have the highest level of strain (since they are a few mol% mono-olein [MO] away from being forced into the cubic phase). Therefore, the change from lamellar to cubic which relieves the strain, also strongly activates the membrane bound PKC. Notice that the level of activation per amount of enzyme bound is closest to one with lamellar phases closest to the lamellar/cubic boundaries.

Although the cubic phase study did not use any PE in the lamellar phase vesicles (which would be expected to have a high level of curvature strain), the results obtained with the study of 18:1/22:6 PE can be used for a discussion on the effects of curvature strain in the lamellar phase. When PE was added to the lamellar phase, the increase in activity was correlated with an increase in binding. Therefore, the ratio of activation:binding would be expected to be close to one. Although the study in manuscript 5 found increases in relative activity of the three PEs that were highest for

vesicles with the least amount of enzyme bound, the overall effect was that the PEs all increased the level of PKC that was bound to the membrane relative to vesicles lacking PE. (However, it should be noted that these studies showed a significant increase in binding at low Ca^{2+} concentrations, but not as much of an effect at higher concentrations. which were used in the cubic phase study). Also, when actual values of the curvature strain associated with vesicles made up entirely of either DPLPE, DOPE, and DVPE at 25°C were calculated using the equation $0.5K_c/(R_o)^2$ (manuscript 5), it revealed that the relative activation obtained with each of these PE species did not correlate with the relative curvature strain of each system. The curvature strain of DPLPE membranes was intermediate between those of DOPE and DVPE, while its activation was the lowest. Although these calculations used R_o values at a temperature of 25°C which is above the T_H of DOPE, but below that of DPLPE and DVPE, the relative values of curvature strain were the same when the R_o values at T_H were used (Epand *et al.*, 1996).

Collectively, these studies suggest that curvature strain is not the only requirement for activation of PKC. There must be a role for the curved morphology itself. However, this requirement also reaches a maximum because the increase in curved morphology in going from the cubic to the hexagonal phase (manuscript 4) did not result in an increase in activity of membrane bound PKC. Therefore, the findings of this study suggest a role for both curvature strain and curved morphology. Note however that the hexagonal phase was much less accessible to PKC than was the cubic phase, which may have influenced the level of activation obtained.

Although there is activation of enzymes in Triton-X100 micelles which do not posses negative curvature, in the case of PKC at least, this activation is not as strong (unpublished data). The mechanism of activation of PKC may be different in LUVs compared with mixed micelles (Epand, 1992). Hexagonal phase promoters are able to activate PKC when added to a micelle (Epand, 1992). The curvature of the membranes is different in micelles and LUVs. It is possible that it is the destabilization itself that activates PKC. If a hexagonal phase promoter added to a micelle acts to destabilize it, this may account for the activation seen when hexagonal phase promoters are added to mixed micelles. However, the finding that it is not only curvature strain that activates PKC but that other factors such as a curved morphology and interfacial polarity must be involved (manuscripts 4, 5), ties in well with the observation of activation caused by hexagonal phase promoters in micelles. They may serve to alter the morphology or the interfacial polarity of the micelle, which may play a role.

Physical properties related to curvature strain R_o and K_c, did not correlate directly with PKC activation, as seen in manuscript 5. Instead, a role for interfacial membrane polarity was established, suggesting that the greater access PKC has to the hydrophobic interior of the membrane, the easier it is to become activated. This fits in with other studies that found PKC activation correlated well with headgroup spacing. (Although headgroup spacing is related to curvature stress, it is not the only factor which contributes to it. Headgroup spacing as measured by the fluorescent probe was not perfectly correlated with the calculated curvature strain of the 3 PEs). It has been suggested that T_H is a good predictor of curvature strain of the system (reviewed in Janes, 1996).

However, neither T_H nor curvature strain is correlated perfectly with activation (manuscripts 1, 4, 5).

The finding that PKC activation correlates well with the access it has to the membrane brings up the question of the depth to which PKC actually penetrates the membrane. This topic has been debated somewhat in the literature. It has been suggested that PKC interacts primarily with the phospholipid headgroup region rather than the acyl chains of the membrane, based on evidence that found fluorescence of tryptophan residues on PKC was quenched by probes labeled near the headgroup region. but not at further depths in the membrane (Mosior and Newton, 1995). Along these same lines is the finding that PKC activation is not correlated with the acyl chain order or fluidity of the membrane (Slater *et al.*, 1994, Bolen and Sando, 1992), as may be expected if the enzyme interacted with the acyl chains. However, it has also been suggested that there is a membrane inserted, irreversibly activated form of PKC, which may penetrate into the bilayer, since it is not easily removed from the membrane after becoming bound (Bazzi and Nelsestuen, 1989a, 1989b), and since activation was observed in the absence of Ca^{2+} in the presence of PA (Epand *et al.*, 1992). Regardless of the actual depth of penetration into the bilayer, the fact that a structural rearrangement must occur reveals that headgroup spacing and overall access to the membrane interior must play a role in the activation process. This is because the enzyme must have room to undergo a conformational change while bound to the membrane. The only way this can happen is if the phospholipid headgroups are arranged in such a way so that the enzyme

