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Sphingosylphosphorylcholine as a novel calmodulin inhibitor

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Abbreviations used: ANS, 8-anilinonaphtalene-1-sulfonic acid; CaM, calmodulin; CD, circular dichroism; CMC, critical micelle concentration; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LT-SPC, L-threo-sphingosylphosphorylcholine; mant-cGMP, 2'-O-(N-methylanthraniloyle)guanosine-3',5'-cyclic monophosphate; PC, phosphatidylcholine; PDE, phosphodiesterase; PE, phosphatidylethanolamine; PNPP, p-nitrophenyl phosphate; PS, phosphatidylserine; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; SPR, surface plasmon resonance

SYNOPSIS

Sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) have been recently recognized as important mediators of cell signaling, regulating basic cellular processes like growth, differentiation, apoptosis, motility, and Ca^{2+} homeostasis. Interestingly, they can act as first and second messengers as well. While their activation of cell surface G protein-coupled receptors is intensively studied, not much is known about their intracellular mechanism of action, their target proteins are yet to be identified. We hypothesized that these sphingolipids might bind to calmodulin (CaM), the ubiquitous intracellular Ca^{2+} sensor. Binding assays utilizing intrinsic tyrosine fluorescence of the protein, dansyl-labeled CaM, and surface plasmon resonance revealed that SPC binds to both apo and Ca^{2+} -saturated CaM selectively, when compared to the related lysophospholipid mediators S1P, lysophosphatidic acid (LPA), and lysophosphatidylcholine (LPC). Experiments carried out with the model CaM-binding domain melittin showed that SPC dissociates the CaM – target peptide complex, suggesting an inhibitory role. The functional effect of the interaction was examined on two target enzymes, phosphodiesterase and calcineurin, and SPC inhibited the Ca^{2+} -CaM-dependent activity of both. Thus, we propose that CaM might be an intracellular receptor for SPC, and raise the possibility for a novel endogenous regulation of CaM.

INTRODUCTION

Sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) have recently been shown to be important modulators of basic cellular processes like growth, differentiation, apoptosis, motility, and Ca^{2+} homeostasis, by activating numerous signal transduction pathways [1, 2]. Most interestingly, the same molecules can act as first messengers activating specific cell surface G protein-coupled receptors exerting diverse effects, or can act as second messengers as well, mediating mitogenic and antiapoptotic responses or Ca^{2+} mobilization from internal stores in a yet unknown manner. While the former is extensively studied, not much is known about the latter, the intracellular target proteins mediating the second messenger functions of S1P and SPC are yet to be identified.

Compared to S1P, limited information is available regarding the metabolism and the mechanism of action of SPC. Extracellularly applied SPC has been shown to modulate cellular processes like growth [3], migration [4], adhesion [5], cytoskeleton rearrangement [6], and Ca^{2+} signaling [7, 8]. SPC is a natural constituent of blood, having a possible physiological role in regulating cardiac function [9]. Furthermore, SPC as a putative second messenger can release Ca^{2+} from internal stores [10, 11]. This effect was shown to require micromolar lipid concentrations, and to be promoted by both the naturally occurring D-erythro and the synthetic L-threo stereoisomer, in contrast to its stereospecific action on G protein-coupled receptors [12-14]. SPC is capable of activating ryanodin receptors [15, 16], however, the exact mechanism of intracellular Ca^{2+} release is still unknown. Though stimulated production of SPC has not yet been demonstrated, the average concentration of the lipid in cardiac muscle has been estimated to be approximately $15.6 \mu\text{M}$ [16]. Hence, local concentrations can be high enough for SPC to act as a real second messenger.

In this report we seek for an intracellular target protein of S1P and/or SPC. We hypothesized that these sphingolipids might interact with calmodulin (CaM) based on the following data: *i*) CaM binds to its target proteins and to its aromatic inhibitors through hydrophobic interactions [17], *ii*) sphingosine has already been shown to be an inhibitor of several CaM-dependent enzymes [18-20], *iii*) S1P and SPC can mobilize Ca^{2+} from internal stores [10, 11], and *iv*) sphingosine kinase binds to and is regulated by CaM [21, 22]. CaM is the ubiquitous intracellular Ca^{2+} sensor of eukaryotic cells [23]. This small (148 amino acids) dumbbell-shaped protein comprises of four α -helical Ca^{2+} -binding EF hand motives and a short flexible linker. CaM regulates the activity of a great number of proteins, including kinases, phosphatases, and ion channels, in a variety of ways. In the most common model, as

a result of Ca^{2+} binding, CaM undergoes a conformational change which leads to the exposure of hydrophobic patches, allowing the protein to bind to basic amphipatic helices on target enzymes, which leads to their activation by release of autoinhibition.

In the present study we examine whether S1P and SPC, and the related signaling glycerolipids lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC), interact with CaM. Binding assays utilizing fluorescence of intrinsic tyrosine residues as well as of dansyl-labeled protein and surface plasmon resonance measurements revealed that SPC binds to both apo and Ca^{2+} -saturated CaM specifically in a concentration dependent manner. SPC disrupted the interaction between CaM and the model CaM-binding domain melittin, suggesting an inhibitory role. The functional effect of the interaction was explored in a Ca^{2+} -CaM-dependent phosphodiesterase and calcineurin assay, and SPC inhibited the Ca^{2+} -CaM-dependent activity of both enzymes. Therefore, we suggest that CaM is an intracellular receptor for the lipid mediator SPC.

EXPERIMENTAL

Preparation of CaM from bovine brain

CaM was purified from bovine brain using isoelectric precipitation and phenyl-sepharose (Amersham Biosciences, 17-0973-05) affinity chromatography according to Gopalakrishna and Anderson [24]. Protein purity was checked by SDS-PAGE, and CaM concentration was determined by circular dichroism spectroscopy (molar residual ellipticity of 158,000 deg cm² at 222 nm [25]).

Preparation of lipid solutions

S1P (860492), LPA (857130), and LPC (845875) were purchased from Avanti Polar Lipids, D-erythro-SPC (1318) and L-threo-SPC (1319) from Matreya. 10 mM stock solutions were prepared in methanol and a small amount of HCl was added in case of S1P to increase solubility. Before each experiment, lipids were added from stock solutions to the appropriate assay buffer (usually 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM EGTA or CaCl₂), and a homogenous solution was created by sonication.

