Inhibition of Phospholipase C-δ_1 Catalytic Activity by Sphingomyelin†

Suzanne Scarlata,*,‡ Rakesh Gupta,§ Pilar Garcia,§ Heather Keach,‡ Shefali Shah,§ Chandraprakash Reddy Kasireddy,|| Robert Bittman,|| and Mario J. Rebecchi*,‡,§

Departments of Anesthesiology and Physiology & Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794, and Department of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, New York 11367-1597

Received May 6, 1996⊗

ABSTRACT: We measured the ability of sphingomyelin (SPM) to inhibit phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] hydrolysis catalyzed by human phospholipase C-δ_1 (PLC-δ_1) in model membranes and detergent phospholipid mixed micelles. SPM strongly inhibited PLC-δ_1 catalytic activity measured in large unilamellar vesicles (LUVs) composed of egg phosphatidylcholine (PC), PI(4,5)P_2, and SPM from brain or egg. At 37 or 45 °C, the rate of PI(4,5)P_2 hydrolysis in PC/SPM/PI(4,5)P_2 vesicles (15:80:5 mol:mol) was less than 25% of that observed in PC/PI(4,5)P_2 vesicles (95:5). By contrast, catalysis was only weakly inhibited by equivalent concentrations of the SPM analog, 3-deoxy-2-*O*-stearoyl-SPM, which lacks hydrogen bond-donating groups at the C-3 and C-2 positions of the sphingolipid backbone. Inhibition by SPM was not observed in detergent/phospholipid mixed micelles. The binding affinity of PLC-δ_1 for vesicles containing PC and PI(4,5)P_2 was slightly diminished by inclusion of SPM in the lipid mixture, but not enough to account for the decreased rate of catalysis. We could find no evidence of specific binding of the enzyme to SPM, which argues against a simple negative allosteric mechanism. To understand the cause of inhibition, the effects of SPM and 3-deoxy-2-*O*-stearoyl-SPM on the bulk properties of the substrate bilayers were examined. Increasing the mole fraction of SPM altered the fluorescence emission spectra of two sets of head group probes, 6-lauronyl(*N,N*-dimethylamino)naphthalene and *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, that are sensitive to water content at the membrane/solution interface. Results obtained with both probes suggested a reduction in hydration with increasing SPM content. Vesicles containing 3-deoxy-2-*O*-stearoyl-SPM produced intermediate changes. Our results are most consistent with a model in which SPM inhibits PLC by increasing interlipid hydrogen bonding and by decreasing membrane hydration; both factors raise the energy barrier for activation of PLC-δ_1 at the membrane/protein microinterface.

Phosphoinositide-specific phospholipase C (PLC)[1] cleaves the phosphodiester bond of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] which links the two intracellular second messenger molecules, inositol 1,4,5-trisphosphate [Ins(1,4,5)-P_3] and diacylglycerol. The PLC isozymes, identified thus far, can be grouped by sequence into three types: β, γ, and δ (Lee & Rhee, 1995; Exton, 1994). Each group appears to be subject to a unique mode of cellular regulation. The β isozymes are controlled by the α or $\beta\gamma$ subunits of heterotrimeric GTP-binding proteins (G-proteins) coupled to receptors possessing seven predicted transmembrane-spanning segments. The PLC-γ isozymes are substrates for receptor tyrosine protein kinases, exemplified by the receptors for platelet-derived and epidermal growth factors. Calcium and/or G-proteins may control the δ isoforms, but pathways linking these enzymes to cell surface receptors are not yet identified.

The properties of the membrane surface exert another level of control on PLC enzymatic activity. Bulk properties of the membrane, such as packing density and degree of hydration, may modulate PLC-catalyzed reactions at various steps, including productive binding to the membrane surface, membrane penetration, and formation of the enzyme/substrate complex. For example, the hydrolysis of PI(4,5)P_2 in phospholipid monolayers catalyzed by all three PLC isoforms can be profoundly inhibited by membrane compression, suggesting that the PLC isozymes, like other lipases, must penetrate the phospholipid surface to access their substrate, undergo a conformational change, or both (Rebecchi et al., 1992a; Boguslavsky et al., 1994; James et al., 1994). These events could underlie the molecular mechanisms by which receptors directly or indirectly activate the PLC isozymes.

Specific properties of the individual lipid components may also affect PLC enzymatic activity. Several early studies demonstrated that the relative surface concentrations of PC,

† This work was supported by National Institutes of Health Grant GM-43422 (M.J.R.), GM-53132 (S.S.), and HL-1660 (R.B.) and the American Heart Association-New York State Affiliate Grant 93-321-GS (M.J.R.).

* To whom correspondence should be addressed: M.J.R. [Telephone: (516) 444-8178. Fax: (516) 444-2907. E-mail: rebecchi@pofvax.sunysb.edu.] and S.S. [Telephone: (516) 444-3071. Fax: (516) 444-3432. E-mail: scarlata@physiology.pnb.sunysb.edu.].

‡ Department of Physiology & Biophysics, SUNY at Stony Brook.

§ Department of Anesthesiology, SUNY at Stony Brook.

|| Queens College of the City University of New York.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; SPM, egg or brain sphingomyelin; egg PC, phosphatidylcholine; Laurdan, 6-lauronyl(*N,N*-dimethylamino)naphthalene; dansyl-PE, *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; 2- and 12-AS, 2- and 12-(9-anthroyloxy)stearic acid; TLC, thin layer chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; LUVs, large unilamellar vesicles.

