Effect of Phospholipid Unsaturation on Protein Kinase C Activation†

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ABSTRACT: To examine the hypothesis that physical features of the membrane contribute to protein kinase C activation, phosphatidylcholine/phosphatidylserine/diolein (70:25:5) vesicles of defined acyl chain composition were tested for their ability to activate the enzyme. Maximal activation was found to correlate with the mole percent unsaturation in the system. Unsaturation could be provided by *either* the phosphatidylserine or the phosphatidylcholine component. Vesicles containing 5 mol % diolein but lacking any unsaturation in the phospholipid did not support activity, indicating that acidic head groups alone are not sufficient for activity. The saturated lipid vesicles could be rendered effective but only at very high (25 mol %) concentrations of diolein. The degree of acyl chain unsaturation and the positioning of the double bond had little effect on the activity, suggesting that the effect of the unsaturation is due to some physical property of the lipid rather than to a specific lipid–protein interaction. Addition of cholesterol to both saturated and unsaturated systems indicated that fluidity, as assessed by fluorescence anisotropy, did not correlate with activity. These results suggest that a physical property of the membrane other than fluidity is important for the activation of protein kinase C. A model for protein kinase C activation involving phase separation and/or head group spacing is discussed.

Activation of protein kinase C (PKC)[1] in vitro requires the presence of phospholipid, Ca^{2+}, and diacylglycerol (Nishizuka, 1986), yet the mechanism by which activation occurs is poorly understood. In particular, the requirement for phospholipid has remained obscure. In vesicle systems, acidic phospholipids are essential for activity, with PS being preferred (Takai et al., 1979). For this reason, bovine PS vesicles have been used extensively for assaying PKC activity. Pure PS vesicles, however, are unstable in the presence of high concentrations of cations such as those employed in the kinase assay (Boni & Rando, 1985). Hannun et al. (1985) observed that Triton/PS mixed micelles would support PKC activity. They determined that four PS molecules and one Ca^{2+} were required for PKC activation and proposed a Ca^{2+} bridge model for activation (Hannun et al., 1985, 1986). The effectiveness of the micelle system and the instability of the PS vesicles under PKC assay conditions led many researchers to the conclusion that intimate membrane–protein interactions were not particularly important for the activation of PKC (Rando, 1988).

Recently, Bazzi and Nelsestuen (1990) presented evidence for PS-dependent Ca^{2+} binding to PKC, further supporting the Ca^{2+} bridge model. McLaughlin and co-workers, on the other hand, have shown that peptides corresponding to the pseudosubstrate region of PKC can bind directly to the phospholipid vesicles through a cooperative binding of the basic amino acids of the peptide to the acidic phospholipids (Kim et al., 1991; Mosior & McLaughlin, 1991). This mechanism for PKC–membrane binding does not involve Ca^{2+} but still features a charge interaction at the membrane surface. We have shown, however, that PC micelles, which do not bind Ca^{2+}, can activate PKC in the absence of acidic phospholipids (Walker & Sando, 1988; Walker et al., 1990). Therefore, depending on the physical state of the lipid, charge interactions are not strictly required for activation.

Several other labs have shown that the physical properties of the membrane can affect the level of PKC activation. Snoek et al. (1988) have observed that the higher the degree of polyunsaturation in the PS, the lower the activation. Murakami et al. (1986) observed that PKC could be activated by DOPS but not by DMPS. Boni and Rando (1985) have shown that activation by different vesicle types is dependent on the available surface area such that LUVs > SUVs > MLVs. Epand and co-workers have shown that the ability to induce hexagonal phase tends to correlate with the ability to activate PKC (Epand, 1987; Epand & Bottega, 1988). Recently, they have presented evidence for the regulation of PKC activity through changes in the bilayer to hexagonal phase transition temperature of the membrane (Epand et al., 1991). These studies, taken together, suggest that the membrane plays a greater role in the activation process than merely to provide a charged surface.

The studies described here were designed to examine the importance of the physical state of the membrane in the activation process. PC/PS/DO (70:25:5) vesicles of defined acyl chain composition were tested for their ability to activate PKC. Mixed PC/PS vesicles containing up to 60% PS have previously been shown (Boni & Rando, 1985) to be stable under the conditions of the assay. In addition, Bazzi and Nelsestuen (1987) have shown that maximal binding of PKC to the membrane occurs with PC/PS vesicles containing 25–30% PS. Our studies show that activation of PKC is dependent on the presence of unsaturation in the acyl chains of the phospholipid. This unsaturation may occur in *either* the PS or the PC component of the PS/PC vesicles. The activation does not

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[1] Abbreviations: PKC, protein kinase C; PC, phosphatidylcholine; PS, phosphatidylserine; DOPC, 1,2-dioleoyl-PC; DOPS, 1,2-dioleoyl-PS; DMPC, 1,2-dimyristoyl-PC; DMPS, 1,2-dimyristoyl-PS; DO, 1,2-dioleoyl-*sn*-glycerol; DLPC, 1,2-dilauroyl-PC; POPC, 1-palmitoyl-2-oleoyl-PC; POPS, 1-palmitoyl-2-oleoyl-PS; OPPC, 1-oleoyl-2-palmitoyl-PC; DEPS, 1,2-dielaidoyl-PS; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSPC, 1,2-distearoyl-PC; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; MLV, multilamellar vesicles; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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appear to result from a specific enzyme–fatty acyl chain interaction but rather appears to be related to some physical property of the membrane which is imparted to it through the unsaturation in the acyl chains.