For certain SPR experiments SPC was incorporated into liposomes, prepared by drying the necessary lipids into glass vials, and resuspending in the assay buffer by vigorous sonication and vortexing. The freshly prepared liposomes were pelleted by centrifugation at 5000×g for 5 min to remove unincorporated lipids, then reconstituted in the assay buffer. For liposome preparation the following lipids were purchased from Avanti Polar Lipids: brain total lipid extract (131101), phosphatidylcholine (PC, 840051) phosphatylethanolamine (PE, 841118), phosphatidylserine (PS, 840032), sphingomyelin (SM, 860061), and cholesterol (700000). Host liposome compositions were as follows: 1) 50 mol% brain total lipid extract, and 50 mol% PC, representing the outer leaflet of the plasmamembrane, 2) 50 mol% brain total lipid extract, 25 mol% PE, and 25 mol% PS, representing the inner leaflet of the plasmamembrane, 3) 50 mol% brain total lipid extract, 25 mol% SM, and 25 mol% cholesterol, representing lipid rafts. SPC was incorporated into host liposomes at ratios of 5, 10, and 20 mol%. Total lipid concentration was 1 mM.

Intrinsic tyrosine fluorescence

Spectra were collected using a Jobin Yvon Fluoromax-3 spectrofluorimeter at 25°C in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM EGTA or CaCl₂. Bandwidths were adjusted to 5 nm, Tyr was excited at 274 nm, emission was monitored from 285 to 370 nm, values were

read at 303 nm. Protein and lipid concentrations were 1 μ M and 100 μ M, respectively. Buffer containing lipids was measured as a blank. After the addition of CaM, the spectrum was scanned again and corrected by subtracting the blank.

Determination of the critical micelle concentration

The critical micelle concentration (CMC) of lipids were measured using the fluorescent hydrophobic probe 8-anilinonaphtalene-1-sulfonic acid (ANS, Fluka, 10417) [26] on a Jobin Yvon Fluoromax-3 spectrofluorimeter at 25°C. ANS was excited at 388 nm, emission was monitored from 400 to 600 nm, the excitation slit was adjusted to 2 nm and the emission slit to 5 nm. The fluorophore concentration in the solution was 5 μ M. Lipids were solubilized as for all other experiments in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM EGTA or CaCl₂. Blank values were subtracted from the fluorescence spectra and the maximum intensities were read. Two straight lines reflecting the aqueous and the micellar environments were traced, and the CMC was defined as the concentration referring to their point of intersection.

Fluorescence of dansyl-labeled CaM

CaM was dansylated by reacting 1 mg of protein with 5 fold molar excess of dansyl-chloride (Fluka, 39220) in 1 ml 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM CaCl₂ for 2 hours at room temperature. Unbound fluorophores were removed by dialysis. Measurement of absorbance at 340 nm (molar extinction coefficient of 3400 M⁻¹cm⁻¹ [27]) gave an incorporation of ~1.1 dye units per CaM molecule. Fluorescence measurements were performed on a Jobin Yvon Fluoromax-3 spectrofluorimeter at 25°C in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM EGTA or CaCl₂. Bandwidths were set to 5 nm, dansyl was excited at 340 nm, emission was monitored from 400 to 600 nm. Protein and lipid concentrations were 0.2 μ M and 100 μ M, respectively. A matching buffer scan (blank) was subtracted from each spectrum, and the maximum intensities were read. Steady state fluorescence anisotropy measurements were conducted similarly, only bandwidths were set to 10 nm and emission was monitored at 510 nm. The fluorescence intensities at vertically and horizontally aligned polarizers and analyzers were recorded. The blank I_{VV} , I_{VH} , I_{HH} , and I_{HV} values were subtracted from the CaM containing values, and anisotropy was calculated from the following equation:

$$\text{Anisotropy} = (I_{VV} - G I_{VH}) / (I_{VV} - 2G I_{VH}), \text{ where } G = I_{HV}/I_{HH}$$

Surface plasmon resonance (SPR)

SPR measurements were performed using a BIACORE X instrument (Biacore, Uppsala, Sweden) at 25°C. CaM was immobilized onto the surface of the carboxymethylated dextran chip (CM5) using standard amine-coupling chemistry in 10 mM acetate buffer, pH 3.8, 1 mM CaCl₂, according to the manufacturer's instructions. The mean amount of immobilized protein was 4000 RU (resonance units). S1P, LPA, LPC, LT-SPC (100 µM), SPC at varying concentrations (10, 20, 30, 50, 100 µM), and liposomes of different compositions (see Preparation of lipid solutions), dissolved in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM CaCl₂, were passed over the chip surface at a flow rate of 10 µl/min. The results were analysed by BIAevaluation 4.1 software (Biacore). The corresponding association and dissociation parts of sensorgrams were fitted to a Langmuir binding model and the equilibrium dissociation constant, K_D , was calculated as the ratio of dissociation to association rate constants.

Circular dichroism spectroscopy

Far-UV CD spectra were recorded with a JASCO J-720 spectropolarimeter using a 1 mm quartz cuvette at 25°C in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM EGTA or CaCl₂. The bandwidth was set to 2 nm. CaM and SPC concentrations were 10 µM and 200 µM, respectively.

Melittin binding assay

Melittin from honey bee venom was purchased from Sigma (M2272). Experiments with dansyl-labeled CaM were performed as described in the previous section. Dansyl-CaM, melittin, and lipid concentrations were 0.2, 0.4 µM, and 100 µM, respectively.

In the complimentary set of experiments, the Trp fluorescence of melittin was monitored using a Jobin Yvon Fluoromax-3 spectrofluorimeter at 25°C in 10 mM HEPES, pH 7.4, 100 mM KCl, and 1 mM CaCl₂. Bandwidths were adjusted to 5 nm, Trp was excited at 280 nm, and spectra were recorded from 300 to 400 nm. Melittin, CaM and lipid concentrations were 0.2, 0.4 and 100 µM, respectively. Emission of the buffer containing lipids and CaM as well, was subtracted from each spectrum.

