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Observation of Topical Catalysis by Sphingomyelinase Coupled To Microspheres

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Abstract: Sphingomyelinase, SMase (EC 3.1.4.12), was coupled onto amino-derivatized acrylate microspheres and was shown to retain its catalytic activity. The immobilized enzyme allows one to carry out topical enzymatic reaction in a controlled manner. Accordingly, these spheres were held with a micropipet and using micromanipulator brought into contact with a giant liposome membrane composed of phosphatidylcholine and sphingomyelin (SOPC/C16:0-SM, 0.75:0.25, molar ratio), representing the substrate for the immobilized enzyme. The macroscopic consequences of the enzyme reaction were visualized using fluorescence microscopy as well as differential interference contrast microscopy. The surface contact of the giant vesicle and immobilized enzyme causes membrane microdomain formation and domain clustering (capping) in the membrane and subsequent shedding of small vesicles from the membrane into the interior of the giant liposome. The method described represents a novel approach to study enzymatic reactions and allows manipulating giant vesicles as well as cultured cells in a spatially controlled manner.

Introduction

Giant vesicles (GVs) with diameters in the range of 10−200 μm represent a new biomembrane model with sizes comparable to most eukaryotic cells,[1] thus enabling the characterization of individual vesicles in real time using microscopy techniques. So far, giant vesicles have been used to investigate the physical properties of membranes,[2] morphological changes such as fusion,[3] lipid lateral organization,[4] as well as DNA,[5] and protein−membrane interactions.[6] Giant vesicles have also been employed to study the consequences caused by enzymes modifying the vesicle lipid composition, such as phospholipase A_2,[7] phospholipase D,[8] and sphingomyelinase (SMase).[9] The latter enzyme is of particular interest because of the involvement of the reaction product, ceramide, as a second messenger in differentiation, cellular stress, growth, and apoptosis.[10] Moreover, SMase and the presence of sphingomyelin in the cell membrane have been found to be important in bacterial[11] and viral[12] invasion into nonphagocytic cells. We have recently shown, using giant liposomes and microinjection, that SMase causes vectorial formation of vesicles in GVs. More specifically, both endocytosis and budding could be seen following the action of SMase on the outer and inner leaflet of the vesicle, respectively.[9] We also demonstrated that the major carrier particle for cholesterol in human plasma, the low-density lipoprotein (LDL), has SMase activity associated with it and induces an endocytosis-like process when contacting a giant liposome surface.[13] We have postulated that the SMase activity of LDL may represent a direct mechanistic link between atherosclerosis and programmed cell death, apoptosis via the reaction product ceramide.[14]

In this study we describe the coupling of SMase onto amino-derivatized acrylate beads. Subsequently, we show that these SMase-derivatized beads can be held by a micropipet and brought to contact with the outer surface of giant liposomes. The resulting localized enzyme−membrane interaction and the consequences of the formation of the reaction product can be

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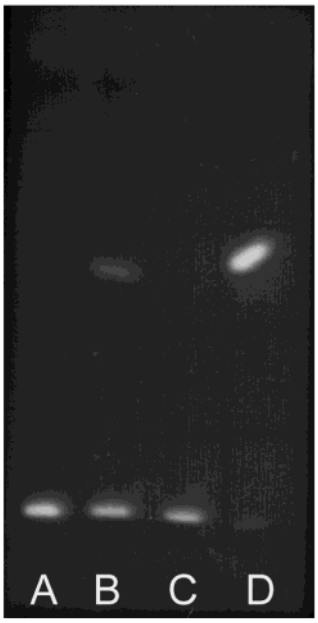

Figure 1. Thin-layer chromatography analysis on silicic acid-coated plates of the reaction catalyzed by SMase coupled to microspheres. Lane A: Bdp-SM. Lane B: Formation of ceramide after the incubation of SOPC/C16:0-SM/Bdp-SM liposomes (at a final lipid concentration of 35 μM) with SMase-derivatized microspheres (0.74 mg) coupled via glutaraldehyde for >48 h at 37 °C in 0.5 mM Hepes, pH 7.4 buffer. Lane C: Same experiment as described for lane B except for using BSA-derivatized microspheres. Lane D: Bdp-ceramide. The TLC plate was developed with 1,2-dichloroethane/methanol/water (90:20:0.5 (v/v/v)) and the lipids localized by illumination with an UV lamp. It should noted that incubation for 48 h at 37 °C was used to ensure sufficient product formation so as to allow the detection of ceramide on TLC, despite losing some of this lipid in extraction.