Materials and Methods

Materials. DMPS was from Calbiochem. Diolein and Triton X-100 were from Sigma. All other lipids were from Avanti Polar Lipids (Birmingham, AL). DPH was from Molecular Probes (Eugene, OR). [γ-^{32}P]ATP was obtained from ICN. Frozen rat brains were purchased from Pel Freeze. Lysine-rich histone was from Sigma (Type IIIS). Anti-PKC α and β antibodies were from UBI (Lake Placid, NY). All other chemicals were reagent grade.

Purification of Protein Kinase C. Specific isozymes of protein kinase C were purified from frozen rat brains by sequential chromatography on DEAE-cellulose, phenyl-Sepharose, Q-Sepharose, phenyl-Superose, and hydroxyapatite (Walker et al., 1990; Homan et al., 1991). Purity was verified by polyacrylamide gel electrophoresis followed by silver staining and by Western blotting with isozyme specific antibodies. Purified type II (β) and type III (α) isozymes were brought up to 30% glycerol (v/v) and stored at −70 °C.

Preparation of Lipid Vesicles. All lipids were prepared just prior to assaying. Phospholipids and diolein were mixed together and dried thoroughly under nitrogen. Lipids were resuspended in 20 mM MOPS buffer, pH 7.5, and vortexed extensively. MLVs formed by vortexing the hydrated lipids gave more reproducible results in the kinase assay and were much simpler to prepare than SUVs which gave variable results depending on the extent of sonication and the length of storage. We also attempted to prepare extrusion vesicles of the different lipids but were unsuccessful due to extensive clogging of the extruder filter with some of the lipids used. Therefore, all studies presented here were performed with MLVs. Lipid samples for the fluorescence measurements were prepared as described for the kinase assay except that the lipid was dried under vacuum overnight. The vesicles were then incubated with DPH (in tetrahydrofuran) for 1.5 h at 40 °C. The final molar ratio of probe to lipid was 1 to 500.

Assay of Protein Kinase C Activity. Kinase activity was assessed by the ability of the enzyme to phosphorylate lysine-rich histone in a reaction mixture (75 μL) containing 0.2 mg/mL histone, 40 μM [γ-^{32}P]ATP (1200 cpm/pmol), 5 mM magnesium acetate, 20 mM MOPS buffer, pH 7.5, 67 μM lipid, and approximately 20 nM PKC. Ca^{2+} concentrations were controlled with Ca^{2+}–EGTA buffers. The reaction was terminated after 5 min at 30 °C by spotting 60 μL onto P-81 ion exchange paper (Whatman). Papers were washed three times in 50 mM NaCl to remove unreacted ATP and dried. Bound radioactivity was determined by scintillation counting. Each figure presented is representative of 3–5 independent experiments.

Fluorescence Measurements. DPH fluorescence anisotropy measurements were made on an SLM 8000C spectrophotometer using the T format. Excitation and emission wavelengths were 360 and 430 nm, respectively. Samples (2 mL) contained 250 μM lipid. A program for calculating anisotropy values was available with the SLM software. The anisotropy, r, is calculated from

$$r = 2P/(3 - P)$$

where P is the polarization and is given by

$$P = [(R_{vert}/R_{horiz}) - 1]/[(R_{vert}/R_{horiz}) + 1]$$

where R_{vert} is the ratio A/B when the excitation polarizer is set to the vertical (0°) position and R_{horiz} is the ratio A/B when the excitation polarizer is set to the horizontal (90°) position, with channels A and B being used to acquire the vertical and horizontal components of the emission, respectively. Lipid samples which did not contain DPH were used as blanks in these measurements. The presence of 250 μM Ca^{2+} in the samples had little effect on the observed anisotropies but caused a slight flocculation of the sample; therefore, all measurements were made in the absence of Ca^{2+}.

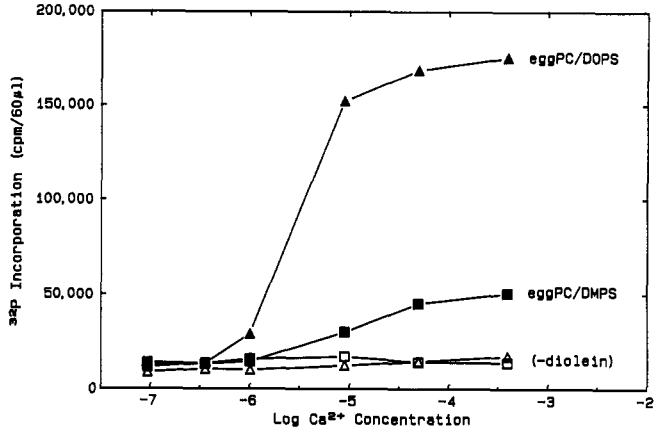

FIGURE 1: Effect of PS acyl chain composition on PKC activity. PKC activity was measured as a function of Ca^{2+} in eggPC/DOPS (▲) and eggPC/DMPS (■) vesicles containing 5 mol % diolein and 25 mol % of the indicated PS. Open symbols indicate the activity in the different vesicles in the absence of diolein.