Phosphodiesterase activity assay

Phosphodiesterase (PDE) activity was measured fluorimetrically by following the cleavage of mant-cGMP as described by Johnson *et al.* [28]. Phosphodiesterase I, 3',5'-cyclic-nucleotide-specific from bovine brain was purchased from Sigma (9529) and mant-cGMP from Calbiochem (370668). The reaction was followed on a Jobin Yvon Fluoromax-3 spectrofluorimeter at 25°C in 10 mM MOPS, pH 7.0, 90 mM KCl, 5 mM MgCl₂, and 1 mM CaCl₂. Mant-cGMP, PDE, CaM and lipid concentrations were 10 µM, 5 nM, 50 nM, and 100 µM, respectively [25]. Mant-cGMP was excited at 280 nm and emission was monitored at 450 nm. The excitation slit was adjusted to 2 nm and the emission slit to 5 nm. Mant-cGMP in the lipid solution was monitored for 5 minutes to check mant-cGMP emission stability and the possible impact of the lipids on the fluorescence of the substrate. After adding 5 nM PDE, the basal activity of the enzyme was monitored for 5 minutes. Finally, 50 nM CaM was added, and the CaM-dependent activity was measured. CaM-dependent and independent activities were calculated as the initial velocities of the phosphodiesterase reaction after correction with blank values.

Calcineurin activity assay

Calcineurin (PPase-2B) was purchased from Promega (V6361), and the assay was carried out based on the protocol of the company. Dephosphorylation of the substrate p-nitrophenyl phosphate (PNPP, Sigma, 1040) was followed by monitoring the increase in absorbance at 405 nm on a Wallac Victor microplate reader. Lipids (100 µM in screening experiments and 50 µM SPC in the CaM dose response experiment) were solubilized in 50 mM Tris-HCl, pH 7.4, 0.5 mg/ml BSA, 20 mM PNPP, and 1 mM NiCl₂. This reaction mixture was divided into 4 separate wells on a 96 well plate and either calcineurin (0.001 unit/µl) or CaM (10 µg/ml), neither or both were added. The plate was incubated at 30°C, and the absorbance was read at 405 nm every 2 minutes for 20 minutes. Blank values (without added calcineurin) were subtracted before calculating the CaM-dependent and independent activities from fitting a linear function to the data.

Data evaluation

In equilibrium binding experiments, the apparent K_D and cooperativity values were estimated by fitting a saturation function to the data according to the Hill equation:

$$F = F^{\text{free}} + F^C \times \frac{L^{n_H}}{K_D^{n_H} + L^{n_H}},$$

where F , F^{free} , and F^C denote the fluorescence intensity measured at a given ligand concentration, that of the ligand-free CaM, and that of the SPC-CaM complex, respectively, L is the concentration of the ligand (SPC), n_H is the Hill-coefficient, and K_D is the apparent dissociation constant.

In “reverse” titration experiments, where the concentration of SPC was held constant and the concentration of CaM was varied, data were fitted to a Langmuir binding model at 1:1 stoichiometry, taken into account that no terms are negligible and that the fluorescence intensity measured (F) are composed of two terms: the fluorescence of free CaM (F^{free}) and the fluorescence of complexed CaM (F^C) as follows:

$$F = F^{\text{free}} + F^C = f^{\text{free}}(CaM^T - CaM^C) + f^C CaM^C,$$

where CaM^T and CaM^C denote the total and the complexed CaM concentrations, and f^{free} and f^C are the molar fluorescence of free and complexed CaM, respectively. CaM^C is given by the following equation:

$$CaM^C = \frac{1}{2}(CaM^T + L + K_D - \sqrt{(CaM^T)^2 + L^2 + K_D^2 + 2(CaM^T + L)K_D - 2CaM^T L}),$$

where L is the ligand (SPC) concentration, and K_D is the equilibrium dissociation constant.

In functional assays, IC_{50} and EC_{50} values were estimated by fitting a sigmoid function in the appropriate form:

$$A = A^{\min} + (A^{\max} - A^{\min}) \times \left(1 - \frac{L^n}{IC_{50}^n + L^n}\right),$$

$$A = A^{\min} + (A^{\max} - A^{\min}) \times \frac{L^n}{EC_{50}^n + L^n},$$

where A , $A^{\min}$ and $A^{\max}$ is the enzyme activity at a given ligand concentration, its minimum, and its maximum value, respectively; L is the ligand (SPC) concentration, n is the cooperativity exponent, and IC_{50} or EC_{50} is the ligand concentration eliciting 50% inhibition or activation.

Statistical analysis

To compare mean values of different measurements, Student's t-tests were performed at the appropriate degree of confidence. In binding experiments, $p < 0.01$ values were accepted as statistically significant, to rule out the small variations in fluorescence intensities due to solvent effects caused by the lipids. In functional assays, the general $p < 0.05$ condition was used.

RESULTS

SPC binds to CaM specifically in a concentration dependent manner – intrinsic tyrosine fluorescence

To examine whether S1P or SPC interacts with CaM, binding assays were carried out with the purified protein. First, changes caused by the addition of these sphingolipids in the intrinsic Tyr fluorescence of the protein were analysed (Figure 1). CaM has two Tyr residues at positions 99 and 138 in the two C-terminal EF-hand motifs. Ca^{2+} binding caused an approximately 2.5 fold increase in fluorescence intensity, consistent with previous data [29, 30]. Effects of S1P and SPC, and of the related glycerolipid mediators LPA and LPC, were inspected at a concentration of 100 μM on both apo and Ca^{2+} -saturated CaM. We found that among these lipids SPC elicited selective effects, causing significant changes in the intrinsic Tyr fluorescence of the apoprotein (Figure 1A). As can be seen on Figure 1B, SPC raised the fluorescence intensity of apoCaM approximately 3 fold, resulting in a similar spectrum to that of the Ca^{2+} -saturated protein. To check whether this effect is concentration dependent, apoCaM was titrated with SPC. The binding of SPC could be described by a saturation curve resulting in an apparent K_D of $46.9 \pm 0.8 \mu\text{M}$ and a Hill-coefficient of 3.3 ± 0.2 (Figure 1C). To address the stereoselectivity of SPC's action, we have also explored the impact of the synthetic L-threo stereoisomer. It is important to note that the naturally occurring stereoisomer is D-erythro-SPC (which we abbreviate SPC). We found that L-threo-SPC modified the intrinsic Tyr fluorescence of CaM in the same manner as SPC (Figure 1A). Previous studies have shown that the effects of extracellularly applied SPC are stereospecific, while intracellularly applied are not [13, 14]. Our results are in line with these findings, indicating that CaM might be a mediator of the intracellular actions of SPC.