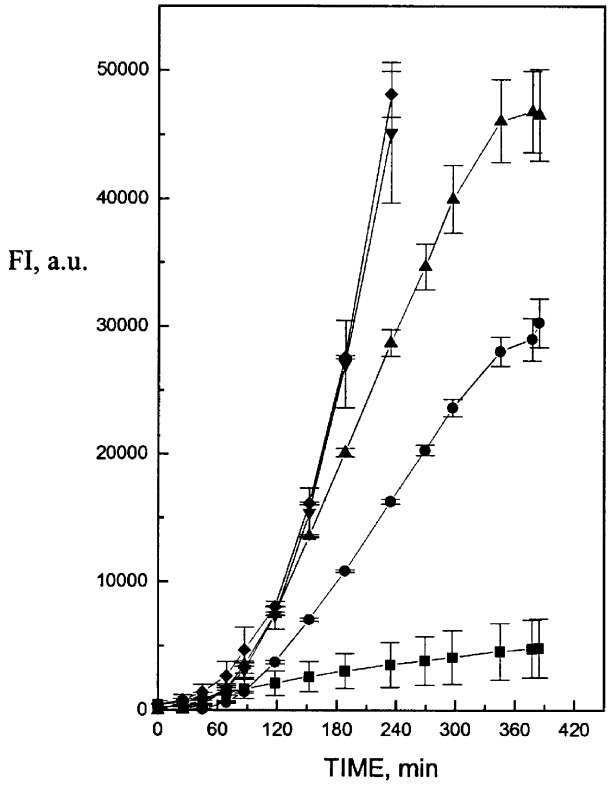

Figure 2. Time course for the increase in fluorescence due to SMase (*S. aureus*) coupled via glutaraldehyde to microspheres measured by the Amplex Red assay performed at +37 °C with stirring at 30 rpm. The reaction catalyzed by 1 μL of the SMase-derivatized microsphere suspension (▼, ◆) is illustrated, with BSA-modified microspheres (■) used as a control. SMase activity of the microspheres was estimated by comparing the extent of product formation in the region of linear progression of catalysis to the change in fluorescence caused by known aliquots of soluble SMase, viz. 0.4 (●) and 0.8 mU (▲). Total volume of the reaction mixture was 200 μL. Enzymatic activity of SMase coupled to microspheres could be calculated to be approximately 3.1 mU/mg of spheres.

monitored by microscopy. This method represents a novel approach to manipulate giant vesicles as well as living cells in a spatially controlled manner.

Results

The activity of SMase coupled to microspheres was first assessed qualitatively. Large unilamellar liposomes (SOPC/C16:0-SM, mole fractions 0.75:0.2, respectively) and also incorporating the Bdp-SM ($X = 0.05$) as a fluorescent tracer were used as a substrate. After 48 h of incubation with SMase-derivatized microspheres at 37 °C the lipids were isolated on TLC and visualized by UV-illumination (Figure 1). Formation of ceramide was evident in the presence of SMase containing microspheres, whereas this lipid was absent for BSA-derivatized microspheres, used as a control.

Likewise, although we did not carry out quantitative analysis reacting SMase coupled to microspheres with C16:0-SM resulted in ceramide formation when analyzed by TLC and iodine staining (data not shown). Amplex Red assay was used to quantitate the activity of the immobilized enzyme (Figure 2). In this assay, the released phosphocholine headgroup is converted to a fluorescent end product, allowing one to monitor the progress of the hydrolytic reaction by the increment in fluorescence with time. Interestingly, similarly to other lipolytic enzymes, including phospholipase A_2[15] and phospholipase C,[16] there is a relatively long lag time of approximately 30 min, which precedes the phase with steady progression of catalysis. SMase activity of the microspheres was estimated by comparing the change in fluorescence in the region of linear progression of catalysis to the reactions measured with known amounts of free enzyme. Enzymatic activity of SMase-derivatized microspheres thus obtained is approximately 3.1 mU/mg of microspheres. Accordingly, the calculated average SMase activity is approximately 2.4 nU/microsphere.