Results

To examine the effect of the physical state of the membrane on PKC activation, PC/PS/DO vesicles of defined acyl chain composition were tested for their ability to activate the enzyme. MLVs were used in these studies for the reasons stated previously and so that the results of these experiments could be compared with results from other physical studies on the structure of the lipid. SUVs gave slightly higher activity under some conditions, but results were generally less reproducible than those with MLVs. Qualitatively, the results with SUVs were similar to those with MLVs. All vesicles contained 70% PC, 25% PS, and 5% diolein unless otherwise noted. Since the diolein was not varied in most of these studies, the vesicles will be referred to simply by their PC/PS compositions. No differences between PKCβ and PKCα were observed, so only experiments with PKCβ are shown.

PKC activity was measured in PC/PS vesicles containing either DOPS (18:1) or DMPS (14:0) with egg PC (mixed acyl chains) as a function of Ca^{2+} concentration. As shown in Figure 1, the DOPS/egg PC vesicles supported significantly higher activity than did the DMPS/egg PC vesicles. The Ca^{2+} dependence, however, was unaffected by the acyl chain composition. The activity, in these vesicles, was completely dependent on the presence of diolein, even at high Ca^{2+} concentrations. This absolute requirement for diacylglycerol in PC/PS mixed lipid vesicles was also observed by Bazzi and Nelsestuen (1987). Similar diacylglycerol dependence is seen in Triton mixed micelles (Hannun et al., 1985) and short-chain PC micelles (Walker & Sando, 1988) as well.

The requirement for unsaturation was further tested with vesicles of defined PC and PS acyl chain composition (Figure 2). Interestingly, the acyl chain composition of both the PC and the PS lipid components affected the activity. These results suggest that, in addition to the specific requirement for a PS head group, an unsaturated lipid is necessary for PKC activation. This is demonstrated most clearly by the obser-

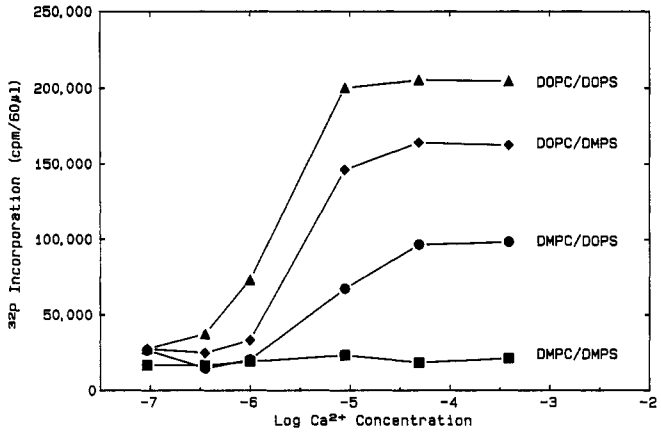

FIGURE 2: Effect of PC and PS acyl chain composition on PKC activity. PKC activity was measured as a function of Ca^{2+} in DOPC/DOPS (▲), DOPC/DMPS (◆), DMPC/DOPS (●), and DMPC/DMPS (■) vesicles containing 5 mol % diolein, 25 mol % of the indicated PS, and 70 mol % of the indicated PC.

Table I: Kinase Activity in Various PC/DMPS Vesicles[a]

PC	PC acyl chain	activity[b]
dioleoyl (DOPC)	18:1 (9-*cis*)	100
dipetroselinoyl	18:1 (6-*cis*)	103 ± 9
dielaidoyl (DEPC)	18:1 (9-*trans*)	54 ± 6
diarachidonoyl	20:4	114 ± 12
dilinolenoyl	18:3	114 ± 14
dierucoyl	22:1	112 ± 9
dinervonoyl	24:1	95 ± 12
dimyristoyl (DMPC)	14:0	16 ± 3

[a] $[Ca^{2+}]$ for these measurements was 217 µM. [b] Activity is given as a percent of maximal activity in DOPC/DMPS.

vation that vesicles containing both a PS head group and 5 mol % diacylglycerol, but lacking unsaturation in the acyl chains, (i.e., DMPC/DMPS) failed to activate PKC.

One interpretation of these results is that a specific activation of the enzyme by the 18:1 acyl chain of the lipid occurs. Activation by unsaturated free fatty acids has, in fact, been demonstrated previously by several others (Shinomura et al., 1991; Fan et al., 1990; Dell & Severson, 1989; Seifert et al., 1988). However, this interaction is not very specific. All of the unsaturated fatty acids that were tested in the previous studies (i.e., 20:4, 18:3, 18:2, and 18:1) were found to activate the enzyme to the same level plus or minus 10% (Fan et al., 1990). This was the case for the phospholipid systems examined here, too. Vesicles containing DMPS and one of the following diacyl chain PC's: 20:4(5,8,11,14-*cis*), 18:3-(9,12,15-*cis*), 18:1(9-*cis*), 18:1(6-*cis*), 18:1(9-*trans*), 22:1-(13-*cis*), or 24:1(15-*cis*) showed little difference in their ability to support PKC activity (Table I). DMPS was chosen for the PS component since this enhanced the dependency of the activity on the unsaturation in the PC. PC/DOPS vesicles gave the same results; the activities were simply shifted up and compressed relative to those in DMPS. The slight increase in activity (15%) with polyunsaturated lipids was not statistically significant. The placement of the double bond in the chain [18:1(9-*cis*) versus 18:1(6-*cis*)] also did not appear to make any difference. The only lipid that was significantly different was the 18:1(9-*trans*) chain PC, which had only 60% of the activity of the others. In addition, the positioning of the unsaturated acyl chain was tested [i.e., POPC (16:0, 18:1) versus OPPC (18:1, 16:0)] and found to have no effect on the observed activity. It is difficult to imagine from these results that a specific interaction between the enzyme and the fatty acyl chain of the lipid is required for activation. Rather it