Micelles are required for binding, the SPC micelle – CaM interaction is strong, characterized by a submicromolar dissociation constant

A question arises whether we are seeing a monomer lipid – protein or a micelle – protein interaction. To give an answer, the critical micelle concentration (CMC) of SPC should be known. Li *et al.* measured the CMC of SPC to be 158 μM using isothermal titration calorimetry [31]. However, since the CMC is very much dependent on the exact conditions (buffer, temperature and solubilization), we measured the CMC under the circumstances which most of our experiments were carried out, using the fluorescent hydrophobic probe ANS. With this method, the CMC of SPC was determined to be $33 \pm 2 \mu\text{M}$ (Figure 2), and

did not depend on the presence or absence of Ca^{2+} . The fact that this value is in the same range as the apparent K_D value obtained from titrating apoCaM with SPC (Figure 1C), and the intensive apparent positive cooperativity of the titration curve suggested to us that there is a quite strong interaction between CaM and SPC micelles, while SPC monomers do not bind efficiently to the protein.

Therefore, the dissociation constant estimated from titrating with the lipid only provides information about micelle formation and does not characterize the strength of the interaction. To estimate the real K_D value, we employed "reverse" titration experiments, where the amount of SPC was fixed above the CMC and the concentration of CaM varied (Figure 1D). This way we excluded the additional effect of micelle formation on the interaction. Nevertheless, to obtain the dissociation constant from fitting an appropriate binding curve, one must know the concentration of SPC micelles in the solution. To avoid guesswork, we also treated this term as a parameter in the fitting equation (see Experimental for the equation). The fitting process yielded an estimated SPC micelle concentration of $4.1 \pm 0.1 \mu\text{M}$ (meaning an average of approximately 20 monomers per micelle) and an estimated K_D value of $0.067 \pm 0.037 \mu\text{M}$. This low K_D value supports our hypothesis that the CaM-SPC interaction is rather strong when CaM can bind to SPC micelles instead of monomers.

To make sure that the specificity of the observed effects does not arise from the different CMC values of the tested lipids, namely that only SPC forms micelles at the concentration our experiments were carried out, the CMC of S1P, LPA, LPC, and LT-SPC was determined as well (data not shown). We found the CMC values of each to lie in the 20-50 μM concentration range. It should be noted, that we faced difficulties measuring the CMC with the ANS method in case of lipids with a phosphate head group, that is S1P and LPA. To validate the obtained CMC values of these two lipids, the more hydrophilic one, LPA, was also measured by isothermal titration calorimetry, yielding a CMC of approximately 50 μM (Jozsef Kardos personal communication). Thus, each tested lipid forms micelles at the concentration of 100 μM , where the screening experiments were conducted.

Dansyl-labeled CaM also reveals a strong and selective binding of SPC to CaM

Changes in fluorescence of dansyl-labeled CaM is another good indicator of ligand binding to the protein (Figure 3). Ca^{2+} binding caused an approximately 1.5 fold elevation in the fluorescence intensity, which was accompanied by an approximately 20 nm blue shift, in good agreement with previous data [32, 33]. The same set of lipids were tested at a concentration of 100 μM . Results were consistent with that of Tyr fluorescence, that is, SPC

and LT-SPC caused significant changes in both the intensity (Figure 3A) and the wavelength of maximum intensity (position of peak) of the spectra (data not shown). LPC caused a uniform upward intensity shift of both forms of the protein, a phenomenon that can be marginally seen for S1P too. However, since LPC and S1P did not show any sign of interaction in the other binding assays, we attribute this effect to the sensitivity of the dansyl fluorophore to hydrophobic environment rather than their binding to CaM. The steady state anisotropy of the fluorophore, which is sensitive to the radius of giration of the molecule carrying the label, also shows that only SPC and LT-SPC bind to the protein (Figure 3B). Here we note that we could not determine the precise anisotropy values in case of S1P (Figure 3B, see high errors for S1P), because S1P greatly elevated the baseline, which we could not correct appropriately.

The effect of SPC on the fluorescence spectrum of the dansyl-labeled protein (Figure 3C) is very similar to the effect on Tyr fluorescence. SPC enhanced the fluorescence intensity of apo and to a smaller extent Ca^{2+} -saturated CaM, resulting in a similar spectrum, regardless of the presence or absence of Ca^{2+} . The SPC-bound spectra were again closer to that of the Ca^{2+} -saturated protein, but differed from it the intensity and in the wavelength of the maximum intensity as well. This suggests that SPC binds to both forms of the protein, and the SPC-bound conformation is closer to the Ca^{2+} -saturated form, though not completely the same. Binding of SPC to apoCaM followed saturation kinetics resulting in an apparent K_D of $21.7 \pm 1.3 \mu\text{M}$ and a Hill-coefficient of 1.8 ± 0.2 (Figure 3D), again being characteristic of micelle formation. In the “reverse” titration experiment (data not shown), in which the SPC concentration is held fixed at $100 \mu\text{M}$, the fitting process yielded an estimated micelle concentration of $1.3 \pm 0.1 \mu\text{M}$ with a K_D value of $0.67 \pm 0.34 \mu\text{M}$, also in line with the hypothesis of a strong interaction between CaM and SPC micelles. L-threo-SPC changed the fluorescence intensity and the steady state anisotropy of the dansyl-labeled protein in the same way as SPC (Figure 3A,B), again indicating that the interaction is not stereospecific.