S0006-2960(96)01072-0 CCC: $12.00

PE, and phosphoinositide critically affected the rate of PI hydrolysis catalyzed by PLC present in brain cytosol (Irvine et al., 1984) or isolated from sheep seminal vesicles (Hofmann & Majerus, 1982; Wilson et al., 1984). More recently, Pawelczyk and Lowenstein (1992, 1993) reported that the catalytic activity of PLC-δ, isolated from rat liver cytosol, is reduced as the concentration of bovine brain SPM is increased in PC/PI(4,5)P_2 vesicles. They suggested that SPM could limit PLC activity by a negative allosteric mechanism or a change in the surface properties of the membrane. The former idea implies that PLC-δ can distinguish SPM from PC. Alternatively, SPM could inhibit PLC by affecting the colligative properties of the membrane.

The unique physical properties of SPM are well-known but not entirely understood. It is known that a bilayer of pure SPM has a higher hydration pressure and a smaller area per phospholipid molecule than PC (Macintosh et al., 1992a,b) and that SPM condenses with cholesterol in membrane bilayers at physiologic temperatures (Lund-Katz et al., 1988). These special properties of SPM have been attributed to its ability to form intra- and intermolecular hydrogen bonds with itself, with other phospholipids, or with cholesterol through the C-3 hydroxyl and C-2 amide of the sphingosine moiety (Boggs, 1987). Although the hydrogen-bonding potential of SPM may influence the hydration and cohesiveness of the region near the polar head groups, the asymmetry and degree of saturation of the ceramide moiety and its ability to align with the planar steroid ring are the most important factors that contribute to the condensation of SPM with cholesterol (Smaby et al., 1994). The predominant interactions involve strong van der Waals attractions between SPM and neighboring cholesterol molecules rather than formation of strong hydrogen bonds (Kan et al., 1991; Grönberg et al., 1991).

In this study, we investigated the mechanism by which SPM inhibits PLC-δ_1 activity. We compared inhibition by brain or egg SPM to that of its analog 3-deoxy-2-*O*-stearoyl-SPM, in which hydrogen replaces the hydroxyl group attached to the C-3 position of sphingosine and an ester linkage replaces the amide at the C-2 position (Bittman et al., 1994). Both natural lipids inhibited PLC-catalyzed hydrolysis of PI(4,5)P_2 in membrane bilayers but not in detergent/phospholipid mixed micelles. 3-Deoxy-2-*O*-stearoyl-SPM and 3-deoxy-2-*N*-stearoyl-SPM were weaker inhibitors than the natural compounds. Because we could find no evidence that SPM binds directly to PLC-δ_1, we considered the possibility that SPM had altered the bulk properties of the membrane. We characterized the lipid free volume, compressibility, and hydration of membranes containing SPM or its analogs using fluorescent probes located in the hydrocarbon and polar head group regions. Our results are most consistent with the hypothesis that SPM inhibits PLC-catalyzed hydrolysis of PI(4,5)P_2 by increasing interlipid hydrogen bonding and by decreasing membrane hydration.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin, gelatin, and SPM of egg origin were purchased from Sigma. SPM from bovine brain, phosphatidylcholine (PC) of egg origin, synthetic phosphatidylserine, and PC (1-palmitoyl, 2-oleyl) were purchased from Avanti (Alabaster, AL). [^{3}H]PI(4,5)P_2 (2−10 Ci/mmol) was from Du Pont-New England Nuclear. Laurdan, dansyl-PE, and 2- and 12-(anthroyloxy)stearic acids were purchased from Molecular Probes, Inc. (Eugene, OR), and used without further purification. PI(4,5)P_2, containing mostly stearic and arachidonic acids esterified at the C-1 and C-2 positions of the glycerol moiety, was isolated from bovine brain Folch fraction I as previously described by Low (1990).

Synthesis of SPM Analogs. The 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-2-*N*-stearoyl-SPM were synthesized and purified according to the procedures described previously (Grönberg et al., 1991; Bittman et al., 1994).

Preparation of Human and Bovine PLC-δ_1. PLC-δ_1 was purified from bovine brain cytosol as previously described (Rebecchi & Rosen, 1987; Rebecchi et al., 1992b). Recombinant human PLC-δ_1 was expressed in *Escherichia coli* and purified as described elsewhere.[2]

Measurements of PLC-δ_1 Activity in Detergent/PI(4,5)P_2 Mixed Micelles. Catalytic activities were measured in reactions with dodecylmaltoside/PI(4,5)P_2 mixed micelles as previously described (Rebecchi & Rosen, 1987; Cifuentes et al., 1993). For the preparation of mixed micelles, [^{3}H]-PI(4,5)P_2 ($4-8 \times 10^{12}$ cpm/mol) and other test lipids in chloroform/methanol (19:1) were dried under a high vacuum for at least 30 min and then solubilized in a solution that consisted of dodecylmaltoside, 100 mM NaCl, and 20 mM HEPES buffer at pH 7.2. The mixtures were flushed with N_2 and bath-sonicated (Bransonic ultrasonic cleaner, Fisher Scientific) for 15 min. Reactions (30 μL volumes) were initiated by the addition of PLC-δ_1 and contained the mixed micelle substrate, dodecylmaltoside/PI(4,5)P_2 (95:5 mol:mol) or dodecylmaltoside/SPM/PI(4,5)P_2 (75:20:5 mol:mol) [[PI-(4,5)P_2] = 60 μM], 0.5−20 ng of PLC-δ_1, 60 μM $CaCl_2$, 100 mM NaCl, 5 mM DTT, 0.1% gelatin, and 20 mM HEPES (pH 7.2) at 37 or 45 °C. The reactions were stopped by the addition of 250 μL of 10% trichloroacetic acid and 25 μL of 20% Triton X-100. The samples were kept on ice, and the precipitates were sedimented at 12000*g* for 30 s in a microfuge. Each supernatant fluid was extracted with 1 mL of chloroform/methanol (2:1), and the upper aqueous phase, containing the [^{3}H]Ins(1,4,5)P_3 product, was transferred to a scintillation vial for counting.