On the basis of the observed catalytic activity of the immobilized SMase on sphingomyelin present in the above two assays, it seems reasonable to assume it will be catalytically active also when presenting the substrate in a giant liposome membrane. In subsequent experiments giant vesicles composed of SOPC, C16:0-sphingomyelin, and Bdp-SM (molar ratio 0.75:0.20:0.05, respectively) were visualized by fluorescence microscopy of the included fluorescent lipid tracer (Figure 3, panel A). Uniform distribution of the fluorescent lipid Bdp-SM was initially evident at the resolution of the optical microscope, in keeping with the miscibility of sphingomyelin in PC membranes,[17] as observed previously.[9] In the interpretation of the microscopy experiments we have assumed Bdp-SM and C16:

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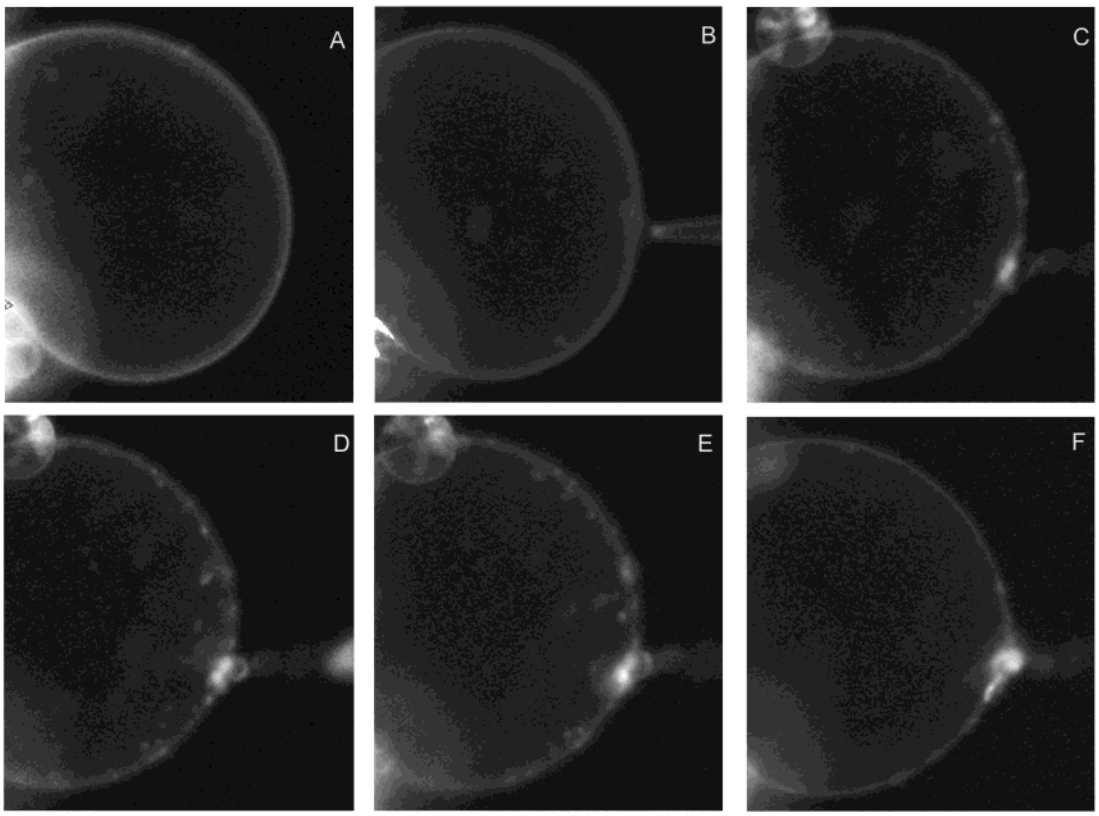

Figure 3. Transformation of a single SOPC/C16/0-SM:Bdp-SM (0.75:0.20:0.05) giant vesicle. Panel A: Following ceramide formation by immobilized Smase. Still fluorescence microscopy image taken before contacting the membrane surface with a SMase derivatized microsphere. Panel B: Image taken immediately after touching the membrane surface with the sphere. The subsequent images were taken 45 (panel C), 50 (panel D), 55 (panel E), and 90 min (panel F) after contacting the vesicle surface with the microsphere. The diameter of the vesicle in panel A is 127 μm.