Surface plasmon resonance as a direct evidence for the SPC-CaM interaction

To characterize the binding of SPC to CaM, we have also utilized surface plasmon resonance. In a Biacore setup, where Ca^{2+} -saturated CaM was immobilized directly to the surface of a CM5 chip by amine coupling, we observed selective (Figure 4A) and dose-dependent (Figure 4B) binding of SPC to the CaM surface. We evaluated the sensorgrams to obtain the dissociation constants at low ($10 \mu\text{M}$) and high ($100 \mu\text{M}$) concentrations of SPC. It should be noted, however, that the estimation at concentrations above the CMC depend on the actual

concentration of the SPC micelles, a value we do not exactly know. Based on the "reverse" titration experiment with Tyr fluorescence, we estimated the concentration of SPC micelles as 4 μM for the evaluation of the sensorgrams. This yielded an estimate of $0.64 \pm 0.07 \mu\text{M}$ for the K_D of the CaM – SPC micelle interaction. It should be noted however, that 4 μM is an upper estimate for the concentration of micelles. If we evaluate the sensorgrams supposing lower micelle concentrations, the analysis resulted in lower K_D values. When only 10 μM SPC was injected over the CaM surface, the analysis resulted in an estimate of $200 \pm 100 \mu\text{M}$ for the K_D of the CaM – SPC monomer interaction. Taken together, we conclude that once the conditions required for binding develop, that is SPC monomers cluster, SPC binds to CaM quite strongly, characterized by a submicromolar dissociation constant. This interaction is not stereoselective, but selective when compared with the related lysophospholipid mediators S1P, LPA, and LPC.

Furthermore, we investigated whether SPC can bind to CaM when incorporated into model membranes. We carried out SPR experiments injecting liposomes of three different composition, resembling the outer and the inner leaflets of the plasmamembrane, as well as lipid rafts, with increasing SPC content from 0 to 20 mol%. The exact composition of the liposomes are given in the Preparation of lipid solutions section in Experimental. We found that SPC containing vesicles bind to CaM in all three cases in a concentration dependent manner, although with different kinetics, while the control vesicles do not. The results obtained with liposomes representing the inner leaflet of the plasmamembrane are depicted in Figure 4C. It is important to note that the response size (RU values) of the sensorgrams are not directly proportional to the amount of binding when analytes having different dielectric constants are compared, as in the case of micellar solutions and liposomes. Thus, the smaller RU values observed when SPC was presented in liposomes do not mean that the binding is weaker in this case. The shape of the sensorgrams unambiguously demonstrate the binding of SPC-containing vesicles to CaM, while it is not possible to calculate the binding strenght, because the bulk concentration of SPC in the case of liposomal presentation is not feasible. The successful vesicular delivery of SPC to CaM makes the interaction likely to occur *in vivo*.

SPC does not exert detergent-like effects, it binds as a real ligand to CaM

To rule out the possibility that SPC exerts detergent-like effects, somehow disrupting the native structure of the protein, circular dichroism (CD) measurements were carried out (Figure 5). The far-UV CD spectrum of CaM shows the mainly α -helical structure of the protein, which is slightly increased after the addition of Ca^{2+} , again indicating that our protein

is well folded, and responds accordingly to previous reports [34]. 200 μ M SPC did not have any effect on the spectrum of either apo or Ca^{2+} -saturated CaM. At high concentrations of 500 μ M, a slight shift of the apoCaM spectrum in the direction of Ca^{2+} -saturated CaM was observed. These results confirmed that the binding of SPC does not perturb the secondary structure of the protein, SPC binds as a real ligand to CaM.

SPC dissociates the CaM – target peptide complex, suggesting an inhibitory role

Melittin is a 26-mer peptide of honey bee venom, that forms a basic amphipathic helix [35], which is the typical structure of the CaM-binding domain. Thus, melittin is used to model CaM-target interactions [36]. We examined whether SPC affects the CaM-melittin interaction by measuring changes in fluorescence of the dansyl-labeled protein and the Trp of the peptide as well (CaM does not contain any Trp residues) (Figure 6). The binding of melittin increased the fluorescence intensity of dansyl-labeled CaM approximately 2 fold and caused an approximately 30 nm blue shift of the spectrum, as observed previously [32]. The addition of SPC resulted in the same spectrum regardless of the presence or absence of melittin, which was typical for the SPC-bound CaM spectrum (Figure 6A). In contrast, S1P, shown as a negative control, did not change the spectrum of either dansyl-CaM alone, or the dansyl-CaM-melittin complex (Figure 6B). In the complimentary experiment, the binding of CaM to melittin brought forth an approximately 10 nm blue shift and a slight intensity elevation of the Trp spectrum of the peptide, according to previous data [37]. SPC caused a substantial drop in the fluorescence intensity of melittin, which was not affected at all by the addition of CaM (Figure 6C). The fact that SPC modified the spectrum of melittin was not surprising, since melittin is known to integrate into lipid bilayers [38]. In case of S1P, the fall in melittin fluorescence, probably caused by the partitioning of the peptide into lipid micelles, could be observed, but in the presence of CaM, S1P did not modify the CaM-bound melittin spectrum (Figure 6D). The effects of LPA, LPC and LT-SPC were also examined, and LPA and LPC resulted in similar spectra to S1P, while LT-SPC resulted in similar spectra to SPC. Taken together, only SPC modified the spectra of either dansyl-CaM or melittin in a manner which is consistent with dissociating the CaM – target peptide interaction, while the other lipids did not. An alternative explanation could be that SPC modifies the spectra of the CaM-melittin complex to mimic this effect, but the fact that only SPC causes this fluorescence change on two different fluorescent groups in two different environments (the dansyl attached to CaM and the Trp of melittin), makes this explanation unlikely. Thus, we conclude that SPC and

LT-SPC interfere with CaM – target peptide binding, suggesting an inhibitory role, while S1P, LPA and LPC do not have any significant effect on the CaM – target peptide complex.

