The hypothesis that protein kinase C (PKC) activity is sensitive to phospholipid head group interactions was tested using lipid bilayers of defined composition with PKC purified from rat brain. The head group interactions were modulated by varying phosphatidylcholine cis-unsaturation, vesicle curvature, and by the addition of phosphatidylethanolamine and cholesterol. With unilamellar vesicles (including 20 mol % brain phosphatidylserine), increased phosphatidylcholine unsaturation potentiated basal and phorbol ester-stimulated PKC activity. By contrast, in the presence of phosphatidylethanolamine, the activity decreased with increasing phosphatidylcholine unsaturation. Weakening phospholipid head group interactions spaces the head group region and increases interstitial water, and this effect was assessed from its effect on the fluorescence intensity of the phospholipid-labeled fluorophore 1-palmitoyl-2-N-(4-nitrobenzo-2-oxa-1,3-diazole)aminohexanoylphosphatidylcholine (C₄-PC). When the PKC activities with vesicles of varying phosphatidylcholine unsaturation, with and without phosphatidylethanolamine, were plotted as a function of the fluorescence intensity of C₄-PC-labeled vesicles, a biphasic profile was obtained, which had an optimum of intensity, relating to head group spacing, that corresponded to a maximal enzyme activity. A similar biphasic curve was also found when PKC activities were plotted as a function of published bilayer intrinsic curvature x-ray diffraction data, a parameter closely related to head group spacing. By contrast, no simple relationship was evident between PKC activity and 1,6-diphenyl-1,3,5-hexatriene anisotropy, taken as a measure of lipid order or fluidity. Therefore, increasing the level of phosphatidylcholine unsaturation, phosphatidylethanolamine, or cholesterol either potentiates or attenuates PKC activity, dependent on whether the initial condition is above or below its optimum.

Protein kinase C (PKC) is involved in the control of signal transduction (reviewed in Nishizuka (1988), Stabel and Parker (1991), and Blumberg (1991)). Following an increase in intracellular Ca²⁺, the enzyme translocates to the inner surface of the cell membrane, where it interacts with phosphatidylinerine. PKC has a regulatory domain that inserts into the membrane hydrophobic region and interacts with diacylglycerol (DAG), released by hormone receptor-regulated phospholipase C. This leads to a conformational change in the enzyme (Bazzi and Nelsestuen, 1988; Brumfeld and Lester, 1990; Snoek et al., 1988; Bosca and Moran, 1993), which causes a region termed the pseudosubstrate (House and Kemp, 1987) to fold out of the catalytic domain, enabling interaction with a serine- or threonine-containing peptide sequence of an adjacent protein, such as an ion channel or other membrane protein. The ensuing phosphorylation of the protein leads to a modulation of its function.

Evidence is emerging that structural features of the membrane lipid bilayer may play a role in modulating the activity of PKC (reviewed in Bazzi and Nelsestuen (1988), Bell and Burns (1991), and Zidovetzki and Lester (1992)). In particular, lipids that would not ordinarily be expected to interact specifically with the enzyme, such as phosphatidylethanolamine (PE) (Kai-uchibi et al., 1981; Epand and Bottega, 1988; Bazzi et al., 1992; Orr and Newton, 1992a) and cholesterol (Bolen and Sando, 1992), and the level of PC unsaturation (Bolen and Sando, 1992; Senisterra and Epand, 1993) are able to influence activity. Whereas PE tends to show polymorphic behavior and naturally occurring PE alone will not form a bilayer at physiological temperature, there is currently no evidence that non-bilayer structures occur in plasma membranes in vivo. Thus, it would appear that there is a structural feature of the lipid bilayer that modulates PKC activity, although the exact nature of this property remains to be elucidated.