0-SM to have similar reactivities with SMase. We consider this to be reasonable since the enzyme binds to the surface of the substrate bilayer, interacting with the headgroup and hydrolytically cleaving the phosphocholine headgroup. The hydrophobic Bodipy-moiety is buried in the hydrocarbon phase and is unlikely to have major impact on the enzyme−bilayer interaction on the surface.

We have demonstrated previously that ceramide forms microdomains in large unilamellar vesicles (LUVs).[18] We have also investigated the consequences of enzymatic conversion by SMase of sphingomyelin to ceramide in giant phosphatidylcholine/sphingomyelin vesicles.[9] Our purpose here was to observe this reaction using an immobilized SMase, which is brought into contact with membrane in a controlled manner using micromanipulation. Touching the liposome with the SMase-derivatized microsphere deforms the membrane surface (Figure 3, panel B). After a relatively long lag time (45 min) the fluorescence intensity at the contact site increased, together with the emergence of smaller more brightly fluorescent spots peripheral to the contact site, causing the vesicle surface to appear somewhat granular. With further progress of the reaction the fluorescent domains increased in size and became brighter. Simultaneously, small vesicles became visible in the GV interior (Figure 3, panel C). With time progressive accumulation of these small vesicles inside the GV was evident (Figure 3, panels D and E). Finally, a large fluorescent area appeared at the contact site of the microsphere with the vesicle surface (Figure 3, panel F). Using either nonderivatized spheres or spheres with coupled BSA did not produce changes in GVs (not shown).

In a giant vesicle with a diameter of 127 μm (Figure 3) the total amount of lipid is approximately 0.12 pmol. A mole fraction of ceramide $X \geq 0.10$ of the total lipid is needed for the emergence of ceramide-enriched microdomains in LUVs.[18b,19] For the giant vesicle illustrated (Figure 3) this would correspond to the formation of approximately 12 fmol of ceramide. Reaching $X_{Cer} = 0.10$ would under optimal conditions require $\approx$4 min if all the enzyme molecules in the contacting microsphere would participate in catalysis. However, we may estimate that the fraction of the enzyme on the microsphere having contact with the liposome surface is less, closer to 1/4. This means that the lag time should be approximately 15 min. Domain formation becomes visible in the fluorescence microscopy images approximately 45 min after contacting the vesicle with the microsphere. Moreover, the microscopy experiments were carried out at $\approx$27 °C, whereas the Amplex Red assays were performed at 37 °C. Taking into account the uncertainties involved, we do consider this matching of the lag times to be surprisingly good, thus providing further evidence for SMase action being responsible for the observed microscopic changes.

The formation of small vesicles was evident also using differential interference contrast optics (Figure 4). Within approximately 20 min after the contact between the giant vesicle surface and the microspheres, small vesicles started to emerge

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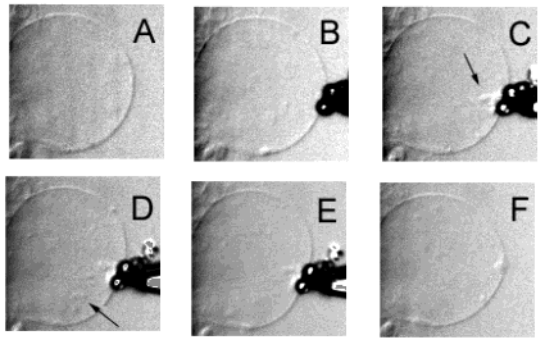

Figure 4. Changes in vesicle morphology induced by contacting the outer surface of SOPC/C16:0-SM (molar ratio, 0.75:0.25) giant vesicle with SMase derivatized microspheres. Differential interference contrast microscopy images were taken before the microspheres contact the vesicle (panel A), immediately after the contact with the giant vesicle (panel B), as well as 20 (panel C), 50 (panel D), and 60 min (panel E) after establishing the contact of the SMase derivatized microsphere with the substrate surface. Panel F illustrates the outcome of the reaction approximately 2 min after the sphingomyelinase coupled microspheres were pulled away. Note that two microspheres are in contact with the giant vesicle. The arrows point to the newly formed vesicles. The diameter of the vesicle in panel A is 50 μm.

near to contact site. Subsequently, the number of these small vesicles increased (Figure 4, panel D). These vesicles remained unaffected after the SMase-derivatized spheres were pulled away (Figure 4, panel F).