Formation of Large Unilamellar Vesicles (LUVs). The purity of all phospholipid stocks was determined by TLC. The concentrations were determined by phosphate assay and by weighing a small portion of the lipid on a Cahn electrobalance (Rebecchi et al., 1992b). Phospholipids dissolved in chloroform/methanol (19:1, v:v) were transferred to a 50 mL pear-shaped glass flask attached to a rotary evaporator. The flask was immersed in a water bath at 45 °C. The sample was dried rapidly under a high vacuum to form a thin film and then kept under a vacuum for an additional 30 min. The lipid film was rehydrated in buffer containing 100 mM NaCl and 20 mM HEPES at pH 7.2 and 45 °C. The mixture was gently agitated for 5 min and then subjected to four cycles of rapid freezing in liquid N_2 and thawing in a 45 °C water bath. The vesicles were then extruded through a 0.2 μm polycarbonate filter as previously described (Rebecchi et al., 1992b), except that the extrusion

[2] E. Tall, G. Dorman, P. Garcia, S. Shah, S., G. D. Prestwich, and M. J. Rebecchi, submitted for publication.

apparatus was prerinsed with a 45 °C buffer. Vesicles were stored under argon at 45 °C for up to 12 h before use.

Binding of PLC-δ_1 to LUVs. Membrane binding was determined by measuring the amounts of PLC-δ_1 bound to LUVs that contained 0.16 M sucrose. Bound enzyme was separated from free by sedimenting the vesicles in an ultracentrifuge as previously described (Rebecchi et al., 1992b; Cifuentes et al., 1993).

Measurements of PI(4,5)P_2 Hydrolysis in LUVs. Vesicles were prepared as described above, except that [^{3}H]PI(4,5)P_2 ($1-1.5 \times 10^{13}$ cpm/mol) was included. The reaction mixtures contained 60 μM PI(4,5)P_2 (exposed in the outer monolayer) in PC/PI(4,5)P_2 (95:5) LUVs, 0.5 μg of PLC-δ_1, 30 μM $CaCl_2$, 0.1% gelatin, 100 mM NaCl, and 20 mM HEPES at pH 7.2. Reactions were initiated by addition of the enzyme equilibrated in buffers at the reaction temperature. Reactions were stopped at 8 or 15 s by the addition of 250 μL of ice-cold 10% TCA and 25 μL of 20% Triton X-100. Samples were kept on ice, and the resulting precipitates were sedimented at 12000*g*, for 30 s in a microfuge. Each supernatant fluid was extracted with 1 mL of chloroform/methanol (2:1), and the upper aqueous phase, containing the [^{3}H]InsP_3 product, was transferred to a scintillation vial for counting.

Fluorescence Measurements. Fluorescence measurements were taken on an ISS K2 spectrophotometer (ISS, Champaign, IL). Emission spectra were acquired without polarizers at a total lipid concentration of 200 μM with 1 mol % Laurdan or 1 mol % dansyl-PE. Samples were placed in 1 cm quartz cuvettes with stirring in a temperature-controlled chamber. Both probes were excited at 340 nm, and the emission was scanned from 380 to 580 nm. Data were corrected for light scattering and dilution. Anisotropy measurements of 2- and 12-(anthroyloxy)stearic acids were taken at excitation and emission wavelengths of 381 and 460 nm, respectively, and were averages of at least eight measurements per sample. Emission spectra under pressure were taken in a home-built pressure cell with supracil quartz windows [see Paladini and Weber (1983)]. For these experiments, higher lipid concentrations (500 μM) were used. Fluorescence intensities were determined by integrating the area under the emission peak and are expressed in arbitrary units (au). Each spectrum was determined at least twice in independent samples. Time studies were performed to ensure that the signal was stable during the experiment and events such as photo-oxidation had not occurred. We report the shift in the emission energy as the center of spectral mass in kilokaisers (kk where 1 kk = 1000 cm^{-1}) which is a unit of energy. The center of spectral mass (CM) takes into account changes in the skew of the peak and is calculated from the emission energy (EE) and intensity (INT) at each wavelength (λ_i) by $CM = \Sigma_i EE_i INT_i / \Sigma_i INT_i$.

Statistical Analysis. The significance of the differences among treatments was determined by analysis of variance.

RESULTS

Effect of SPM on the Rate of PI(4,5)P_2 Hydrolysis. LUVs were formed from mixtures of egg PC, brain or egg SPM, and [^{3}H]PI(4,5)P_2. The initial rates of PI(4,5)P_2 hydrolysis, catalyzed by recombinant human PLC-δ_1, were measured as a function of the mole fraction of SPM. The bulk concentration of PI(4,5)P_2 and the calcium concentration in these reaction mixtures were well above the levels required to achieve V_{max} (Cifuentes et al., 1993). Studies were conducted at 37 and 45 °C to remain above the 30 °C gel/liquid crystalline phase transition temperature reported for similar mixtures of egg PC and egg SPM (Epand & Epand, 1980). We found that the initial rate of PLC-catalyzed hydrolysis of PI(4,5)P_2 decreased as the mole fraction of egg SPM was increased from 40 to 80 mol % with little change below 40% (Table 1, lines 1−3 and 5−7). Inhibition was essentially unchanged when the temperature was raised from 37 to 45 °C or when SPM from bovine brain was substituted for SPM from egg (lines 10−14). Lowering the concentration of vesicles 10-fold did not affect the inhibition observed (results not shown). Similar data were also obtained for PLC-δ_1 isolated from bovine brain cytosol (results not shown). These studies are in accord with the data reported by Pawelczyk and Lowenstein (1992).