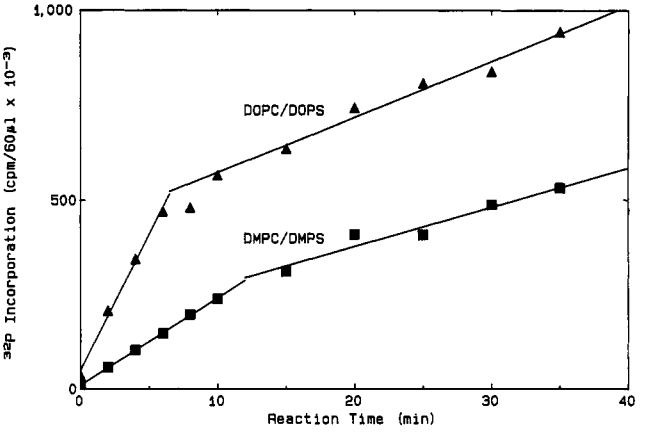

FIGURE 3: Time dependence for activation of PKC in DOPC/DOPS (▲) and DMPC/DMPS (■). Vesicles contained 5 mol % diolein, 25 mol % of the indicated PS, and 70 mol % of the indicated PC, and 217 µM Ca^{2+}.

appears that some other physical property of the membrane which is imparted to the bilayer through the unsaturation in the acyl chains is required for activation.

The most obvious physical difference between the saturated and unsaturated lipid systems is fluidity. The phase transition temperatures for DMPC and DMPS are 24 and 38 °C, respectively, whereas the transition temperatures for DOPC and DOPS are −20 and −11 °C, respectively (Silvius, 1982). Since the activity assay is performed at 30 °C, the DOPC/DOPS system is comfortably above its phase transition while the DMPC/DMPS system is marginally in the liquid-crystalline phase. In addition, lipid and enzyme are incubated briefly at 0 °C before initiation of the reaction and, therefore, the DMPC/DMPS system is in the gel phase rather than the liquid-crystalline phase for this period of time. It is possible that the DMPC/DMPS system requires a longer incubation at 30 °C, in order to compensate for this shift from gel to liquid crystalline, to reach maximum activity. To test this possibility, the activity in DMPC/DMPS and DOPC/DOPS was measured as a function of time, and the results are shown in Figure 3. It is clear from these results that both systems reach a pseudomaximum at approximately the same point in time and then continue in parallel after that point. The DMPC/DMPS system never reaches the activity observed in the DOPC/DOPS system. Another concern with the DMPC/DMPS system is that these vesicles may lack appropriate vesicle structure since the phase transition of the DMPS component is higher than the temperature (22 °C) at which the vesicles are prepared and held with the exception of a brief warming to 40 °C during vortexing. In order to dismiss this concern, DMPC/DMPS and DOPC/DOPS vesicles were prepared, and held, at 40 °C before assaying at 30 °C. No difference in activation was observed between vesicles made at 40 °C and those made at room temperature (data not shown). In addition, electron micrographs showed that both systems did form vesicle structures, although they were not identical in their appearance (data not shown). To further eliminate the concern about the structure of the DMPC/DMPS vesicles, Triton X-100 mixed micelles containing either DMPS or DOPS as the PS component were used to measure the activity as a function of Ca^{2+}. The results from these experiments are shown in Figure 4. Again, the same dependence of activity on unsaturation as seen in the PC/PS vesicles was observed in the micelles. Since both the DMPS- and the DOPS-Triton micelles lack a vesicle structure, the inability of the DMPS-Triton micelles to activate PKC can not be attributed to the lack of vesicle structure.

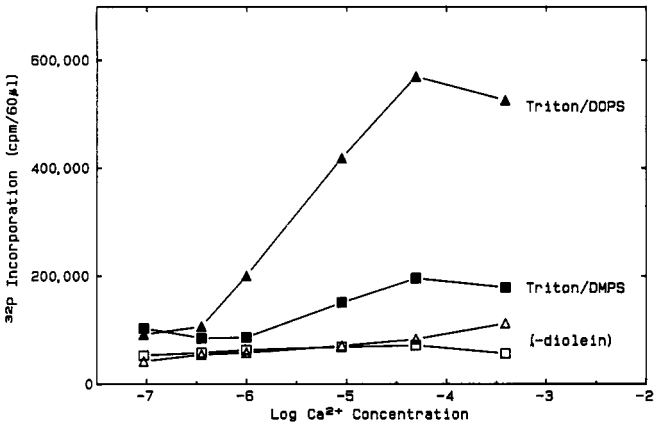

FIGURE 4: Effect of PS acyl chain composition in Triton mixed micelles. PKC activity was measured as a function of Ca^{2+} in Triton/DOPS (▲) and Triton/DMPS (■) mixed micelles containing 2 mol % diolein and 10 mol % of the indicated PS. The concentration of Triton in the samples was 0.1% (w/v). Open symbols indicate activity in the absence of diolein.