Discussion

The reaction product of SMase, ceramide, has been shown to form ceramide-enriched microdomains in both fluid and gel state phosphatidylcholine (PC) membranes.[19,20] The segregated domains have physical properties very different from the membrane composed of phosphatidylcholine. Segregation of ceramide has been suggested [9,14,18b] to result from efficient intermolecular hydrogen bonding between the headgroups of this lipid.[18a] The headgroup of ceramide is also weakly hydrated, which allows tight lateral packing in the membrane. Not only domain formation but also release of "endocytotic" vesicles into the interior of the vesicle could be seen in our experiments (Figure 3). These results conform with our earlier observations,[9] where SMase was injected onto the vesicle surface, resulting in domain formation and vectorial budding. Asymmetric formation of ceramide in a bilayer, i.e., either in the outer or inner leaflets, will cause an area difference between the two monolayers. Ceramide has also a tendency to form inverted nonlamellar phases.[21] These properties, area difference between adjacent monolayers, and negative spontaneous curvature for ceramide containing domains together with the high bending rigidity of the ceramide-enriched domain provide the driving force for vectorial formation of vesicles in the membrane.[9]

Previous studies on the behavior of ceramide in model membranes as well as the physical properties of this lipid thus comply with the observations reported here. Importantly, no changes were observed in control vesicles not subjected to any reagents, thus excluding spontaneous aggregation of the probes. Likewise, nonderivatized microbeads and those coupled with BSA were without effect.

Definitive evidence for enzymatic degradation of sphingomyelin taking place in giant vesicles contacted by microspheres with covalently coupled SMase would be to analyze the lipid composition of the vesicle. In a giant vesicle with a diameter of 127 μm (Figure 3) the total amount of lipid is approximately 0.12 pmol. Accordingly, the amount of the substrate, sphingomyelin at $X = 0.25$, is 30 fmol. Our previous studies suggest that ceramide-enriched microdomains emerge in LUVs when the mole fraction of this lipid exceeds 0.1.[18b,19] For the giant liposome illustrated in Figure 3 this corresponds to the formation of approximately 12 fmol of ceramide. Unfortunately, analysis of the lipid composition in this concentration range is not possible with the techniques available to us at present. Moreover, the vesicles are fragile, and their capture for analysis would be extremely demanding.

We have shown earlier that when soluble SMase has been injected onto the surface of a giant vesicle, a similar vesicle formation is evident,[9] as in the present study using an immobilized SMase. We have also observed the action of microinjected phospholipase C on a giant vesicle. Interestingly, this enzyme causes a gradual stepwise shrinkage of the giant vesicle.[22] On the other hand cytochrome *c* produces no morphological changes in SOPC GVs (Holopainen and Kinnunen, unpublished) and neither do $CaCl_2$ and $MgCl_2$ at concentrations of 7.4 and 7.7 mM, respectively. Also applying lysozyme to the surface of GV composed of zwitterionic lipids induced no morphological changes.[7] The above findings demonstrate that the changes observed in the GVs depend on the reagent used.

In the experiments using microinjection of SMase the formation of ceramide was catalyzed by an uncontrolled amount of enzyme, which with time becomes diluted into the surrounding medium due to diffusion.[9] Importantly, in the experiments shown here the enzymatic catalysis can be restrained topically to the site of contact between the vesicle and the derivatized microsphere. The formation of ceramide-enriched fluorescent microdomains in the membrane reveals that the reaction product diffuses laterally away from the contact site. Thereafter, when the content of this lipid is sufficiently high, nucleation of clusters[18b] is observed, distantly from the contact site (Figure 3). As mentioned above, our earlier data suggest that at this stage the mole fraction of ceramide in the involved leaflet of the bilayer exceeds 0.10.[18b,19] Subsequently, the ceramide-enriched domains detach as small vesicles into the interior of the giant vesicle, similarly to our previous results with microinjected SMase.[9]

The resemblence of our present in vitro findings with the in vivo observations on ceramide formation in cells[23] is striking. In brief, Grassmé et al. observed that the formation of ceramide by acidic SMase is essential for the clustering of the FAS-ligand reseptor, CD95, an integral membrane protein. Small patches of CD95 first emerged which then fused to form a large cap. Further studies showed that ceramide was concentrated in distinct membrane domains resembling these CD95 and acid SMase containing clusters. This kind of process was observed