SPC inhibits the phosphodiesterase activating ability of CaM

CaM was first identified as a regulator of phosphodiesterase I (PDE1), an enzyme that terminates intracellular cAMP and cGMP signaling by converting the cyclic nucleotides to the inactive 5'AMP and 5'GMP forms. For a recent review on PDE1 see [39]. Since then, this enzyme is most often used to measure CaM activity by several methods, our choice was a continuous fluorescence assay using a fluorescent cGMP derivative, mant-cGMP. We have explored the effect of lysophospholipids on both the basal and the Ca^{2+} -CaM-dependent activity of PDE. Unfortunately, the results obtained are quite difficult to interpret, since certain lipids activated PDE, which has previously been shown by other groups as well [40, 41]. It seems that the measured activity is the outcome of two distinct effects, certain lipids increase the basal activity of PDE to varying degrees, while others inhibit the activator CaM. Taking both effects into consideration, results depicted in Figure 7 can be explained by the following molecular events. At the concentration of 100 μM , LPC fully activates PDE, after which the addition of CaM can not have any further stimulating effect. S1P and LPA also activate the enzyme, though not completely. Since lipids and CaM have been shown to act competitively [40, 41], the addition of CaM has no notable effect, even though maximal activation is still not reached. SPC and LT-SPC, at the concentration of 100 μM , do not activate the enzyme, however, inhibit its activation by CaM.

We tried to measure the concentration dependence of SPC's effect, and the emerging picture became even more complicated, since we found that monomer SPC activates PDE as well. Hence, the effect of SPC on CaM-dependent phosphodiesterase is influenced by both the enzyme-lipid and the CaM-lipid interaction. Moreover, while the former seems to require monomers, the latter requires micelles, therefore, after reaching the CMC, we see a completely different scenario. To sum up, the PDE-CaM-SPC system is so complex, that further investigation is needed in order to thoroughly understand it, which is not the purpose of this paper. However, it can be concluded, that SPC at concentrations above the CMC is an efficient inhibitor of the Ca^{2+} -CaM-dependent activity of phosphodiesterase.

SPC inhibits the calcineurin activating ability of CaM

To assess the functional effect of the CaM-SPC interaction, calcineurin, also known as Ca^{2+} -CaM-dependent protein phosphatase 2B, was chosen as a second target enzyme. It is a

heterodimer of a 61 kDa catalytic and CaM-binding subunit, calcineurin A and a 19 kDa Ca^{2+} -binding regulatory subunit, calcineurin B, which is an EF-hand protein very similar to CaM. For a review on calcineurin see [42]. The enzyme reaction can be easily followed by monitoring the increase in absorbance at 405 nm, caused by the dephosphorylation of the substrate p-nitrophenyl phosphate (PNPP). As can be seen in Figure 8A, CaM increased the activity of calcineurin approximately 2.5-fold, which was substantially inhibited in case of SPC and LT-SPC, while was not affected significantly in case of S1P, LPA, and LPC. For clarity, the fold activation by CaM is shown, because the tested lipids slightly influenced, S1P increased, while SPC, LPC, and LT-SPC decreased the basal activity of calcineurin.

The concentration dependent inhibition of both the CaM-dependent and independent calcineurin activity by SPC is depicted in Figure 8B. The half-inhibitory concentration (IC_{50}) was estimated to be $27.4 \pm 2.6 \mu\text{M}$ with a cooperativity coefficient of 2.3 ± 0.4 in case of the CaM-dependent, and $29.8 \pm 3.5 \mu\text{M}$ with a cooperativity coefficient of 1.7 ± 0.2 in case of the basal activity. Since interaction between the two subunits, calcineurin A and CaM-like calcineurin B, is essential for activity, we hypothesize that the decrease in basal activity is a consequence of the fact that SPC causes the dissociation of these two subunits similarly to CaM-target interactions. To prove that the inhibition of the CaM-dependent calcineurin activity is a result of CaM inhibition, calcineurin was titrated with CaM both in the presence and absence of $50 \mu\text{M}$ SPC (Figure 8C). The fact that excess CaM can bring back the activation of calcineurin in the presence of SPC, and that SPC causes a right shift of the saturation curve, indicates that inhibition by SPC is indeed due to the dissociation of the CaM-calcineurin complex by a competitive mechanism. The thousand-fold difference of the EC_{50} values ($2.5 \pm 0.4 \text{ nM}$ for CaM alone versus $2.9 \pm 0.2 \mu\text{M}$ in the presence of $50 \mu\text{M}$ SPC) also implicates the strong binding of SPC to CaM. In conclusion, SPC is a potent inhibitor of the Ca^{2+} -CaM-dependent calcineurin system.

DISCUSSION

Sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) have recently emerged as important mediators of cellular signaling. They are characterized with the unique feature to be first as well as second messengers. While their action on plasma membrane G protein-coupled receptors are extensively studied and quite well elaborated, not much is known about their intracellular site and mechanism of action, even the identity of their target proteins are to be defined. Most publications in the field of sphingolipid signaling present data obtained from live cell experiments, the results of which are often hard to reconcile to interactions of individual partners. Moreover, since sphingolipid metabolism is very rapid, one can never be sure that the observed effect can be attributed to the added lipid, not one of its metabolites. Hence, to identify the intracellular target proteins through which these lipids exert their actions as second messengers, and to characterize their interaction without the disturbing effects of other cellular components, an approach using purified proteins might be more efficient [43]. Several published data [10, 11, 17-22] suggested to us that S1P and/or SPC might bind to calmodulin (CaM). We used fluorescence spectroscopy and surface plasmon resonance to examine whether these sphingolipids, as well as the related glycerolipid mediators lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC), bind to the protein, and carried out activity assays with target enzymes phosphodiesterase (PDE) and calcineurin to find out whether these interactions have functional effects. Our results show that among S1P, SPC, LPA, and LPC only SPC binds to CaM, and inhibits its activity in the functional assays.

In this report we demonstrate by intrinsic tyrosine (Figure 1) and dansyl-labeled protein (Figure 3) fluorescence, that SPC binds to both apo and Ca^{2+} -saturated CaM, generating a conformation which is closer to the Ca^{2+} -saturated form. Far-UV circular dichroism spectroscopy revealed that SPC does not perturb the native conformation of the protein (Figure 5), we are dealing with a real ligand-protein interaction, not any detergent effect of the lipid. The fact that SPC interacts with apoCaM also, suggests that the mechanism and site of SPC binding is different from the classical aromatic CaM inhibitors such as trifluoperazine, since the latter bind in the hydrophobic pocket which is only open in case of Ca^{2+} -saturated CaM [44]. Our further research will be focused on giving structural insight into the SPC-CaM interaction.