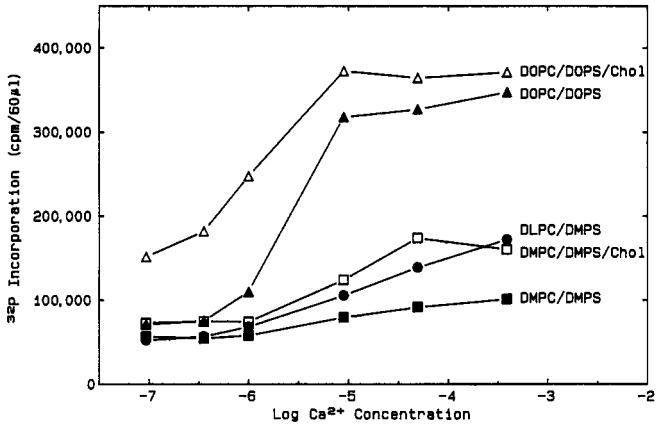

FIGURE 5: Effect of changes in the membrane fluidity on PKC activity. PKC activity was measured as a function of Ca^{2+} in DOPC/DOPS (▲, △), DMPC/DMPS (■, □), and DLPC/DMPS (●) vesicles in the presence (open symbols) and absence (closed symbols) of cholesterol. Vesicles contained 5 mol % diolein, 25 mol % PS, 40 mol % PC, and 30 mol % cholesterol or 5 mol % diolein, 25 mol % PS, and 70 mol % PC in the absence of cholesterol.

The question remained then as to what physical property of the membrane was related to the PKC activation. To test whether a correlation between fluidity and activity existed, the fluidity of the DOPC/DOPS and DMPC/DMPS systems was altered by the addition of 30 mol % cholesterol, and PKC activity was measured as a function of Ca^{2+} concentration. As shown in Figure 5, the addition of cholesterol enhanced rather than decreased the activity observed in both systems. To further test the fluidity hypothesis, a DLPC/DMPS system was tested. DLPC (di-12:0 PC) has a transition temperature of 0 °C; so even though it is saturated, it should be more fluid than DMPC at both the preincubation at 0 °C and the measurement of activity at 30 °C. As is shown in Figure 5, the DLPC/DMPS system supports a slightly, but not significantly, higher activity than the DMPC/DMPS system, again arguing against the fluidity hypothesis. In order to verify that the predicted fluidity changes had been achieved, the anisotropy of the different systems was measured using the fluorescent probe DPH. The anisotropy is related to the viscosity of the membrane such that the higher the anisotropy (theoretical maximum of 0.4), the more rigid the membrane, while the lower the anisotropy, the more fluid the membrane. As shown in Figure 6, the addition of cholesterol to

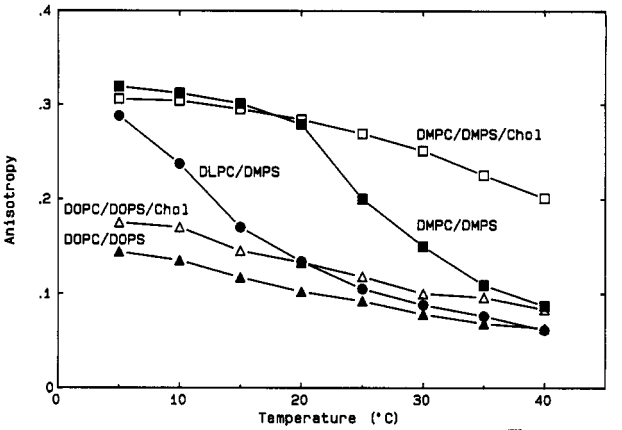

FIGURE 6: DPH anisotropy in various lipid systems. The anisotropy was measured as a function of temperature for DOPC/DOPS (▲, △), DMPC/DMPS (■, □), and DLPC/DMPS (●) vesicles in the presence (open symbols) and absence (closed symbols) of cholesterol. Vesicle compositions were the same as in Figure 5. Samples were 250 µM lipid and had a probe to lipid ratio of 1 to 500.

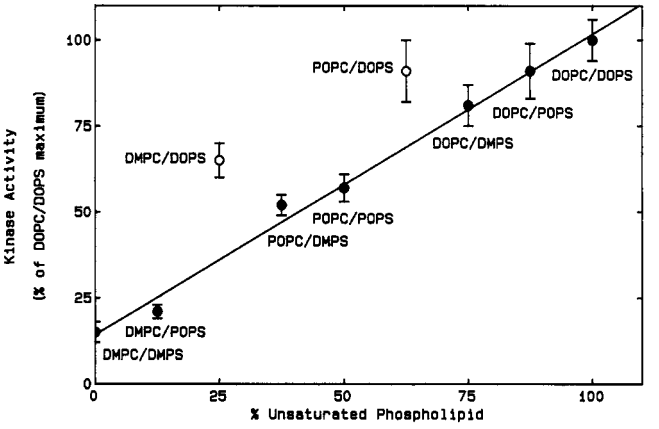

FIGURE 7: Effect of phospholipid acyl chain unsaturation on PKC activity. PKC activity in the different lipid vesicles was plotted as a function of the percent of unsaturated phospholipid in the system. The percent of unsaturated phospholipid was calculated as follows: [74(number of unsaturated acyl chains in the PC component) + 26(number of unsaturated acyl chains in the PS component)]/2. For example, the DMPC/DOPS system has a percent unsaturation of [74(0) + 26(2)]/2 = 26 while the DOPC/DMPS system has a percent unsaturation of [74(2) + 26(0)]/2 = 74. Linear regression of the line defined by the closed symbols gave a correlation coefficient of 0.996. All vesicles contained 5 mol % diolein.