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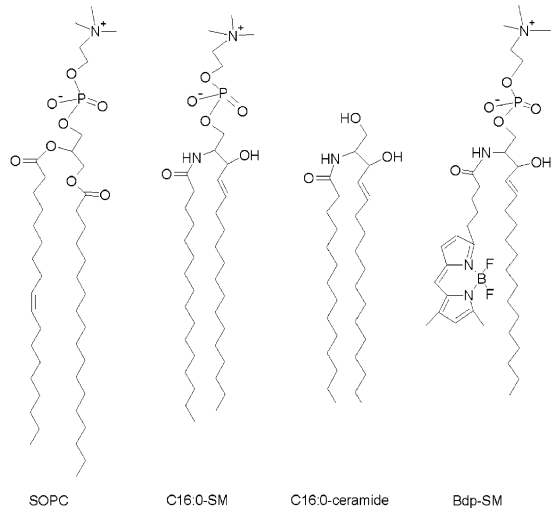

Figure 5. Chemical structures of the lipids used.

also in the present study (Figure 3, panel F). Finally, the topology of the SMase reaction and ceramide formation in apoptotic cells is not known. Our results suggest the possibility that also in cells ceramide could form at restricted, specific sites in the plasma membrane, in keeping with the lateral organization of membranes into compositionally and functionally specified domains.[24]

Materials and Methods

Materials. *N*-Palmitoylsphingomyelin (C16:0-SM) was obtained from Northern Lipids (Vancouver, BC, Canada), SOPC from Avanti Polar Lipids (Alabaster, AL), and bodipy-sphingomyelin (Bdp-SM) from Molecular Probes (Eugene, OR). The purity of the above lipids was verified by thin-layer chromatography (TLC) on silicic acid-coated plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:25:4, by volume). The concentrations of C16:0-SM and SOPC were determined gravimetrically with a high-precision electrobalance (Cahn 2000, Cahn, Cerritos, CA) and that of Bdp-SM spectrophotometrically, using 77 000 cm^{-1} at 505 nm as its molar extinction coefficient. Amino-modified microspheres (average diameter $\approx 10.8\ \mu m$) were obtained from Bangs Laboratories, Inc. (Fishers, IN). Methyl-*N*-succinimidyl adipate (MSA), 1-ethyl(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and hydroroxylamine were purchased from Pierce. SMase from *Staphylococcus aureus* (297 units/(mg of protein)), and glutaraldehyde were from Sigma. Pro analysis grade solvents were from Merck and other chemicals from standard sources. Structures of the lipids used are illustrated in Figure 5.

Preparation of LUVs. Appropriate amounts of the lipid stock solutions were mixed in chloroform to obtain the desired compositions with Bdp-SM as a fluorescent probe (SOPC/C16:0-SM/Bdp-SM, at respective mole fractions of 0.7:0.25:0.05). The resulting mixtures were evaporated to dryness under a stream of nitrogen and traces of solvent subsequently removed by evacuating under reduced pressure for at least 12 h. The lipid residues were hydrated at 50 °C in 0.5 mM Hepes, pH 7.4 , to yield the desired lipid concentration of 35 μM. The lipids were maintained at this temperature for 30 min prior to irradiation for 2 min in a bath type ultrasonicator (NEY Ultrasonik 104H, Yucaipa, CA). The resulting dispersions were subsequently processed to large unilamellar vesicles by extrusion through Millipore (Bedford, MA) 0.1 μm pore size polycarbonate filters using a Liposofast-Pneumatic (Avestin, Ottawa, Canada) gas pressure driven homogenizer.

Coupling of SMase to Microspheres. The reaction conditions were adapted from the protocols provided by Bangs Laboratories Inc. and