Lipids such as PE and the endogenous activator DAG can be thought of as cone-shaped (Israelachvili et al., 1980), owing to the small head group size. Under appropriate conditions, a monolayer containing a sufficiently high level of such lipids will spontaneously curve, so as to minimize the head group packing energy (Kirk et al., 1984). In the extreme case, the bilayer collapses, leading to the formation of the hexagonal and other non-bilayer phases. However, if the bilayer is unable to curve, because of extrinsic constraints induced by the presence of other phospholipids, such as PC, that prefer the bilayer phase, the system will have an associated potential energy or a propensity to form the hexagonal phase (Kirk et al., 1984; Gruner, 1985; Hui and Sen, 1989). This so-called “propensity” has been equated with the radius of curvature that a bilayer would adopt if unconstrained and is termed “the intrinsic radius of curva-
toyl-2-docosahexaenoylphosphatidylcholine; PE, phosphatidylethanol-
amine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; PS, phosphatidylseri-
ine; SUV, small unilamellar vesicles; TPA, 12-O-tetradecanoylphorbol-13-acetate.
ture" (Kirk et al., 1984; Gruner, 1985). Recently, a correlation between intrinsic curvature and the conductance properties of the ion channel alamethicin has been demonstrated (Keller et al., 1993). If the curvature is not "expressed," there will be a compensatory head group spacing effect in the phospholipid head group region, leading to an increased level of interstitial hydrogen-bonded water (e.g. see Lis et al. (1982), Epand and Leon (1992), and Slater et al. (1993a)). The ease by which an intrinsic membrane protein such as PKC may insert into the membrane could be potentially be influenced by the strength of phospholipid head group interactions, as governed by the propensity to form non-bilayer phases, and of other head group spacing effects such as increased acyl chain unsaturation (e.g. see Demel et al. (1972)) and the presence of cholesterol (Ghosh, 1988). Thus, the finding that under appropriate conditions PE, for example, increases PKC activity, suggests that the enzyme is sensitive to a function of the energetics of phospholipid head group interactions. Further, this apparent correlation between PKC activation and factors that are related to the propensity to form non-bilayer structures is reinforced by the finding that bilayer stabilizing compounds inhibit PKC activity (Epand et al., 1993). Also, alcohols and anesthetics have recently been found to directly inhibit PKC, but with a potency dependent on lipid bilayer properties (Slater et al., 1993b).

Another major determinant of lipid bilayer properties and structure-function relationships in membranes is lipid acyl chain order or fluidity. Thus, the first aim of the present study was to determine if lipid order and/or lipid head group spacing properties were important features regulating PKC activity. The second aim was to determine to what extent the activity of PKC may be modulated by major membrane lipid components, apart from the essential lipid co-factors, DAG and phosphatidylserine. Accordingly, the relation of PKC activity to parameters relating to head group spacing and lipid order/fluidity were explored by varying PC unsaturation, vesicle size, and by the addition of PE and cholesterol. PKC activity was found to be modulated by a structural feature of the lipid bilayer that is closely related to head group spacing. It was found that there was an optimal activity, as a function of head group spacing, suggesting that increased levels of PC unsaturation, PE, and cholesterol in the vicinity of membrane-bound PKC, may either potentiate or attenuate activity, depending on whether the initial condition is above or below this optimum.

EXPERIMENTAL PROCEDURES

Materials—PKC was purified from rat brain as previously described (Kitano et al., 1986) or obtained from Lipidex (Westfield, NJ) purified using the same procedure. Myelin basic protein-peptide (MBP-peptide) was purchased from Life Technologies Inc. [γ-32P]Adenosine 5'-triphosphate (ATP) (3000 Ci/mmol) was from DuPont NEN. Phospholipids were obtained from Avanti Polar Lipids, and TPA, choleralysin, and DAG were from Sigma. DPH was from Molecular Probes (Eugene, OR). All other chemicals used were of analytical grade and were obtained from Fisher Scientific.

The polyunsaturated PC species were handled so as to minimize oxidation. Vesicle preparations were used within a few hours of preparation and kept at 4 °C in the dark, etc. No oxidation products were detected, as monitored by thin layer chromatography, conjugated diene tests, and gas-liquid chromatography analysis of the fatty acyl composition of vesicles after experimental handling, as previously described (Staubbs et al., 1981; Cox et al., 1992).