DOPC/DOPS increased the anisotropy, indicating a decrease in fluidity. Addition of cholesterol to DMPC/DMPS likewise increased the anisotropy (at 30 °C), again indicating a decreased fluidity. The DLPC/DMPS system was found to have a lower anisotropy than the DMPC/DMPS system; however, it did not support a significantly increased PKC activity. The DLPC/DMPS system also had a lower anisotropy than the DOPC/DOPS cholesterol system (at 30 °C), whereas it supported significantly less activity than the latter. The inclusion of 5 mM Mg^{2+} and 300 µM Ca^{2+} in the samples did not alter the anisotropy results, with the exception of a small (2 °C) shift in the temperature dependence of the anisotropy in the DMPC/DMPS vesicles (data not shown). Therefore, it is apparent that activity is not related in any simple way to the fluidity of the membrane.

There does, however, appear to be a correlation between the percent of unsaturated phospholipid in the system and the activity of the enzyme. This is shown in Figure 7. PKC activity was assayed in nine different lipid systems. All systems exhibited similar Ca^{2+}-dependent responses but with different

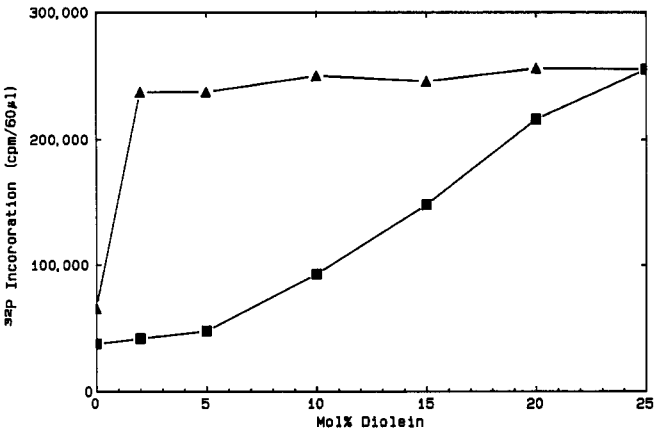

FIGURE 8: Effect of diolein concentration on PKC activity. PKC activity was measured as a function of diolein concentration in DOPC/DOPS (▲) and DMPC/DMPS (■) vesicles containing 25 mol % of the indicated PS. The mole percent PC was adjusted according to the diolein content and ranged between 50 and 75 mol % of the indicated PC. Samples were 217 μM Ca^{2+}.

maximal activities. The maximal activity (as a percentage of the maximum seen in the DOPC/DOPS system) was then plotted as a function of the percentage of unsaturated acyl chain in the system. As shown in Figure 7, there is a linear relationship between the observed activity and the percent of unsaturated phospholipid in the system. Two systems, DMPC/DOPS and POPC/DOPS, yielded activities greater than that predicted based on their mole percent unsaturation. This suggests that the composition of the PS may be more important than that of the PC.

The question still remained as to what physical property of the membrane was important for the activation of PKC. One property imparted to the membrane by unsaturation is increased head group spacing. The addition of diacylglycerol to membranes also has this effect (Cunningham et al., 1989). Since we had previously observed that addition of diacylglycerol to pure DMPS vesicles rendered them effective, whereas pure DOPS vesicles provided substantial activity without diacylglycerol (Walker et al., 1990), attempts were made to achieve activation in the saturated DMPC/DMPS vesicles by increasing the diolein concentration. Figure 8 shows the activation of PKC by DOPC/DOPS and DMPC/DMPS vesicles containing 0–25 mol % diolein. While the unsaturated lipid vesicles require ≤2 mol % diolein to reach full activity, the saturated lipid system reaches full activity only at very high (25 mol %) diolein. This suggests that the activation in the saturated DMPC/DMPS system is due, at least in part, to a structural change in the lipid which is effected by the diolein.

Discussion

The results presented here strongly support the hypothesis that the physical state of the membrane is important for activation of PKC. Activation of PKC in PC/PS/DO (70/25/5) vesicles required the presence of unsaturated phospholipid; the presence of a PS head group alone was not sufficient for activation. This unsaturation could be provided by either the PS or the PC component of the vesicles. Polyunsaturated lipids were not significantly better in activating the enzyme than monounsaturated ones. In addition, the placement of the double bond on the acyl chain had little effect on the activity. A very strong correlation was observed between the PKC activity and the percent of unsaturated phospholipid in the system. These results suggest that some physical property of the membrane is important for the activation of PKC. The most obvious difference between saturated and unsaturated membranes is fluidity; therefore, the hypothesis that membrane fluidity correlated with PKC activity was tested. Activities in both DOPC/DOPS and DMPC/DMPS ± 30 mol % cholesterol and in DLPC/DMPS were compared with the fluidity of the different systems as determined by DPH anisotropy measurements. These experiments showed, however, that activation did not correlate with fluidity.