Table 1: Effect of SPM on the Rate of PI(4,5)P_2 Hydrolysis in LUVs Catalyzed by PLC

composition (mol:mol)	*T* (°C)	rate[a] (% of control)	SD	*n*
1 PC/SPM/PI(4,5)P_2 (55:40:5)[b]	37	115	30	7
2 PC/SPM/PI(4,5)P_2 (35:60:5)[b]	37	37[f]	21	7
3 PC/SPM/PI(4,5)P_2 (15:80:5)[b]	37	22[f]	16	7
4 PC/NSPM/PI(4,5)P_2 (15:80:5)[d]	37	79[f]	22	4
5 PC/SPM/PI(4,5)P_2 (55:40:5)[b]	45	108	22	7
6 PC/SPM/PI(4,5)P_2 (35:60:5)[b]	45	38[f]	24	7
7 PC/SPM/PI(4,5)P_2 (15:80:5)[b]	45	20[f]	14	7
8 PC/NSPM/PI(4,5)P_2 (15:80:5)[d]	45	64[f]	16	8
9 PC/OSPM/PI(4,5)P_2 (15:80:5)[e]	45	74[f]	5	5
10 PC/SPM/PI(4,5)P_2 (85:10:5)[c]	45	89	4	2
11 PC/SPM/PI(4,5)P_2 (75:20:5)[c]	45	94	5	3
12 PC/SPM/PI(4,5)P_2 (55:40:5)[c]	45	72[f]	27	10
13 PC/SPM/PI(4,5)P_2 (35:60:5)[c]	45	38[f]	18	7
14 PC/SPM/PI(4,5)P_2 (15:80:5)[c]	45	19[f]	12	9

[a] Expressed as percent of the PI(4,5)P_2 hydrolysis rate in PC/PI(4,5)P_2 (95:5) control vesicles catalyzed by recombinant human PLC-δ_1 determined in each experiment. [b] Egg SPM. [c] Bovine brain SPM. [d] NSPM = 3-deoxy-*N*-stearoyl-SPM. [e] OSPM = 3-deoxy-*O*-stearoyl-SPM. [f] Significance was determined by one-way analysis of variance using Dunnett's method: ($P < 0.05$).

Table 2: Effect of SPM or PC on the Rate of PI(4,5)P_2 Hydrolysis in Dodecylmaltoside/Phospholipid Mixed Micelles

composition (mol:mol)	*T* (°C)	rate (% of control)	SD	*n*
dodecylmaltoside/PI(4,5)P_2 (95:5)	37	100	8	9
dodecylmaltoside:PC/PI(4,5)P_2 (75:20:5)	37	89	12	10
dodecylmaltoside:SPM/PI(4,5)P_2 (75:20:5)	37	105	19	10

The rates of PI(4,5)P_2 hydrolysis were also measured in dodecylmaltoside/PI(4,5)P_2 mixed micelles (Table 2). No inhibition by SPM was observed, although the bulk concentrations of substrate and SPM were comparable to those in the reaction mixtures described in Table 1. Thus, inhibition was observed only when the lipids were in a bilayer state. These results differ from the inhibition of PLC-δ by SPM observed in deoxycholate/PI(4,5)P_2 mixed micelles reported by Pawelczyk and Lowenstein (1992; see Discussion).

To verify that the observed effects of SPM on PLC activity were not simply due to a change in membrane binding, we measured the affinity of PLC-δ_1 for vesicles composed of pure PC and PC/SPM mixtures or PC/SPM (3:2) and 5% PI(4,5)P_2 as previously described (Rebecchi et al., 1992;

Cifuentes et al., 1993). Binding to vesicles composed of PC or PC and SPM was barely detectable at millimolar lipid concentrations, showing that the binding affinity for SPM, like that for PC (Rebecchi et al., 1992), is extremely weak (apparent K_d of $>10^{-3}$ M). Binding to PC/SPM/PI(4,5)P_2 (K_d = 1.7 μM) was weaker than to vesicles composed of PC/PIP$_2$ [K_d = 0.8 μM; this assumes a 1:1 binding to PI(4,5)P_2]. The concentration of PI(4,5)P_2 in the activity assay, however, was more than 20 times the apparent dissociation constant measured in vesicles containing SPM (see Experimental Procedures). Thus, it is unlikely that the extensive inhibition of PLC activity caused by SPM is due to changes in PI(4,5)P_2 binding.

Our results differ significantly from those reported by Pawelczyk and Lowenstein (1993; see Discussion), who found that increasing the mole percent of SPM enhances binding of PLC-δ isolated from rat liver cytosol.

Effects of 3-Deoxy-2-O-stearoyl-SPM and 3-deoxy-N-stearoyl-SPM on the Activity of PLC-δ_1. To test the structural requirements for inhibition, we measured the activity of PLC in membranes containing either 3-deoxy-2-*O*-stearoyl-SPM or 3-deoxy-2-*N*-stearoyl-SPM (Table 1, lines 4, 8, and 9). These analogs differ from SPM by replacement of the hydroxy group at C-3 with a hydrogen and replacement, in 3-deoxy-2-*O*-stearoyl-SPM, of the amide-linked acyl chain at C-2 with an ester linkage.

3-Deoxy-2-*O*-stearoyl-SPM and 3-deoxy-2-*N*-stearoyl-SPM were less effective than brain or egg SPM in reducing the rate of PI(4,5)P_2 hydrolysis in LUVs formed from mixtures of egg PC and PI(4,5)P_2. At 80 mol %, 3−deoxy-2-*O*-stearoyl-SPM only reduced the reaction rate by 26% at 45 °C (Table 1, line 9) compared with an 80% reduction by egg or brain SPM (lines 7 and 14). At 37 and 45 °C, 3-deoxy-*N*-stearoyl-SPM decreased PLC activity by 21 and 36% (Table 1, lines 4 and 8). The reduced potencies of these analogs suggest that the C-3 hydroxyl and the C-2 amide groups of SPM are important determinants of inhibition.