Preparation of Vesicles—Chloroform solutions of the required lipids were mixed, and the solvent was removed under a stream of nitrogen. The lipids (750 µm) were then co-dispersed to form multilamellar vesicles, by vortexing in 50 mm Tris-HCl buffer, pH 7.4, containing 0.1 mm EGTA. Small unilamellar vesicles (SUV) were prepared by sonication for a total of 4 min, maintained at a temperature of 10 °C (50% maximum power on a Fisher Scientific Sonic Dismembrator model 300, fitted with microtip). Large unilamellar vesicles (LUV) were made by the ecrusson technique, using either a Lipex extruder (Lipex Biomedical industries, Vancouver, British Columbia) as previously described (Hope et al., 1985) or an Avestin Liposofast Extruder (MM Developments, Ottawa, Canada) also as previously described (MacDonald et al., 1997). Both techniques gave similar results.

PKC Assay—PKC activity was determined by measuring the incorporation of 32P from [γ-32P]ATP into a peptide corresponding to the consensus sequence of myelin basic protein, based on a previously described method (Kitano et al., 1986). Briefly, the reaction mixture (75 µl) consisted of 150 µl lipids diluted from 750 µm, 50 µm Tris-HCl, pH 7.4, 15 mm MgCl2, 18 µM ATP, 0.1 mM CaCl2, 50 µM MBP-peptide, and 0.6 µM TPA, where added. The assay was initiated by the addition of PKC (≈0.01 µg), and the mixture was then incubated for 30 min at 30 °C. The reaction was quenched by the addition of 100 µl of 175 µm phosphoric acid, and 100 µl was spotted onto squares of Whatman P81 ion exchange paper. These were then washed (twice) in 75 mM phosphoric acid, and the incorporation of 32P into MBP-peptide was measured by scintillation counting. The linearity of the assay was confirmed, as shown in Fig. 1.

Fluorescence Measurements—The fluorophore-labeled phospholipid, 1-palmitoyl-2-[(4-N-nitrobenzo-2-oxa-1,3-diazol)aminohexanoyl]-PC (CN-NBD-PC), was added (0.25 µg) to preformed lipid vesicles (50 µm) from a stock suspension in buffer. The fluorescence intensity at 530 nm, upon excitation at 470 nm, was measured under conditions that were identical with the PKC assay (see above) using a PFI Alphascan fluorimeter (Photon Technology Instruments, Princeton, NJ). The steady state anisotropy of DPH was determined using an SLM 48000 fluorimeter in the T-format as previously described (Conroy et al., 1986).

RESULTS

The effect of varied cis-PC unsaturation on PKC activity was investigated by using 1-palmitoyl-2-oleylphosphatidylethanolamine (POPC), 1-palmitoyl-2-arachidonoylphosphatidylethanolamine (PAPC), and 1-palmitoyl-2-docosahexaenoylphosphatidylethanolamine (PDPC). These lipids have one, four, and six sn-2 cis-double bonds, respectively, and were selected because they are major naturally occurring PC molecular species as well as being the essential lipid co-factors, DAG and phosphatidylserine (BPS) vesicles (4:1 molar) is established as a previously described (Boni and Rando, 1985). Both basal and TPA-stimulated PKC activity increased in the order POPC, PAPC, and PDPC, showing potentiation of activity with increased unsaturation. An increased activity with PC unsaturation was observed previously with diunsaturated PC (Bolen and Sande, 1992), but not with naturally occurring PC molecular species as used here, where the saturation is confined to the sn-2 chain.

One likely explanation of the ability of increased PC unsaturation to elevate PKC activity is its head group spacing effect (see, for example, Demel et al. (1972)). To investigate this possibility, PE, another major membrane lipid constituent that also has a head group spacing effect, due to its small head group volume compared to PC (see Epand and Leon (1992)), but not with naturally occurring PC molecular species as used here, where the unsaturation is confined to the sn-2 chain.
Effect of Lipid Bilayer on PKC

**Fig. 2.** Effect of varying PC unsaturation on PKC activity, with and without PE. **A,** the effect of increasing PC sn-2-acyl cis-unsaturation from one (POPC), four (PAPC) to six (PDPC) double bonds/PC on PKC activity, with (open circles) and without TPA (filled circles) for BPS/PC vesicles (BPS/PC, 1/4 molar, SUV). **B,** in the presence of PE (BPS/PC/POPE, 1:2:2 molar, SUV). Data are expressed as the mean of triplicate determinations (±S.D.) taken from one of over three replicate experiments. Details are otherwise as described under "Materials and Methods."