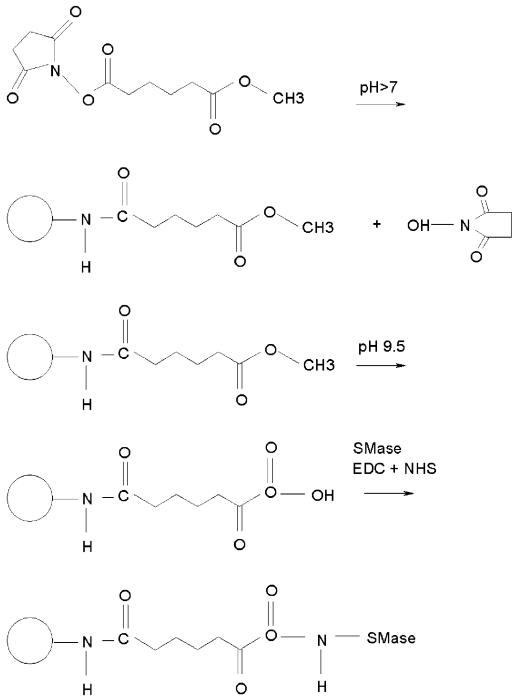

Figure 6. Schematic illustration of the coupling of sphingomyelinase to amino-modified microspheres by methyl-*N*-succinimidyl adipate-linker (MSA). In the first step *N*-hydroxysuccinimide ester of MSA reacts with the amino groups on the surface of the acrylate microsphere. Subsequently, the carboxyl group of MSA was unmasked by increasing to pH 9.5 and then activated with EDC and NHS, thus allowing coupling of SMase via its amino groups to the carboxyl moiety of MSA.

Pierce. Amino-modified microspheres were purified prior to use according to the manufacturer's instructions. Two coupling methods were used as follows. In the first reaction MSA, the amine-reactive reagent containing a masked carboxyl group, was used as a linker between SMase and the acrylate microspheres (Figure 6). Briefly, MSA was first dissolved in 140 μL of DMSO to a concentration of approximately 1.4 M and then added to the microsphere suspension (10 mg of spheres/mL $\approx 10^6$ spheres/mL) in 2.5 mL of buffer A (0.1 M Na_2HPO_4, 0.15 M NaCl, pH 7.2), followed by an incubation for 5−10 h at room temperature with continuous shaking. Subsequently, the microspheres were washed three times with 1 mL of buffer A and the carboxyl groups of MSA were unmasked by incubating the mixture for 30 min in 5 mL of buffer B (0.1 M Na_2HPO_4, 0.15 M NaCl, pH 9.5) at room temperature. Thereafter, the microspheres were washed three times with 1 mL of buffer B. For coupling the carboxyl group of MSA-linker to the amino group of SMase (i.e. the ϵ-NH_2 of lysine or amino terminus), 2 mM 1-ethyl(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in buffer C (0.1 M MES, 0.5M NaCl, pH 6.0) was added and 5 mM NHS was used to enhance the coupling reaction.[25] Excess EDC was inactivated by reacting with 1.4 μL of 2-mercaptoethanol (final concentration, 20 mM) for 15 min at room temperature, whereafter the microspheres were washed three times with 1 mL of buffer C. Subsequently, 50 μg (approximately 15 U) of *Staphylococcus aureus* (*S. aureus*) SMase (Sigma) was added to the microsphere suspension (10 mg/mL) in 1 mL of buffer A, followed by an incubation at room temperature for 2 h with continuous stirring. The reaction was terminated by adding 1 mL of 10 mM hydroxylamine in buffer A. Microspheres were washed several times with 1 mL aliquots of buffer A and stored at +4 °C (10 mg/mL) for maximally three weeks prior to their use.

Alternatively, we employed glutaraldehyde to couple SMase to the amino-modified microspheres (Figure 7). Final concentration of glut-

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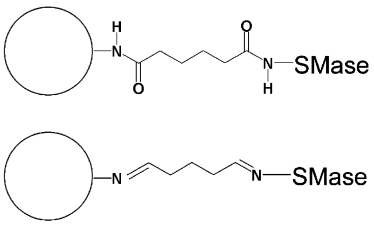

Figure 7. Chemical structures of the linker molecules MSA (top) and glutaraldehyde (bottom) used in the coupling SMase to microspheres. See text for details.

araldehyde in the coupling solution was 1 M (total volume, 1 mL) and the spheres were incubated for 2 h at room temperature with continuous stirring. After the spheres were washed three times with 1 mL of buffer A, 50 μg of *S. aureus* SMase was added (total volume, 500 μL), and incubation was continued for further 2−4 h. Free amino groups of the beads were blocked with 40 mM glycine and 0.1% bovine serum albumin, BSA (in 1 mL of buffer A). Control microspheres were prepared essentially as described above but using BSA instead of SMase.