Titration of CaM with SPC resulted in a saturation curve showing strong cooperativity and an apparent K_D of approximately 30 μM , which is near the critical micelle concentration

of the lipid (see Figure 2 for the determination of the CMC). This suggested to us that the interaction requires micelles, and the apparent K_D obtained in this manner only shows micelle formation, and does not characterize the strength of the interaction. Therefore, to obtain the real dissociation constant, we had to exclude the additional effect of micelle formation, which we have done by keeping the SPC concentration fixed above the CMC and titrating with the protein (Figure 1D). Assuming a 1:1 stoichiometry of the SPC micelle – CaM interaction as the simplest binding model, we estimated the equilibrium dissociation constant to be $0.067 \pm 0.037 \mu\text{M}$ in case of intrinsic tyrosine fluorescence, and $0.67 \pm 0.34 \mu\text{M}$ in case of the dansyl-labeled protein. Nevertheless, if the binding stoichiometry is not 1:1, this simple fitting cannot be valid for the whole concentration range, but can approximate a part of the titration acceptably. Here we could not apply a more complex model, because the molar fluorescence factors of different CaM species can be determined experimentally only for free CaM and CaM in the presence of high molar excess of SPC. Based on the fact that the simplest 1:1 binding model resulted in a ten-fold difference in the estimated K_D values obtained by tyrosine and dansyl fluorescence, we think that more than one CaM molecules can bind to an SPC micelle with different dissociation constants. Based on surface plasmon resonance (Figure 4) the upper limit for the K_D is estimated to be $0.64 \pm 0.07 \mu\text{M}$. These methods provided an estimate of the binding strength, for the determination of the exact stoichiometry and K_D values we plan to utilize additional techniques in the future. In conclusion, the high SPC concentration is only required for the development of the appropriate conditions for the interaction *in vitro*, namely that the sphingolipid should be clustered. Once SPC clusters exist, binding occurs, resulting in a strong interaction between SPC and CaM, characterized by a submicromolar dissociation constant.

The binding of SPC to CaM has functional effects as well, since SPC inhibited the Ca^{2+} -CaM-dependent activity of two target enzymes, phosphodiesterase (Figure 7) and calcineurin (Figure 8). The binding assay carried out with melittin, a model peptide for the CaM-binding domain, revealed that SPC interferes with the CaM-target interaction (Figure 6), elucidating its inhibitory effects. The activation dose response titration of calcineurin by CaM in the absence and presence of SPC (Figure 8C) also supports a competitive type of inhibition and implicates a strong binding of SPC to CaM as well. Thus, SPC is a potent inhibitor of CaM action *in vitro*, suggesting that CaM might be an intracellular receptor for SPC *in vivo*. This is supported by the finding that the CaM-SPC interaction is not stereospecific, as previously shown in live cell studies for the intracellular actions of SPC [13, 14]. The identification of the signaling pathways that are actually modified in this manner *in vivo* still

awaits. The most well studied intracellular action of SPC is the liberation of Ca^{2+} from internal stores through the activation of ryanodine receptors [15,16]. Since these Ca^{2+} -channels are regulated by CaM it is tempting to speculate that the SPC-CaM interaction plays a major role in SPC induced Ca^{2+} release.

An important question is, whether the interaction between SPC and CaM has any physiological relevance. One might argue that lipid micelles do not exist *in vivo*, however, if we think of micelles as lipid clusters, dynamically changing according to local monomer concentrations, we find that these conditions might easily occur *in vivo*. Since in order to be a second messenger, SPC has to be synthesized upon stimulation in bulk quantities, a process resulting in its clustered production and locally high concentrations. Thus, the circumstances that are necessary for the SPC-CaM interaction to occur *in vitro* may already be given *in vivo*. Moreover, we demonstrated that SPC can bind to CaM when incorporated into model membranes (Figure 4C), making the interaction even more likely to occur under physiologic conditions. Still, the question is open, is SPC formed upon stimulation in large numbers? We do not know yet, but the fact that Betto *et al.* [16] measured the overall SPC concentration in cardiac muscle to be approximately 15.6 μM , is very promising.

To sum up, we have shown that SPC binds to both apo and Ca^{2+} -saturated CaM specifically, and inhibits the protein's activity on the target enzymes phosphodiesterase and calcineurin. Our data are consistent with earlier findings on SPC's intracellular actions *in vivo*. Thus, we propose that CaM might be an intracellular receptor for SPC, and raise the possibility for a novel regulation of CaM.

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FIGURE LEGENDS

Figure 1 Effect of SPC on the intrinsic tyrosine fluorescence of CaM

(A) Effect of lysophospholipids on apo (light bars) and Ca^{2+} -saturated (dark bars) CaM. Protein and lipid concentrations were 1 μM and 100 μM , respectively. Mean and standard error values were calculated from at least three independent experiments. SPC and LT-SPC treated apoCaM values show significant differences ($p < 0.01$, see asterisks) with regard to the apoCaM control, based on Student's t-test. (B) The average spectra for SPC. 1 μM apoCaM ($\circ$), 1 μM Ca^{2+} -saturated CaM ($\bullet$), 1 μM apoCaM with 100 μM SPC ($\triangle$), and 1 μM Ca^{2+} -saturated CaM with 100 μM SPC ($\blacktriangle$). (C) Titration of apoCaM with SPC. Protein concentration was 1 μM and SPC concentration was varied from 5 μM to 500 μM . See inset figure for the individual spectra. Data points represent three independent determinations (Mean $\pm$ S.E.M.). Fitting a sigmoid function to the data resulted in an apparent K_D of 46.9 ± 0.8 μM and a Hill-coefficient of 3.3 ± 0.2 . (D) Titration of 100 μM SPC (above the CMC) with apoCaM to obtain the real binding parameters. Data points represent three independent determinations (Mean $\pm$ S.E.M.). The fitting process (see Experimental for the equation) yielded an estimated SPC micelle concentration of 4.1 ± 0.1 μM (meaning an average of approximately 20 monomers per micelle) and an estimated K_D value of 0.067 ± 0.037 μM for the CaM – SPC micelle interaction. Dashed lines represent extrapolated values for free and SPC-bound CaM, calculated from the fluorescence intensities of 1 μM apoCaM (---) and 1 μM apoCaM with 200 μM SPC (— —).