PKC activity (nmol ATP min⁻¹ µg prot⁻¹) vs. Number of sn-2 double bonds.

**Fig. 3.** PKC activity has a biphasic relationship with a fluorescence parameter related to head group spacing for varying phospholipid unsaturation, with and without PE, but not with lipid order. **A,** PKC activities (from Fig. 2, A and B) for vesicles were made consisting of POPC/BPS/PC (circles), PAPC/BPS (triangles, point down); PDPC/BPS (squares) (PC/BPS, 4:1 molar, SUV), or POPC/POPE/BPS (triangles, point up); PAPC/POPE/BPS (diamonds); PDPC/POPE/BPS (asterisk) (PC/POPE/BPS 2:2:1 molar). In the presence (solid symbols) or absence of 0.6 µm TPA (hollow symbols), the fluorescence intensities of C₆-NBD-PC (1 mol %, excitation 470 nm, emission 530 nm) (F₀), indicating different levels of hydration that reflect varied head group spacing, are normalized to the value for BPS/PC/POPE vesicles (Fᵢ). The data shown in Fig. 2B indicate that the presence of POPE in BPS/PC/POPE (1:2:2, molar, SUV) resulted in a markedly increased basal and TPA-stimulated PKC activity, compared to vesicles of BPS/POPE alone.

The effects of increasing PC unsaturation in the presence of POPE is shown in Fig. 2B. Firstly, the activity of PKC with POPE in BPS/PC/POPE (1:2:2, molar, SUV) was considerably higher than without POPE. Increasing phospholipid unsaturation progressively lowered the activity (Fig. 2B). This contrasted with the elevated activity seen without PE (Fig. 2A). This apparent "inhibitory" effect of increasing unsaturation in the presence of PE was observed for both basal and stimulated PKC. Increased PC unsaturation potentiated PKC activity, as shown previously with diunsaturated PC (Bolen and Sando, 1992; Senisterra and Epand, 1993); the "inhibition" effect of PE has not previously been reported.

Apart from the head group spacing effect of increased phospholipid unsaturation and PE, both these lipids modulate acyl chain order, another lipid bilayer feature likely to modulate PKC activity. To investigate whether head group spacing or lipid order exerted dominant regulatory effects on PKC activity, three approaches were used. These involved determination of the relationship of PKC activity with head group spacing as probed by the fluorophore C₆-NBD-PC, published x-ray diffraction intrinsic bilayer curvature, and variation of vesicle diameter.

The average location of the NBD fluorophore of C₆-NBD-PC has been previously determined to be at the glycerol backbone region (Chattopadhyay and London, 1988), allowing it to sense the hydration effects induced by an increased head group spacing (Slater et al., 1993a). Since increased water in the excited state solvent cage of the NBD fluorophore leads to a decrease of its excited state lifetime and, hence, fluorescence intensity, this may be used as an indicator of head group spacing. The effect of varying PC unsaturation on the PKC activity, with and without PE, was plotted against the fluorescence intensity of C₆-NBD-PC (Fig. 3A). A biphasic or bell-shaped curve was obtained, revealing an optimum value of the head group spacing for maximal PKC activity. By sharp contrast to the correlation of C₆-NBD-PC fluorescence with PKC activity, no apparent simple correlation between PKC activity and lipid order was found (Fig. 3A).