Fluorescence Studies. Because we could find no evidence for specific binding of SPM to PLC-δ_1 and because the levels required to observe inhibition were within the range where changes in bulk lipid properties are possible, we conducted a fluorescence characterization of the membranes used to measure catalytic activity. We first incorporated the fluorescent probe Laurdan into LUVs containing sphingomyelin; Laurdan positions itself in the polar head group region of the membrane surface and is highly sensitive to the polarity of its environment (Weber & Farris, 1979; MacGregor & Weber, 1991). Without SPM, the emission spectrum of Laurdan was composed of two poorly resolved peaks (not shown). Increasing egg SPM to 80 mol % caused a large increase in fluorescence intensity and a shift in the relative population from the lower- to the higher-energy species (Figure 1). 3-Deoxy-2-*O*-stearoyl-SPM and 3-deoxy-2-*N*-stearoyl-SPM caused a less extensive shift in the emission energy of Laurdan. Both egg SPM and bovine brain SPM showed similar behavior (results not shown).

If SPM, which can condense with various lipids [see Smaby et al. (1994)], causes Laurdan to penetrate into the bilayer more deeply, then the effects we observed on emission could be traced to repositioning of the probe. This relocation should make the probe less accessible to aqueous soluble quenchers. This possibility was tested by the addition of the collisional quencher KI. We found that the accessibility of KI to Laurdan did not change significantly when the membrane content of SPM was raised from 0 to 80 mol % (results not shown). This demonstrates that the location of the probe with increasing SPM concentration was not altered.

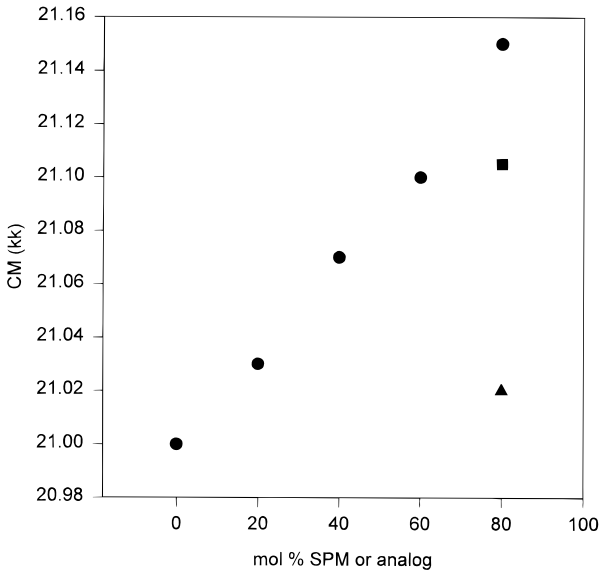

FIGURE 1: Effects of SPM on the fluorescence emission of Laurdan. Fluorescence emission spectra of Laurdan (added to a concentration of 1 mol % of the total lipid) were measured from LUVs composed of 5% PI(4,5)P_2 and either 95% PC, 20−80 mol % egg SPM (●), 80 mol % 3-deoxy-*O*-stearoyl-SPM (▲), or 80 mol % 3-deoxy-*N*-stearoyl-SPM (■). SPM or its analogs replaced PC. Spectra were acquired at 37 °C. Changes in the center of spectral mass (CM, see Experimental Procedures) are expressed in kk or kilokaisers, where 1 kk = 1000 cm^{-1} of Laurdan in the membranes.

Changes in the membrane surface were monitored using a second probe, dansyl-PE. The emission of this probe is efficiently quenched by water [see Ho et al. (1995)]. When dansyl-PE was incorporated into vesicles that contained increasing amounts of SPM, the emission intensity and energy increased (Figure 2). Figure 2 shows the normalized fluorescence intensity of the dansyl-PE emission with increasing SPM or 3-deoxy-2-*O*-stearoyl-SPM. In accord with the effects on Laurdan fluorescence and PLC activity, the SPM analog does not protect the quenching of the dansyl group by water to the same degree as the egg SPM. The decrease in quenching may be correlated to a decrease in head group hydration. Similar behavior was observed at 45 °C (results not shown), which is consistent with the activity measurements (Table 1, compare lines 1−3 to lines 5−7 and line 7 to line 9).

To decide whether the apparent dehydration of the membrane surface is due to condensation of the bilayer, we measured the fluorescence anisotropy of two probes incorporated into the lipid bilayer. The probes, 2-AS and 12-AS, occupy positions close to the lipid head groups and near the center of the bilayer, respectively (Thulborn & Sawyer, 1978). It is well-known that SPM promotes membrane condensation and phase separation in mixtures of PC, SPM, and cholesterol near or below the transition temperatures (Lund-Katz et al., 1988) or when the acyl chain lengths of PC and SPM are quite different (Van Dijck, 1979). Increasing mole fractions of egg SPM in PC vesicles did not significantly change the fluorescence anisotropy of the two probes at the temperatures used in our experiments (results

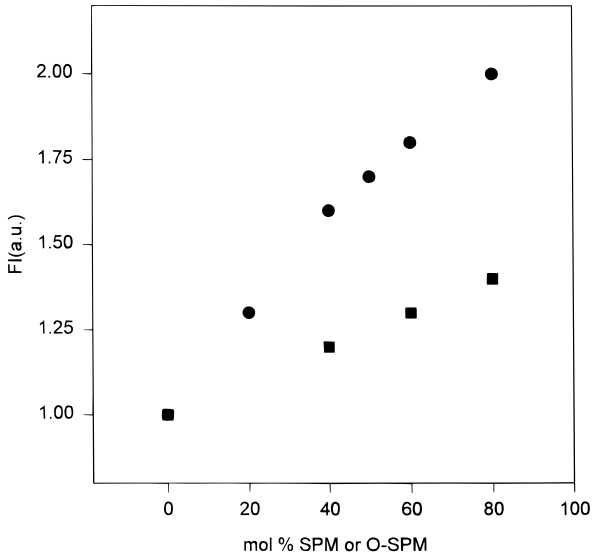

FIGURE 2: Effects of SPM on the fluorescence of dansyl-PE. Fluorescence emission spectra of dansyl-PE (which comprised 1 mol % of the total lipid) were measured from LUVs composed of 5% PI(4,5)P_2 and either 95% PC, 20−80 mol % egg SPM (●), 40−80 mol % 3-deoxy-*O*-stearoyl-SPM (■). SPM or its analog replaced PC. Spectra were acquired at 37 °C. Results are expressed as the relative increase in the fluorescence intensity (FI) of vesicles formed without SPM or its analog. The intensities were determined by integrating the areas under the emission peaks and were normalized to the emission peak area (set to 1.00) of membranes formed from PC/PI(4,5)P_2 (95:5) which contained dansyl-PE.