can rearrange itself without having to become dissociated. This wouldn't be likely if the headgroups were too tightly packed and restricted enzyme movement.

The studies described in manuscripts 1, 4 and 5 are all in agreement with those previously reported, in that a direct relationship between PKC activation and curvature strain was not obtained. In the study by Slater *et al.*, (1994), it was found that the activity of PKC is biphasically dependent on curvature stress. Addition of PE to PS/PC SUVs increased the activation seen, however, progressive addition of unsaturated PC to the PE-containing membranes reduced the activation obtained with the PE itself. PKC activation was measured relative to the curvature stress in these membranes, as measured by C6-NBD-PC fluorescence intensities, or relative to a curvature index calculated from $1/R_o$ values as determined by X-ray diffraction (Slater *et al.*, 1994, Keller *et al.*, 1993). Activation was also plotted relative to acyl chain order as measured by DPH anisotropy. A biphasic relationship between activity and both NBD fluorescence and $1/R_o$ was observed, while no relationship between activity and anisotropy was seen. (Note that R_o values used in this comparison are not a direct indication of curvature strain, since K_c is also a factor). The surprising finding is that if the activation obtained with the 3 PEs in manuscript 5 were plotted against values of curvature strain calculated from R_o and K_c values, a graph with a minimum would be obtained. The difference may be due to the fact that the values used as an indication of curvature strain in the above studies were not exact measures of curvature strain, but of factors related to this term (R_o and headgroup spacing). Although the values of curvature strain listed in manuscript 5 are to describe a bilayer made up entirely of the PE, and therefore are not absolute in magnitude to

describe the LUVs used in the assay, they can serve as relative values for these LUVs, since each LUV contained the same mol% PE, and all other variables were the same in all three cases.

If the values for PKC activation in manuscript 5 were plotted against $1/R_o$ values at 25°C, the result would be a graph which may represent the tail end of a peak (constant activation levels, then a decrease). This is consistent with the shape of the graph obtained in Slater's study (Slater *et al.*, 1994, figure 4b), at $1/R_o$ values of around 0.028 - 0.030 A, corresponding to 50-60 mol% PE.

An interesting finding in that study (Slater *et al.*, 1994), was that the position of the biphasic curve obtained may depend on the type of activator used. The stimulation of PKC activity induced by addition of PE to membranes containing DAG was much more potent than that of PE in the presence of phorbol esters. It has been suggested that DAG and phorbol esters bind to distinct sites on PKC, and that the conformation of active PKC induced by the two may be different. Therefore, each form of active PKC may have its own optimal curvature associated with it. This may explain why addition of PE would have different effects on activation of the two systems (reviewed in Stubbs and Slater, 1996). When the PKC activation levels caused by the three PEs in manuscript 5 were compared with and without the addition of DAG, their relative values were similar. An interesting experiment would have been to compare them in the presence of phorbol esters, to see if their relative activation potencies would have been different.

A recent study looked at the effects of non-lamellar phases on PKC activation, by addition of 25 mol% of each of DAG and unsaturated fatty acids (Goldberg and

Zidovetzki, 1998). It was found that formation of non-lamellar phases in general led to about a 50% reduction in PKC activity. However, the effects on binding were not accounted for, so that the drop in activation may be due mostly to a decrease in binding. so that activation of membrane-bound PKC may still be high.

It has been suggested that the addition of H_{II} forming lipids to the bilayer may cause the sn-2 acyl chain to extend outwards, past the headgroup region to alleviate the curvature stress (Kinnunen, 1996). This results in anchorage of a protein to the membrane, without it actually penetrating the bilayer. It requires the presence of a hydrophobic binding pocket or groove on the protein which forms the binding pocket for the acyl chain so that it is not exposed to a hydrophilic environment. Cytochrome c and annexin V binding have been suggested to occur through this mechanism (reviewed in Kinnunen, 1996).