**In a recent x-ray diffraction study, intrinsic bilayer curvature data were published for DOPC/DOPC mixtures (Keller et al., 1996). PKC activities were therefore determined using the same vesicle composition as used in this study, sufficient PKC activity being attained without added PS. When PKC activity was plotted as a function of fluorescence intensity of C₆-NBD-PC (Fig. 4A), again a biphasic curve was obtained, revealing an optimum value of the head group spacing for maximal PKC activity. When the effect of increasing the mole fraction of DOPE in DOPC SUV on PKC activity was plotted as a function of the intrinsic bilayer curvature, a biphasic relationship was again revealed (Fig. 4B), with an optimum PKC activity expressed at a curvature corresponding to approximately 40% DOPE. The experiment was carried out to 60% DOPE. Beyond 70% DOPE it was not possible to obtain vesicle bilayer suspensions. Lipid order was again assessed from DPH anisotropy measurements on the vesicles, as shown in Fig. 4C. Although PE addition to POPC (also PAPC and PDPC) increased lipid order (Fig. 3B), the lipid order of DOPE was much less sensitive to added DOPE. The reason for this is due to the very different physical properties of the mono- and di-unsaturated phospholipids. For example, DOPE has a much higher gel-liquid crystalline phase transition temperature of -26°C compared to that for DOPE of -16°C (Van Dijck et al., 1976). Also at the assay temperature of 30°C, POPE is well below its bilayer-hexagonal phase transition temperature of 71.4°C (Epand, 1985); by contrast, that for DOPE is 10°C, below the
FIG. 4. PKC activity has a biphasic relationship with a parameter relating to head group spacing, derived from fluorescence or x-ray diffraction data, but not lipid order, shown with DOPE/DOPC mixtures. A, the PKC activities were determined using vesicles (SUV) consisting of DOPC/DOPE mixtures (the proportions of DOPE, left to right, were 0, 10, 20, 30, 40, 50, and 60% DOPE), under the standard conditions as described under “Materials and Methods” (without TPA). PKC activities are expressed as the mean of triplicate determinations (± S.D.) taken from one of the three replicate experiments. The fluorescence intensities of C6-NBD-PC were measured as described in the legend to Fig. 3, measurements were made under the same conditions as used for the PKC assays. A reduced intensity (left to right) is taken to indicate increased hydration or increased head group spacing, the results showing a biphasic relationship with fluorescence (head group spacing). B, the PKC activity plotted as a function of bilayer “intrinsinc curvature” data from published x-ray diffraction data (head group spacing). C, the PKC activity plotted as a function of lipid order. The fluorescence anisotropies of DPH, reflecting acyl chain order (excitation 360 nm, emission 430 nm, mean of triplicate determinations, ± S.D.). Measurements were made under the same conditions as used for the PKC assays. An increased anisotropy (left to right) is taken as a measure of decreased acyl chain fluidity, no simple fluidity/PKC-activity relationship being apparent. Details are otherwise as described under “Materials and Methods.”

Fig. 5. The relationship of PKC activity with vesicle diameter. Vesicles (POPC/BPS, 4:1 molar) of varying size were made using extrusion filters of 50, 100, and 400 nm pore size, except for 25 nm vesicles which were obtained by ultrasonication. Data are means of triplicate determinations (± S.D.) taken from one of three replicate experiments. Details of vesicle preparations and assay details are described under “Materials and Methods.”

In this study, it was found that membrane lipid components that do not interact directly with PKC significantly modified its activity. The data suggest that the spacing or energetics of membrane phospholipid head group interactions govern the ability of the PKC to interact with the membrane, rather than lipid acyl chain order. Three different physical approaches, fluorescence, x-ray diffraction, and variation of vesicle diameter, each yielded parameters which closely relate to head group spacing, the first two showing a narrow optimal value for maximum PKC activity.