Many other properties are imparted to the membrane through the unsaturation in the lipid. The Ca^{2+}-bridge model of Hannun and Bell hypothesizes the aggregation of, minimally, four PS lipid molecules for the activation of PKC. In a mixed lipid system then, PS phase separation may be required for activation of PKC. The degree of unsaturation of the membrane may be related to the ability of the PS to phase separate and form appropriate size patches of acidic lipid for activation. A significant volume of literature exists on the phase separation of PS in the presence of Ca^{2+} [see Silvius (1990)]; however, this hypothesis is supported best by the work of Haverstick and Glaser (1987), who demonstrate the movement of NBD-PS in PC vesicles to a single large patch on the surface of large unilamellar vesicles as a result of treatment with Ca^{2+}. The authors observed that the presence of 33 mol % cholesterol had little effect on the ability of the acidic phospholipids to phase separate; however, the Ca^{2+} concentration at which patching occurred was slightly lower. In addition, the size and distribution of the Ca^{2+}-induced domains was found to be dependent on the phospholipid composition. Our results correspond well with theirs in that we also observed a decreased requirement for Ca^{2+} in the presence of cholesterol and a dependence for PKC activation on the phospholipid composition. The composition of the vesicles in the study by Haverstick and Glaser, however, was not well defined; therefore the contribution of the acyl chain composition to the observed phase separations cannot be determined. Recently, Bazzi and Nelsestuen (1991) have presented evidence suggesting that PKC itself can induce lateral phase separations of PS in PC/PS vesicles. As in the previous case, this work was done using phospholipids of mixed acyl chain composition, and therefore the effect of the acyl chains on the phase separation can not be determined.

The relationship between acyl chain composition and the ability of acidic phospholipids to phase separate has not been examined extensively, but two recent papers suggest that the correlation is, in fact, just the reverse of what we observe for the activation of PKC (Casal et al., 1987; Silvius, 1990). Casal et al. report that the affinity of PS for Ca^{2+} is influenced by the acyl chain composition of the lipid such that DMPS > POPS > DOPS. Silvius has found that the "residual solubilities" of the different PS species in brominated PC bilayers in the presence of Ca^{2+} vary with the structure of the PS component in the order DMPS < DEPS < POPS < brain PS < DOPS. This suggests that DOPS is least effective at phase separation while DMPS is the most effective. In addition, phase separation in these systems required millimolar concentrations of Ca^{2+} while activation of PKC required only micromolar concentrations of Ca^{2+}. Silvius also points out that Ca^{2+}-induced lateral segregation of PS in multilamellar PC/PS vesicles is observed only when the vesicles contain at least 30–35 mol % PS. The vesicles in the studies presented here contained only 25 mol % PS; however, they also contained 5 mol % diolein which may lower the percent of PS required for phase separation.

Another physical property of the membrane that is affected by the unsaturation in the acyl chains is the head group spacing. A certain head group spacing might be required for

PKC activation for, e.g., promotion of a membrane insertion event. The packing arrangement of a membrane composed solely of saturated lipids may not provide the minimum spacing required for insertion. A membrane composed of both saturated and unsaturated lipid would have a head group spacing that is dependent on the mole percent unsaturated lipid in the system. This hypothesis may explain why the activity in the PS/Triton micelles was the same as that seen in the PC/PS vesicles in spite of the lack of membrane structure in the micelles. Even within the mixed micelle, the PS lipid might aggregate to form some type of structure that is incapable of supporting kinase activity by virtue of the fact that the head group spacing is inappropriate. This head group spacing model implies that molecules (such as cholesterol) which disrupt acyl chain packing (thereby increasing head group spacing) should enhance activation of the enzyme. This model is supported by the work of Mattai and co-workers, who have examined the interactions of both monovalent and divalent cations with PS's of differing acyl chain unsaturation (Mattai et al., 1989). While they conclude that the binding of Ca^{2+} to the different PS's is relatively insensitive to the acyl chain composition, the molecular area for the different PS's corresponds precisely with the order we observed for PKC activation. In the presence of Ca^{2+}, the molecular areas (Å^2/molecule) for DOPS, POPS, DEPS, and DMPS were 62.5, 56.6, 48.0, and 40.2, respectively. While these values are for pure PS systems, the same order would be expected for the PC/PS lipids as well.

A recent study by Epand et al. (1991) has shown that the addition of branched chain analogs of DSPC to PS vesicles caused inhibition or activation of PKC depending on the ability of the analog to lower the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine. Di(8-methylstearoyl)-PC raised the bilayer to hexagonal phase transition temperature and inhibited PKC activation, while di(8-*n*-butylstearoyl)-PC was found to lower the bilayer to hexagonal phase transition temperature and enhanced PKC activation. The authors suggest that the differing effects of these compounds on the activity results from their alteration of the membrane, rather than from binding to a specific site on the protein, since the two compounds have such similar structures. Both compounds contain a branched side chain in the middle of the acyl chain which would be expected to disrupt the hydrocarbon packing and increase the hydrocarbon volume, as pointed out by Epand and co-workers. However, since DSPC itself raised the bilayer to hexagonal transition temperature (i.e., acts to stabilize the bilayer), the disruption by a methyl group side chain was not great enough to counteract this stabilizing effect. The disruption in the hydrocarbon packing by the butyl group, however, was sufficient to overcome this effect and cause a significant decrease in the bilayer to hexagonal phase transition temperature.