Changes in the levels of non-lamellar forming lipids *in vivo* can modulate physiological functions. Cubic membranes have been observed, although they are probably only transient in the plasma membrane, (Hyde *et al.*, 1997) since their permanent presence would no longer allow the formation of a barrier between the inside and outside of a cell. Although hexagonal phases on their own are generally not observed in vivo, hexagonal phase promoting lipids are present and have effects on membrane structure. It can be argued that PKC binding and activation can occur in the absence of PE or other H_{II} promoters, and that the mol% of DAG is so low that its effects are due mainly to binding to PKC, rather than an indirect affect on membrane properties. However, it has been shown that PKC activation requires some unsaturation in the

membrane. If assays are done with only saturated lipids, no activity is seen (Bolen and Sando, 1992). Therefore, there is a direct requirement for unsaturation, which can be provided by unsaturated PE's for example. The role of unsaturation in PKC functioning is however, only one of the many roles of unsaturation in membrane function carried out by a variety of lipids.

Hexagonal phases have been observed in the retinal rod outer segment disk membranes from frogs (Fetter and Costello, 1986). The major component of these are ethanolamine plasmalogens (ether link phospholipids) (Greiner *et al.*, 1994), which are hexagonal phase promoters when present in a lamellar phases (reviewed in Lohner, 1996). Some species of bacteria have been shown to increase the level of plasmalogens in their membranes in response to decreases in environmental temperature (Khuller and Goldfine, 1974). They have also been shown to be a major component of membranes that undergo cell fusion, such as synaptic vesicles (Breckenridge *et al.*, 1973). Membrane fusion requires the presence of hexagonal phase promoters, since the outer monolayers of two opposing membranes need to adopt negative curvature. Therefore, H_{II} promoters are essential for processes such as endocytocis, exocytocis, and neurotransmission.

Osmotic stressing agents can be used to monitor the activity of water surrounding a macromolecule. This can lead to important information about the role of water in functioning of that macromolecule. Studies of this sort have been carried out with enzymes, DNA, ion channels and proteins (reviewed in Parsegian *et al.*, 1995). From these studies, it is possible to determine whether water is added or removed during

functioning of the macromolecule. With PKC, it was possible to determine whether water was added or removed along each step of it's reaction pathway, both with cofactor dependent and independent substrates. By using the appropriate assay conditions, it was possible to determine the effect of water on binding of PKC to membranes, binding of substrate to PKC, and the conformational change in the steps going to the enzyme-substrate transition state.

Studies of this sort have largely been done with soluble enzymes. Only one case was reported using an enzyme/protein complex that can function in the presence or absence of membranes (McGee and Teuschler, 1995). The generation of coagulation factor Xa from its precursors can occur either in aqueous medium or in the presence of membranes. In this case, it was found that there were large differences in the number of water molecules that were associated with the reaction in each case. In the presence of membranes, about 800 water molecules were added to the enzyme/protein complex, while in their absence over 5000 water molecules were removed. Studies with hexokinase found that only 65 water molecules were removed from the enzyme surface upon binding glucose. (Rand *et al.*, 1993). Although this is much lower than the number obtained for PKC binding to histone, this is to be expected due to the small size of glucose and the fact that the binding pocket for glucose would also be small if this substrate is to fit well. There would not be much of an interaction surface between the two molecules, resulting in only a small change in the number of water molecules. The site of interaction of PKC with substrate (active site plus other surfaces of interaction between enzyme and substrate) is expected to be much larger, so that the number of water

molecules associated with it would be high. Also, the relatively large conformational change associated with removal of the pseudosubstrate site undoubtedly exposes new hydrophilic areas on PKC which will need to be hydrated (Bruins and Epand 1995b). Also, the actual number of water molecules was calculated based on the assumption that the volume of each molecule is 10A. However, if the water molecules are forming a cage-like structure, so that each is surrounded by some space, the number of molecules calculated in manuscript 3 will be higher than the actual number.

Given what is known about the structure of PKC, and the interactions that occur between the separate domains, it is not surprising that large conformational changes can occur which will result in differences in hydration states of the enzyme at different points during its activation and catalytic cycle.