A direct consequence of increased head group spacing was an elevated level of hydration in the head group region. In a previous study, hydration was assessed by its effect on the fluorescence intensity of the fluorophore-labeled phospholipid, C6-NBD-PC (Slater et al., 1993a). When PKC activities obtained with increasing PC unsaturation, in the presence and absence of PE, were combined, by using as an abscissa C6-NBD-PC fluorescence, a biphasic curve was obtained, revealing an optimum PKC activity in terms of the physical parameter being sensed by the fluorophore. It possible that, while the NBD fluorophore position has been determined (Chatterjee and London, 1988), it may alter its position in response to varied phospholipid composition. Thus, while lipid order showed no apparent correlation with PKC activity, it was necessary to substantiate the biphasic relationship of head group spacing with activity by another technique. The x-ray diffraction data on DOPC/DOPE mixtures (from Keller et al. (1993)) yield a parameter, the intrinsic bilayer curvature, which is closely related to head group spacing, and, again, the biphasic relationship with PKC activity was revealed. Thus, using different vesicle systems and two different physical approaches to assess

DOPC function of bilayer "intrinsic curvature" data from published x-ray diffraction fluorescence (head group spacing). PKC activities are expressed as the mean of triplicate determinations (± S.D.) taken from one of three replicate experiments. The fluorescence intensities of C6-NBD-PC were measured as described in the legend to Fig. 3, measurements were made under the same conditions as used for the PKC assays. A reduced intensity (left to right) is taken to indicate increased hydration or increased head group spacing, the results showing a biphasic relationship with fluorescence (head group spacing). B, the PKC activity plotted as a function of bilayer “intrinsinc curvature” data from published x-ray diffraction data (head group spacing). C, the PKC activity plotted as a function of lipid order. The fluorescence anisotropies of DPH, reflecting acyl chain order (excitation 360 nm, emission 430 nm, mean of triplicate determinations, ± S.D.). Measurements were made under the same conditions as used for the PKC assays. An increased anisotropy (left to right) is taken as a measure of decreased acyl chain fluidity, no simple fluidity/PKC-activity relationship being apparent. Details are otherwise as described under “Materials and Methods.”

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head group interactions, a biphasic curve was obtained. Altered head group spacing achieved by different vesicle diameters also modulated activity. Thus, the conclusion is that PKC activity is primarily sensitive to changes in head group spacing, rather than lipid order.

The data are therefore consistent with a model in which there is an optimal configuration of the lipid bilayer for the insertion and conformational change of PKC and for the expression of maximal activity. The fact that decreasing vesicle size, which allowed head group spacing to be increased, while keeping vesicle composition constant, also both increased PKC activity and decreased the fluorescence intensity showed that the effects of PE and PC unsaturation were not due to specific interactions. Thus, the addition of PE, altered unsaturation, or vesicle curvature, is considered to shift the value of the head group spacing or energetics of interaction along the abscissa of Figs. 3d or 4, A and B. By moving from a position to the left of the optimum (i.e. by increasing head group spacing), PKC activity increases; after moving past the optimum, this will have the opposite effect of decreasing PKC activity, as observed.

The observation that there is maximal PKC activity expressed over a narrow range of phospholipid head group spacing is supported by recent monolayer data, in which an optimal surface pressure for PKC activity with PE in LUV. Effect of increasing PC unsaturation, from one (POPC), four (PAPC), or six (PDPC) double bonds/PC in BPS/PC/POPE (1:2:2, molar, LUV), with (open circles) and without (filled circles) TPA. Data are means of triplicate determinations (± S.D.). Assay details are described under “Materials and Methods.”
apparent at this DAG concentration. From this, and other studies using x-ray diffraction (Das and Rand, 1984, 1986) or differential scanning calorimetry (Epand and Bottega, 1988), one may infer that in the system as used here, only very marginal if any effects on bilayer destabilization may be expected at this level of DAG.

The observation that PKC expresses an optimal activity, corresponding to a discrete value of head group spacing and bilayer curvature, has important consequences for the regulation of the enzyme in vivo. The narrow range over which activation of the enzyme can be achieved indicates that the enzyme may be highly sensitive to bilayer modifications that lead to changes in head group spacing. Such modifications include, but are not limited to, altered levels of phospholipid unsaturation, PE, and cholesterol, and also the presence of membrane perturbants such as drugs, alcohol, and anesthetics.

REFERENCES

Bell, R. M., and Burns, D. J. (1991) J. Biol. Chem. 266, 4661-4664
Findlay, J. E., and Barton, P. G. (1978) Biochemistry 17, 2400-2405