Enzymatic Activity of SMase-Derivatized Microspheres. Catalytic activity of the SMase-derivatized microspheres was first verified by thin-layer chromatography. A 2 μL aliquot of microspheres (0.74 mg) was added to SOPC/C16:0-SM/Bdp-SM liposomes (final lipid concentration, 35 μM) in 0.5 mM Hepes, pH 7.4 (total volume, 2 mL). After approximately 48 h at 37 °C with continuous stirring 1 mL of chloroform:methanol (2:1, by volume) was added. The lower chloroform phase was collected and dried under nitrogen. The residue was then dissolved in 10 μL of chloroform and applied onto silicic acid coated plates, which were developed with 1,2-dichloroethane/methanol/water (90:20:0.5, by volume) and visualized by UV illumination. SMase activity of the microspheres was quantitated with a commercial indirect assay (Amplex Red, Molecular Probes, Eugene, OR) performed at 37 °C according to the manufacturer's instructions in 0.1 M Tris-HCl, 10 mM $MgCl_2$, pH 7.4, with continuous stirring at 30 rpm. The reaction mixture contained 5 mM 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red**,** a fluorogenic probe sensitive for H_2O_2), 2.5 mM sphingomyelin, and 2% Triton X-100 (v/v), together with a cascade of enzymatic reactions in which phosphocholine, the reaction product of SMase, is first converted to choline and subsequently to betaine and H_2O_2. A 1 μL aliquot of SMase-derivatized microspheres was added to start the reaction. Microspheres derivatized with BSA were used as a control. Fluorescence intensities were measured with SPECTRAFluor Plus reader (Tecan AG, Hombrechtikon, Switzerland) using excitation and emission wavelengths of 535 and 590 nm, respectively.

Formation of Giant Vesicles and Micromanipulation Techniques. The indicated lipids were mixed in chloroform. This solvent was evaporated under a stream of nitrogen and further maintained under reduced pressure for 12 h to remove traces of solvent. The dry lipid residue was subsequently dissolved in diethyl ether:methanol (9:1, vol/vol) to yield a final total lipid concentration of 1 mM. For differential interference contrast and fluorescence microscopy, SOPC:C16:0-SM (0.75:0.25 molar ratio) and SOPC:C16:0-SM:Bdp-SM (0.75:0.20:0.05 molar ratio) stock solutions were used.

Approximately 1−3 μL of one of the above lipid solutions were transferred on the surface of the two Pt electrodes and then dried under a gentle stream of nitrogen for at least 10 min. Possible residues of organic solvents were removed by evacuation in a vacuum for at least half an hour. A glass chamber with the attached electrodes and a quartz window bottom was placed on the stage of an Olympus IX70 inverted microscope (Olympus Optical co., Ltd., Tokyo, Japan). An AC field (sinusoidal wave function with a frequency of 4 Hz and an amplitude of 0.2 V) was applied prior to adding 1.3 mL of buffer (0.5 mM Hepes, pH 7.4). During the first minute of hydration the voltage was increased to 1.0−1.2 V. The AC field was turned off after 2−4 h , and GVs were observed with differential interference contrast optics with a 10×/0.30 or 20×/0.40 objective. The sizes of the GVs were measured using calibration of the images by motions of the micropipet as proper multiples of the step length (50 nm) of the micromanipulator (MX831 with MC2000 controller, SD Instruments, Grants Pass, OR). Images were recorded with a Peltier-cooled 12-bit digital CCD camera (C4742−95, Hamamatsu, Japan) interfaced to a computer and operated by the software (HiPic 5.0.1 or Aquacosmos 1.2) provided by the camera manufacturer.

After GVs were grown, approximately 0.5−1 μL of the microsphere suspension was transferred into the chamber and buffer containing either $CaCl_2$ or $MgCl_2$ was added to yield final concentrations of 7.4 and 7.7 mM, respectively. The microspheres were allowed to sediment to the bottom of the chamber (2−5 min) and were then collected onto the tip of the micropipet using negative pressure of the pneumatic microinjector (PLI-100, Medical Systems Corp., Greenvale, NY). Micropipets with inner tip diameters of >0.5 μm [26] were made from borosilicate capillaries (1.2 mm outer diameter) by a microprocessor-controlled horizontal puller (P-87, Sutter Instrument Co., Novato, CA). Microspheres attached to the micropipet were positioned onto the surface of a single GV and kept there for the indicated periods of time (30−90 min). For easier handling only vesicles attached to the electrode surface were used. All experiments were made at room temperature ($\approx$27$\pm$ 2 °C). Fluorescence experiments were repeated three times and confirmed with DIC microscopy.

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