Although the study on hydration did not focus directly on the effect of membrane properties on PKC activation, the findings still tie in well with those of manuscripts 1, 4 and 5. For example, the fact that there is a hydration step involved in the binding of PKC to the membrane (PKC + H_20 + Lipid -> PKC:Lipid) may help to explain why addition of PE to LUVs increases the percentage of PKC bound to them. Addition of PE will increase the headgroup spacing in the vesicle. Water can penetrate further into the headgroup region (Lis *et al.*, 1982, Epand and Leon 1992, Slater *et al.*, 1993). The increase in hydration of the membrane in this region may make it easier for PKC to bind, shifting the equilibrium to the bound state. The effects of PE and hydration on PKC are related but not identical. It is not possible at this point to precisely define what role each is playing. PE is affecting the properties of the membrane to which PKC must bind. It

may alter the headgroup spacing as well as alter the free energy contained in the membrane, so that PKC can bind. Hydration is required for PKC binding to the membrane. It is likely that water molecules bind directly to the enzyme, possibly as a result of a conformational change resulting in new areas becoming exposed which need to be hydrated. PE may make it easier for water to access the region of the membrane where PKC binds, making it easier for the enzyme to become hydrated.

It is worth mentioning that all assays were performed using LUVs or SLVs rather than mixed micelles, MLVs or SUVs. There are several advantages to the LUV system, including the fact that it is closer to a physiological state than is a mixed micelle. Also, the Triton X-100 can inhibit PKC activity, which would interfere with the assay (Bazzi and Nelsestuen 1987). The DAG, Ca^{2+}, and phospholipid dependencies for PKC activation are similar in both the LUV and mixed micelle assay (Boni, 1992). LUVs are chosen over SUVs and MLVs since SUVs are intrinsically less stable than LUVs due to the strain caused by the formation of a highly curved structure and tend to fuse into MLVs (Epand, 1994). MLVs have limited phospholipid exposure, since any of the inner rings are not accessible to enzyme. Therefore, more lipid will be required for each assay (Epand, 1994). There are however a few drawbacks to using LUVs. Although they are formed by extrusion of MLVs, it is possible that the size of the vesicles is not always uniform, and some vesicles in the sample may be a little larger or smaller than others (Boni, 1992).

Overall, these studies extend previously observed findings that hexagonal phase promoters activate PKC while those which stabilize the bilayer inhibit the enzyme by

providing new concepts about the mechanisms by which enzyme inhibition occurs by studying inhibitors such as LPG, which function in novel ways, by using lipids such as mono-olein, which are not in the lamellar phase, and for the first time, examining the role of water in PKC activation and binding.

Chapter 6 Conclusions

The studies presented in this thesis focus on the roles of membrane structure in regulating the binding and activation of protein kinase C. In summary, membrane structure was found to play an important role in modulation of PKC. Generally, substances which stabilized the bilayer were found to be inhibitors, while those that promoted the formation of non-lamellar phases were activators. Although this general finding had been established previously, these studies extend these ideas by providing new concepts about the mechanisms by which enzyme inhibition occurs, as well as investigating the properties of the membrane which may be responsible for activation by using lipids which are not in the lamellar phase, which has not been studied previously. This is also the first time that the role of water in PKC activation and binding is examined.

18:1/22:6 PE is an activator of PKC, and was more potent than other, less unsaturated PEs. This was a consequence of increasing the enzyme's partitioning to the membrane at low Ca^{2+} concentrations. Although the relative activation seen by each of the PEs correlated with their ability to promote formation of the hexagonal phase in model membranes, the correlation was not one to one. This activation may compensate for any decreases in PKC activity caused by lowered environmental temperatures *in vivo*.

PKC activation caused by three PEs of varying position of acyl chain unsaturation generally correlated with the relative T_H of the PEs, but again, as in the above findings,

this was not a one to one correlation. Also, R_o, K_c, or overall curvature strain did not correlate with activation. However, interfacial membrane polarity did correlate well, suggesting that the depth to which water can penetrate the membrane can serve as an indication of PKC activation in this system.

Related to this, cubic phases with low PS can support a greater level of PKC activity than the lamellar phase of similar composition. This occurs by increasing the activity of the membrane bound enzyme. Hexagonal phases can also support PKC activity, but this activity correlates with an increase in translocation of PKC to the lipid. These findings suggest that curvature strain as well as a curved morphology can activate PKC, and both seem to be important.

LPG is a structurally unique PKC inhibitor, which can inhibit the membrane bound form of the enzyme, even if it is present on the opposite monolayer to which PKC binds, suggesting that the properties imparted on the membrane by LPG can be transferred from one monolayer of a bilayer to the other. It is possible that the mechanism of action of LPG is similar in intact cells. However, this is not known for certain, since other proteins are present in cellular membranes, which are not present in the *in vitro* assays described here. Another protein may mediate a signalling pathway initiated by LPG.