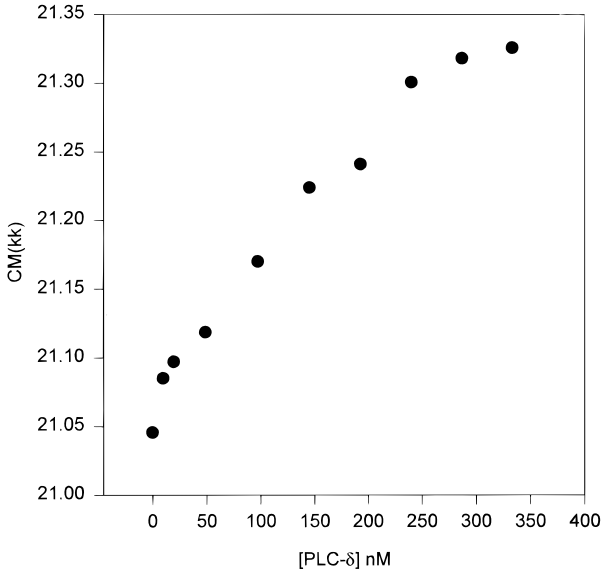

FIGURE 3: Effects of PLC-δ_1 on the fluorescence emission spectrum of Laurdan. Emission spectra of 1% Laurdan incorporated into PC/PS/PI(4,5)P_2 (64:33:2) LUVs were obtained at each increment in the concentration of human recombinant PLC-δ_1. Changes in the center of spectral mass (CM, see Experimental Procedures) are expressed in kk or kilokaisers, where 1 kk = 1000 cm^{-1} of Laurdan in the membranes. To prevent hydrolysis of PI(4,5)P_2, 5 mM EGTA was included in the buffer and the spectra were obtained at 4 °C.[3]

not shown). Since the anisotropy of these probes can be related to the free volume of the lipid (Scarlata, 1991), the lack of change suggests that SPM does not condense the hydrocarbon region of the bilayer at these concentrations. Thus, under our conditions, any condensing effect of SPM must be confined to the membrane surface.

We have previously suggested from monolayer pressure studies that PLC-δ_1 penetrates the membrane surface to hydrolyze its substrate (Rebecchi et al., 1992a; Boguslavsky et al., 1994). Penetration may activate PLC or allow the enzyme to access the substrate or both. Since it is possible that the inhibition of PLC-δ_1 activity observed in bilayers containing SPM or its analog was caused by an increased resistance of the lipid head groups to compression by the enzyme as it penetrates the interface, we measured the compressibility of these surfaces by hydrostatic pressure. In these studies, we followed the fluorescence properties of Laurdan incorporated into vesicles composed of pure PC or PC and 80 mol % egg SPM. If the membrane surfaces had different compressibilities, we would expect that these differences should be reflected in the changes of the membrane surface polarity under pressure. Thus, the emission of Laurdan under pressure should differ in PC vs PC/SPM LUVs. We instead found that the emission of Laurdan in both pure PC and 80% SPM bilayers behaves identically as the pressure is raised from 1 to 1000 atm, suggesting that the surfaces of these membranes have similar compressibilities (results not shown).

If hydration of the membrane surface plays a role in PLC-δ_1-catalyzed PI(4,5)P_2 hydrolysis, then we might predict that the microinterface formed between the enzyme and the membrane surface undergoes some change in water activity that is favorable to catalysis. To test this idea, we measured the effect of increasing amounts of PLC-δ_1 on the fluorescent emission of 1 mol % Laurdan incorporated into PC/PS/PI(4,5)P_2 (64:33:2) membranes (Figure 3). Sufficient PI(4,5)P_2 was present to promote binding of nearly all the added enzyme (Rebecchi et al., 1992b). Increasing the amount of PLC-δ_1 bound to the membranes caused a marked blue shift in the center of spectral mass.[3] We interpret this change to suggest that the membrane region beneath the bound enzyme, the membrane/protein microinterface, is less hydrated.

DISCUSSION

In this study, we investigated the inhibition of PLC-δ_1 by SPM and two SPM analogs that lack key hydrogen-bonding groups. We also explored the hydration and compressibility of these bilayers using fluorescent probes of the polar head group region. Our results are most consistent with the hypothesis that a change in the bulk properties of membranes induced by SPM inhibits PLC-δ_1-catalyzed hydrolysis of PI(4,5)P_2. Three observations support this idea. First, SPM inhibited PLC-δ_1 activity only in bilayer membranes and not detergent/phospholipid mixed micelles. Second, surface concentrations of SPM greater than 40 mol % were required for significant inhibition of PLC-δ_1 activity. Third, inhibition by the SPM analogs correlated well with changes in bulk membrane properties.