The disruption in hydrocarbon packing by the branched acyl chain results not only in an increase in the hydrocarbon volume but also in an increase in the head group spacing. Likewise, the introduction of a double bond into a saturated acyl chain increases the hydrocarbon volume and increases the head group spacing. Both of these disruptions appear to correlate with increased PKC activity and support the hypothesis that a specific head group spacing is required for activation of PKC. The decreased effectiveness of 9-*trans* as opposed to 9-*cis* unsaturated PC (Table I) is consistent with this hypothesis. The trans unsaturation would provide less disruption of acyl chain packing and thus less head group spacing than the cis isomer. Similarly, the effectiveness of saturated short chain PS micelles (Walker et al., 1990) may be related to the increased head group spacing of the lipid in the micelle with respect to that in a vesicle. This may also explain the slight difference in activities between the SUVs and the MLVs since the head group spacing in the vesicles would be affected by the radius of curvature. This head group spacing model is also supported by the work of Bazzi and Nelsestuen (1988) and Souvignet et al. (1990), who have shown that PKC activity is highly dependent on surface pressure in lipid monolayers.

It is clear from Figures 7 and 8 that unsaturation in phospholipid acyl chains is not the only contributor to activation of PKC. Lipids containing two unsaturated acyl chains in the PS component yielded higher activity than predicted on the basis of the percent of unsaturated phospholipid in the system. The presence of two unsaturated acyl chains in a single molecule would further increase the hydrocarbon volume and consequently the head group spacing. The increased effectiveness of PS as opposed to PC with this structure argues for some specificity of the PS head group. Specificity for PS head groups has been observed in most PKC activating systems and is supported most strongly by the observation of stereoselectivity in PS head group structure (Lee & Bell, 1989). Whatever the nature of the PS head group specificity, it can be bypassed entirely with the use of short chain PC micelle systems (Walker & Sando, 1988).

The effect of diolein on PKC activation was also greater than expected on the basis of its contribution to total acyl chain unsaturation. Saturated DMPC/DMPS vesicles supported full activity in the presence of high concentrations of diolein (25%, Figure 8). While diacylglycerols have been shown to compete with phorbol esters for a specific binding site on PKC (Sharkey et al., 1984), they have also been shown to cause major structural changes in PC bilayers. In particular, at low concentrations they spread apart the polar head groups, whereas at high concentrations they induce hexagonal phases (Cunningham et al., 1989). Das and Rand (1986) suggested that the spreading of the polar groups by diacylglycerol may affect the binding of PKC. Coorssen and Rand (1990) have shown that the presence of cholesterol greatly enhances the perturbing effects of diacylglycerol. Shinomura et al. (1991) have recently demonstrated a synergistic action of unsaturated fatty acids and diacylglycerol in the activation of PKC although, as they comment, the biochemical mechanism of activation by this lipid–protein interaction is still not understood (Shinomura et al., 1991). We would suggest that this mechanism may be due in part to the formation of a specific lipid head group spacing which is required for insertion of the enzyme into the membrane and is a critical event in the activation of PKC.

Acknowledgments

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CORRECTIONS

Assignment of the Natural Abundance ^{13}C Spectrum of Proteins Using ^{13}C ^{1}H-Detected Heteronuclear Multiple-Bond Correlation NMR Spectroscopy: Structural Information and Stereospecific Assignments from Two- and Three-Bond Carbon–Hydrogen Coupling Constants, by Poul Erik Hansen, Volume 30, Number 43, October 29, 1991, pages 10457–10466.

Throughout the paper residues 7, 31, and 49 should be Glu-7, Gln-31, and Glu-49. In Table I of the supplementary material, the resonances should read as follows: 175.4 Asn-43 Cγ, 177.3 Glu-49, 178.6 Gln-31 Cδ, 181.5 Glu-7 Cδ, and 182.1 Glu-49 Cδ.

Mapping the Lipid-Exposed Regions in the *Torpedo californica* Nicotinic Acetylcholine Receptor, by Michael P. Blanton and Jonathan B. Cohen*, Volume 31, Number 15, April 21, 1992, pages 3738–3750.

Page 3742. Due to a printing error, pertinent information is missing in Figure 3. The figure should appear as follows:

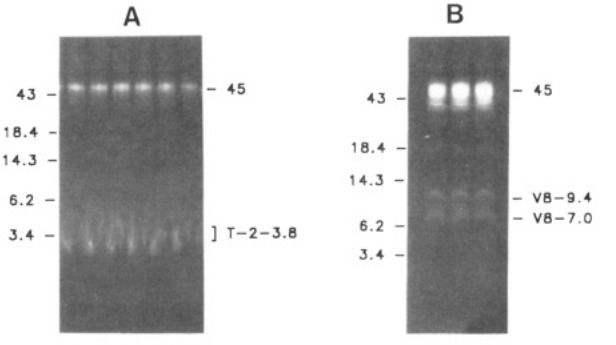

FIGURE 3: Proteolytic mapping of the sites of 1-AP incorporation in the AchR α-V8-20 fragment using trypsin and *S. aureus* V8 protease. The 20-kDa V8 protease fragment (500 μg) of the α-subunit was digested with either trypsin or V8 protease, and the digests were resolved on 16.5% T/6% C Tricine gels as described under Experimental Procedures. The fluorescence of 1-AP-labeled fragments was visualized by irradiation at 365 nm, with the principal bands assigned on the right of each panel for the tryptic digest (A) and for the V8 protease digest (B). Prestained molecular weight standards (BRL) are indicated on the left (see Experimental Procedures).