Figure 2 Determination of the critical micelle concentration of SPC

The CMC of SPC was determined using the fluorescent hydrophobic probe ANS. Two straight lines reflecting the aqueous and the micellar environment were traced, and the CMC was defined as the concentration referring to their point of intersection, yielding a CMC of 33 ± 2 μM for SPC. Mean and standard error values were calculated from three independent experiments.

Figure 3 Effect of SPC on the fluorescence of dansyl-labeled CaM

(A,B) Effect of lysophospholipids on the fluorescence intensity and the steady state anisotropy of apo (light bars) and Ca^{2+} -saturated (dark bars) dansyl-labeled CaM. Protein and lipid concentrations were 0.2 μM and 100 μM , respectively. Mean and standard error values

were calculated from at least three independent experiments. Both in the case of apo and Ca^{2+} -saturated CaM, SPC and LT-SPC show significant differences ($p < 0.01$, see asterisks) with regard to the corresponding controls in both panels. LPC also increased the dansyl intensity values, see Results for explanation. **(C)** The average spectra for SPC. 0.2 μM apoCaM ($\circ$), 0.2 μM Ca^{2+} -saturated CaM ($\bullet$), 0.2 μM apoCaM with 100 μM SPC ($\triangle$), and 0.2 μM Ca^{2+} -saturated CaM with 100 μM SPC ($\blacktriangle$). **(D)** Titration of apoCaM with SPC. Protein concentration was 0.2 μM and SPC concentration was varied from 2 μM to 200 μM . See inset figure for the individual spectra. Data points represent three independent determinations (Mean $\pm$ S.E.M.). Fitting a sigmoid function to the data resulted in an apparent K_D of $21.7 \pm 1.3 \mu\text{M}$ and a Hill-coefficient of 1.8 ± 0.2 .

Figure 4 Surface plasmon resonance measurement of the interaction between SPC and CaM

Selective **(A)** and dose-dependent **(B)** binding of SPC to Ca^{2+} -saturated CaM immobilized on the surface of a CM5 sensor chip by amine coupling. Calculating with an estimated SPC micelle concentration of 4 μM , the fitting process yielded an estimate of $0.64 \pm 0.07 \mu\text{M}$ for the dissociation constant of the SPC-CaM interaction. **(C)** Liposomal presentation of SPC to Ca^{2+} -saturated CaM. Increasing amounts of SPC (0, 5, 10, 20 mol%) were incorporated into vesicles composed of 50 mol% brain total lipid extract, 25 mol% PE and 25 mol% PS, representing the inner leaflet of the plasma membrane. In each panel, typical traces of three experiments are shown.

Figure 5 Far-UV circular dichroism spectrum of SPC-bound CaM

The far-UV CD spectrum of SPC-bound CaM does not differ from the spectrum of free CaM, indicating that the lipid does not perturb the native conformation of the protein. 10 μM apoCaM ($\circ$), 10 μM Ca^{2+} -saturated CaM ($\bullet$), 10 μM apoCaM with 200 μM SPC ($\triangle$), and 10 μM Ca^{2+} -saturated CaM with 200 μM SPC ($\blacktriangle$). The averages of three independent measurements are shown.

Figure 6 Effect of SPC on the CaM – target peptide complex

(A, B) The change in dansyl-CaM (0.2 μM) fluorescence caused by melittin (0.4 μM) binding in the presence of SPC and S1P (100 μM), respectively. Symbols used are: dansyl-CaM ($\circ$), dansyl-CaM in the presence of melittin ($\bullet$), dansyl-CaM with lipid ($\triangle$), and dansyl-CaM in

the presence of melittin with lipid ($\blacktriangle$). (C, D) The change in the fluorescence of melittin's (0.2 μ M) Trp residue caused by CaM (0.4 μ M) binding in the presence of SPC and S1P (100 μ M), respectively. Symbols used are: melittin ($\square$), melittin in the presence of CaM ($\blacksquare$), melittin with lipid (∇), and melittin in the presence of CaM with lipid ($\blacktriangledown$). The averages of three independent measurements are shown.

Figure 7 Effect of SPC on the phosphodiesterase activating ability of CaM

PDE activity was followed fluorimetrically by monitoring the cleavage of the substrate mant-cGMP. Mant-cGMP, PDE, CaM, and lipid concentrations were 10 μ M, 5 nM, 50 nM, and 100 μ M, respectively. Light bars depict the basal and dark bars depict the Ca^{2+} -CaM-dependent activity of PDE. See Results for the interpretation. Mean and standard error values were calculated from four independent experiments.

Figure 8 Effect of SPC on the calcineurin activating ability of CaM

Calcineurin activity was measured by monitoring the increase in absorbance at 405 nm due to the dephosphorylation of the substrate PNPP (20 mM). Calcineurin and CaM concentrations were 0.001 unit/ μ l, and 10 μ g/ml, respectively. (A) Effect of lysophospholipids (100 μ M) on the calcineurin activating ability of CaM. Mean and standard error values were calculated from four independent experiments. Student's t-test revealed significant differences ($p < 0.05$, see asterisks) for SPC and LT-SPC with regard to control. (B) Concentration dependent inhibition of the Ca^{2+} -CaM-dependent ($\bullet$) and independent ($\circ$) activity of calcineurin by SPC. Data points represent five independent determinations (Mean $\pm$ S.E.M.). Fitting a displacement function to the data yielded an IC_{50} value of 27.4 ± 2.6 μ M with a cooperativity coefficient of 2.3 ± 0.4 in case of the Ca^{2+} -CaM-dependent, and an IC_{50} value of 29.8 ± 3.5 μ M with a cooperativity coefficient of 1.7 ± 0.2 in case of the basal activity. (C) Dose response curves for the activation of calcineurin by CaM in the presence ($\bullet$) and absence ($\circ$) of 50 μ M SPC. Data points represent four independent determinations (Mean $\pm$ S.E.M.). Fitting a dose response function to the data yielded an EC_{50} value of 2.5 ± 0.4 nM in case of CaM alone, and an EC_{50} value of 2.9 ± 0.2 μ M in the presence of 50 μ M SPC.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8