We did not observe inhibition of PLC by SPM in dodecylmaltoside/PI(4,5)P_2 mixed micelles. This contrasts with the report of Pawelczyk and Lowenstein (1992), who found that SPM added to deoxycholate/PI(4,5)P_2 mixed micelles inhibits PLC-δ isolated from rat liver cytoplasm. A possible explanation for this discrepancy may relate to the considerable differences in the physical properties of the

[3] It was necessary to perform these experiments at 4 °C to prevent the hydrolysis of PI(4,5)P_2 by PLC. Equivalent experiments were not performed with SPM because of phase separation at this low temperature.

detergent/phospholipid mixed micelles used in the two studies. Deoxycholate, a negatively charged detergent, forms small micelles (~14 detergent molecules/micelle) and is only effective as a phospholipid diluent in the PLC assay over a very narrow concentration range (0.075−0.125%) (Fukui et al., 1988). By contrast, dodecylmaltoside has no charge, is an effective diluent over a very wide concentration range (0.008 to >1.000%), and forms large, well-mixed micelles (~100 detergent molecules/micelle) that exchange their lipid content more rapidly than the enzyme's k_{cat} (Cifuentes et al., 1993).

We could find no evidence that PLC-δ_1 binds specifically to surfaces enriched in SPM. Thus, the proposition that PLC-δ_1 is inhibited by SPM through an allosteric mechanism that involves high-affinity binding of this lipid to the protein appears to be untenable.[4] Our results differ from those of Pawelczyk and Lowenstein (1993), who reported that increasing the mole percent of SPM in LUVs composed of PE and PC (9:1) increased binding of PLC-δ isolated from rat liver cytosol. Two important differences in methodology that may account for this discrepancy are LUV composition and temperature. The major lipid component in their membranes was the hexagonal phase forming lipid PE, whereas we used PC, which forms stable bilayers (Gruner, 1992). In addition, our LUVs were formed at 45 °C, which is 15 °C above the phase transition temperature of the egg SPM/PC mixtures (Epand & Epand, 1980); in our experiments, the lipid samples were always well above this critical temperature. By contrast, the previous studies were conducted at 22 °C, which is well below the transition temperatures of similar bilayers containing egg or brain SPM. It is quite possible that this lower temperature leads to phase separation of SPM from PE and PI(4,5)P_2 or other lipids and the formation of a highly heterogeneous mixture of aggregates for which an accurate assignment of binding constants would not be possible.

The effects of SPM on the bulk properties of the membranes used here may not be related to its well-known propensity to condense the membrane hydrocarbon region. Under our conditions, SPM did not cause significant condensation of the lipids as measured with the fluorescent probes 2-AS and 12-AS; their anisotropy, a measure related to the relative free volume of the lipid, was no different in membranes containing SPM and membranes containing only PC and PI(4,5)P_2.

The effects of SPM on PLC seem unrelated to the nature of the hydrocarbon chains of the SPM used, since brain SPM, which contains approximately 58% stearic acid and 15% nervonic acid, inhibits PLC activity to the same extent as egg SPM, which contains 78% palmitic acid and only 2% nervonate (from the manufacturer's analysis). The absence of any relationship between the character of the hydrocarbon region and suppression of PLC activity provides additional evidence that changes in the membrane bilayer caused by SPM are localized to the polar head group region under our conditions.

Experimentally, the physical changes in PC membranes associated with incorporation of SPM were investigated using fluorescent probes located at the membrane/solution interface. The emission energy of the first probe, Laurdan, is sensitive to the polarity or dielectric constant of its environment (Weber & Farris, 1979; MacGregor & Weber, 1981). This probe showed a decrease in the polarity of the membrane surface with increasing SPM. The emission intensity of the second probe, dansyl-PE, is sensitive to water in its immediate environment [see Ho et al. (1995)]. This probe reported an apparent decrease in the water content of the membrane surface with increasing SPM. Taken together, these studies suggest that SPM reduces hydration of the membrane surface. Our interpretation is consistent with the known properties of SPM. Pure SPM bilayers have a lower hydration pressure (i.e., the pressure required to remove water from between two bilayer surfaces) than PC bilayers (McIntosh et al., 1992b). Thus, the hydration of PC/SPM/PI(4,5)P_2 bilayers should be lower than those composed of PC/PI(4,5)P_2.

Inclusion of SPM in the membrane may perturb the polar head group region through its ability to self-associate, associate with water, or associate with other lipids through hydrogen-bonding networks (Boggs, 1987). Although such a network is clearly unrelated to the condensing effects of SPM on the membrane hydrocarbon (Kan et al., 1991; Grönberg et al., 1991; Smaby et al., 1994), the binding and penetration of interfacial enzymes, such as PLC, into the polar head group region, is likely to be affected. Thus, both interlipid hydrogen bonding and the reduced activity of water at the interface could contribute to the inhibition of PLC that we observed. The forces involved are weak compared with the energies involved in stabilizing membrane bilayers in aqueous solutions; they need not be large, however, if activation of PLC at the membrane surface only involves the displacement of a few polar head groups by the enzyme (Rebecchi et al., 1992a).

We propose a simple idea that can account for the effects of membrane compression and SPM on PLC enzymatic activity and the apparent dehydration of a membrane/PLC microinterface (Figure 3). On the basis of previous work, including X-ray crystallographic studies, PLC-δ_1 is clearly tethered to the membrane surface through an extensive network of hydrogen bonds formed between the PI(4,5)P_2 polar head group and the PH domain of this isozyme; binding promotes the weaker interactions of the catalytic domain with the membrane surface. In PC/PI(4,5)P_2 membranes, these interactions may be driven by displacement of bound water and could involve the insinuation of amino acid side chains of the catalytic, and possibly the C-2 domain, among the lipid polar head groups; in some respects, this is analogous to a model proposed by Jain and Vaz to explain the relationships between binding, enzymatic activity, and water at the membrane/PLA_2 microinterface (Jain & Vaz, 1987). Displacement of water and penetration of the polar head group region may be sufficient to activate PLC-δ_1, to allow the enzyme to access substrate, or both. In the presence of SPM, however, interlipid hydrogen-bonding networks must be disrupted before sufficient penetration can take place. Additionally, the reduction in water bound to the interface diminishes the entropy gain due to dehydration. Weakening of the interlipid hydrogen-bonding networks by removal of the C-3 OH group and C-2 amide bond of SPM reduces considerably the barrier to membrane penetration as does the increment in hydration. Therefore, PLC operates more efficiently on membrane surfaces where *O*- or *N*-deoxy-SPM