Of related structure to LPG, are polyethyleneglycol-linked phosphatidyl ethanolamines, which also inhibit the membrane bound form of PKC. However. free PEG, when used as an osmotic stressing agent was found to activate PKC. Osmotic stress increases the rate of PKC phosphorylation of histone. PKC becomes hydrated when it binds to membranes, and upon binding histone, and dehydrated in going to the

enzyme-substrate transition state. However, dehydration occurs upon binding protamine, and no change occurs in going to the enzyme-substrate transition state. Therefore, membrane structure as well as hydration play an important role in modulation of PKC.

What is it about the "stressed" bilayer that causes activation of PKC? Membranes containing hexagonal phase promoters, are stressed, and therefore are of higher free energy than those without H_{II} forming lipids. It is possible that PKC uses some of this energy in binding and undergoing the conformational change associated with activation. Binding to membranes in the presence of PS, Ca^{2+} and DAG may be sufficient for the enzyme to undergo the necessary conformational change, but the extra energy supplied by a stressed bilayer could serve to stimulate this process. In combination with this, the curved morphology or interfacial polarity of the membrane also plays a role. Curved morphology refers to the actual shape of the membrane, and not the amount of energy associated with it (how far it is from its' phase transition). Maximal activation may be obtained with the proper combination of these factors. It seems as though a balance between curvature stress and a curved morphology are required for maximal activation of PKC, which may be found in the cubic phase.

Chapter 7 Unresolved Issues and Future Work

Some important issues have not yet been resolved by the manuscripts presented. If time had permitted, it would have been interesting to investigate several aspects. Manuscript 1 shows that one of the functions of alteration in membrane structure due to environmental temperature may be the modulation of membrane bound enzymes. This suggests that the activation level of PKC should be similar at both high and low temperatures, if the corresponding membranes are used. This would have been an interesting theory to test, and would have strengthened the conclusion that was drawn in Manuscript 1.

Another question is the mechanism by which LPG can inhibit PKC from the opposite monolayer. Obviously the stabilization of the inner monolayer of LUVs caused by LPG can be sensed by PKC bound to the outer monolayer. One question is whether this mechanism is the same as that which occurs *in vivo*. It is entirely possible that the effects observed will carry through to living cells. However, it cannot be ruled out that other factors come into play. It is possible that there are other proteins involved which can be part of a signalling mechanism initiated by LPG, acting to inhibit the enzyme. Future studies in whole cells may resolve this issue. Despite the outcome, the finding that PKC can be inhibited by compounds present on the opposite monolayer is novel and important from the biophysical perspective.

An extension of the studies in Manuscript 3 would have been to determine the actual change in the number of water molecules associated with PKC upon binding the substrate protamine, and to compare this to the number required for binding histone to see if large differences exist between the two substrates, since there are differences in the direction of water movement (hydration vs. dehydration of PKC). Differences may reflect the binding of protamine to active PKC while histone binds to inactive PKC. In the future, it may be possible to compare the results found in manuscript 3 with the results obtained from a possible crystal structure of the entire enzyme, if it is ever solved.

The cubic phase study revealed that there are multiple factors related to membrane structure involved in activation of PKC. It would be interesting to continue these studies to determine exactly what factors are involved. Since there seems to be a balance between the effects of curvature strain and an actual curved morphology or interfacial polarity, it would be useful to know exactly how and to what extent each of these factors is involved. This may be attempted by using membranes which differ only in level of curvature strain to compare the rate of PKC catalysis. However, as was seen in manuscript 4, it is difficult to set up a system in which membranes that differ only in curvature strain are used. There are always multiple factors involved, since the actual phospholipid also changes. This task will be difficult to accomplish, but if done, very informative and useful in predicting the effects of certain compounds on PKC activation.

Studying the effects of membrane structure on the binding and activation of PKC which has been mutated could lead to an understanding of what regions of the enzyme are responsible for the effects seen. The studies already carried out in manuscripts 1-5

could be repeated in part using mutants. If a certain mutation abolished the effects already seen in wild type PKC, it could indicate that this region is mediating the effects. In this way, the regions of PKC responsible for certain effects could be determined.

Membrane biophysical structure is important for activation of PKC, and can serve to greatly enhance enzyme activity. An interesting question to answer would be to what extent membrane structure modulates enzyme activation *in vivo* above that brought about by physiological activators such as DAG.

Studies on the regulation of PKC by membrane structure have been in progress for the past decade, and have led to many interesting findings. It is unlikely that the modulation of activation and binding can be described by one variable alone, since mechanisms are complex. Future studies will none the less bring about a greater understanding of the interplay between different properties of the membrane in PKC regulation.

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