[4] We cannot eliminate the possibility that under special circumstances, such as when calcium ions are bound and PI(4,5)P_2 tethers the enzyme at the membrane surface, SPM binds to PLC and inhibits enzymatic activity through a negative allosteric mechanism.

analogs replace SPM. The apparent dehydration of the membrane/PLC microinterface may be associated with an activated conformation of the enzyme similar to the E to E* transition observed when bacterial PLC (Volwerk et al., 1994) or PLC-δ_1 (unpublished observation) binds to phospholipid dispersions. This model is consistent with the recently reported three-dimensional structures of PLC-δ_1 (Essen et al., 1996) and PLC from *Bacillus cereus* (Heinz et al., 1995) in which the regions surrounding the catalytic site contain hydrophobic residues that could penetrate the polar head group region, forming a microinterface that would exclude a significant quantity of bound water.

REFERENCES

Bittman, R., Kasireddy, R., Mattjus, P., & Slotte, J. P. (1994) *Biochemistry 33*, 11776−11781.

Boggs, J. M. (1987) *Biochim. Biophys. Acta 906*, 353−404.

Boguslavsky, V., Rebecchi, M., Morris, A., Jhon, D.-Y., Rhee, S.-G., & McLaughlin, S. (1994) *Biochemistry 33*, 3032−3037.

Cifuentes, M., Honkanen, L., & Rebecchi, M. (1993) *J. Biol. Chem. 268*, 11586−11593.

Epand, R. M., & Epand, R. F. (1980) *Chem. Phys. Lipids 27*, 139−150.

Essen, L.-O., Perisic, O., Cheung, R., Katan, M., & Williams, R. L. (1996) *Nature 380*, 595−602.

Exton, J. H. (1994) *Annu. Rev. Physiol. 56*, 349−369.

Fukui, T., Lutz, R. J., & Lowenstein, J. M. (1988) *J. Biol. Chem. 263*, 17730−17737.

Grönberg, L., Ruan, Z.-S., Bittman, R., & Slotte, J. P. (1991) *Biochemistry 30*, 10746−10754.

Gruner, S. M. (1992) Nonlamellar Lipid Phases, in *The Structure of Biological Membranes* (Yeagle, P., Ed.) pp 211−250, CRC Press, Boca Raton, FL.

Heinz, D. W., Ryan, M., Bullock, T. L., & Griffith, O. H. (1995) *EMBO J. 14*, 3855−3863.

Ho, C., Simon, S. J., & Stubbs, C. (1995) *Biochemistry 34*, 6188−6195.

Hofmann, S. L., & Majerus, P. W. (1982) *J. Biol. Chem. 257*, 14359−14364.

Irvine, R. F., Letcher, A. J., & Dawson, R. M. C. (1984) *Biochem. J. 218*, 177−185.

Jain, M., & Vaz, W. (1987) *Biochim. Biophys. Acta 905*, 1−8.

James, S. R., Demel, R. A., & Downes, C. P. (1994) *Biochem. J. 298*, 499−506.

Kan, C.-C., Ruan, Z.-S., & Bittman, R. (1991) *Biochemistry 30*, 7759−7766.

Lee, S. B., & Rhee, S. G. (1995) *Curr. Opin. Cell Biol. 7*, 183−189.

Low, M. (1990) in *Methods in Inositide Research* (Irvine, R. F., Ed.) pp 145−151, Raven Press, Ltd., New York.

Lund-Katz, S., Laboda, H. M., McLean, L. R., & Phillipis, M. C. (1988) *Biochemistry 27*, 3416−3423.

MacGregor, R., & Weber, G. (1981) *Ann. N. Y. Acad. Sci. 366*, 140−149.

McIntosh, T., Simon, S., Needham, D., & Huang, C.-H. (1992a) *Biochemistry 31*, 2012−2020.

McIntosh, T., Simon, S., Needham, D., & Huang, C.-H. (1992b) *Biochemistry 31*, 2020−2024.

Paladini, A., & Weber, G. (1981) *Rev. Sci. Instrum. 52*, 419−429.

Pawelczyk, T., & Lowenstein, J. (1992) *Arch. Biochem. Biophys. 297*, 328−333.

Pawelczyk, T., & Lowenstein, J. (1993) *Biochem. J. 291*, 693−696.

Rebecchi, M., & Rosen, O. (1987) *J. Biol. Chem. 262*, 12526−12532.

Rebecchi, M. J., Boguslavsky, V., Boguslavsky, L., & McLaughlin, S. (1992a) *Biochemistry 31*, 12748−12753.

Rebecchi, M. J., Peterson, A., & McLaughlin, S. (1992b) *Biochemistry 31*, 12742−12747.

Rhee, S.-G., & Choi, K. (1992) *J. Biol. Chem. 267*, 12393−12396.

Scarlata, S. (1991) *Biophys. J. 60*, 334−340.

Smaby, J., Brockman, H., & Brown, R. (1994) *Biochemistry 33*, 9135−9142.

Thulborn, K., & Sawyer, W. (1978) *Biochim. Biophys. Acta 511*, 125−140.

Van Dijck, P. W. M. (1979) *Biochim. Biophys. Acta 555*, 89−101.

Weber, G., & Farris, F. (1979) *Biochemistry 18*, 3075−3081.

Wilson, D. B., Brown, T. E., Hofmann, S. L., & Majerus, P. W. (1984) *J. Biol. Chem. 259*, 11718−11724.

BI961072Z