

Spider and Bacterial Sphingomyelinases D Target Cellular Lysophosphatidic Acid Receptors by Hydrolyzing Lysophosphatidylcholine*[§]

Received for publication, December 29, 2003,
and in revised form, January 16, 2004
Published, JBC Papers in Press, January 19, 2004,
DOI 10.1074/jbc.C300563200

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Bites by *Loxosceles* spiders can produce severe clinical symptoms, including dermonecrosis, thrombosis, vascular leakage, hemolysis, and persistent inflammation. The causative factor is a sphingomyelinase D (SMaseD) that cleaves sphingomyelin into choline and ceramide 1-phosphate. A similar enzyme, showing comparable bioactivity, is secreted by certain pathogenic corynebacteria and acts as a potent virulence factor. However, the molecular basis for SMaseD toxicity is not well understood, which hampers effective therapy. Here we show that the spider and bacterial SMases D hydrolyze albumin-bound lysophosphatidylcholine (LPC), but not sphingosylphosphorylcholine, with K_m values (~ 20 – $40 \mu\text{M}$) well below the normal LPC levels in blood. Thus, toxic SMases D have intrinsic lysophospholipase D activity toward LPC. LPC hydrolysis yields the lipid mediator lysophosphatidic acid (LPA), a known inducer of platelet aggregation, endothelial hyperpermeability, and pro-inflammatory responses. Introduction of LPA₁ receptor cDNA into LPA receptor-negative cells renders non-susceptible cells susceptible to SMaseD, but only in LPC-containing media. Degradation of circulating LPC to LPA with consequent activation of LPA receptors may have a previously unappreciated role in the pathophysiology of secreted SMases D.

Envenomation by *Loxosceles* spiders, endemic to temperate and (sub)tropical regions of the Americas, Africa, and Europe,

* This work was supported by the Dutch Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Figs. 1 and 2.

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can lead to local skin injury as well as to serious systemic toxicity, including thrombus formation, vascular leakage, hemolysis, and persistent inflammation (1–3). In severe cases, the hematologic complications can lead to renal failure and death, especially in children (2, 3). Treatment is difficult; antivenoms are not very effective, and the use of corticosteroids or anti-inflammatory medication is controversial (3). The toxin responsible for the local and systemic effects of *Loxosceles* venom is an unusual sphingomyelinase D (SMaseD)¹ that converts sphingomyelin (SM) in the outer leaflet of the plasma membrane to ceramide 1-phosphate (*N*-acylsphingosine 1-phosphate) (4–7). Strikingly, while SMaseD is not found elsewhere in the animal kingdom, a similar enzyme is produced as an exotoxin by some pathogenic bacteria, notably *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans*, and *Arcanobacterium* (formerly *Corynebacterium*) *hemolyticum* (8–10). *C. pseudotuberculosis* causes lymphadenitis in animals but is also pathogenic for humans, while *C. ulcerans* and *A. hemolyticum* are pathogens of pharyngitis and other human infections (11); in no case is the molecular basis for virulence known (12). The SMaseD from *C. pseudotuberculosis*, also named SM-specific phospholipase D (PLD), is an essential virulence determinant that contributes to the persistence and spread of the bacteria within the host (13). The *Loxosceles* and *C. pseudotuberculosis* SMases D have the same molecular mass (31–32 kDa) and share about 30% sequence similarity (see "Results"). In model systems, the spider and bacterial enzymes provoke remarkably similar pathophysiological effects, including platelet aggregation, endothelial hyperpermeability, complement-dependent hemolysis, and neutrophil-dependent skin necrosis (4–7, 9, 14–16).

Despite decades of study it remains unclear how SMaseD can elicit such a wide variety of biological effects, particularly, since ceramide 1-phosphate is not known as a signaling molecule. In contrast to ceramide, which may reorganize lipid microdomains and associated signaling complexes (17, 18), ceramide 1-phosphate is a bilayer-preferring phospholipid that is unlikely to significantly perturb membrane structure. Furthermore, mammalian cells treated with SMaseD from either *Loxosceles deserta* or *C. pseudotuberculosis* do not convert newly formed ceramide 1-phosphate to ceramide nor does SMaseD treatment affect membrane permeability or cell viability (19, 20).

Given the lack of understanding of SMaseD bioactivity, we set out to re-examine the substrate specificity and cellular effects of the enzyme. Our interest was stirred by a report of more than 30 years ago, showing that partially purified SMaseD from *C. pseudotuberculosis* (*ovis*) can catalyze the release of choline from lysophosphatidylcholine (LPC) but not from phosphatidylcholine (PC) (21). LPC is an abundant plasma component and removal of its choline headgroup yields lysophosphatidic acid (LPA), now known as a pleiotropic lipid mediator acting on specific G protein-coupled receptors in numerous cell types (22, 23). Yet, the possibility that degradation of plasma LPC might contribute to SMaseD toxicity has re-

¹ The abbreviations used are: SMaseD, sphingomyelinase D; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; PLD, phospholipase D; MAP, mitogen-activated protein; GFP, green fluorescent protein; Lox-SMaseD, SMaseD from *L. laeta*; Cp-SMaseD, SMaseD from *C. pseudotuberculosis*; Sc-PLD, PLD from *S. chromofuscus*; DMEM, Dulbecco's modified Eagle's medium; HBS, HEPES-buffered saline; HA, hemagglutinin; PS, phosphatidylserine.

ceived little attention to date. On the other hand, the reported K_m value for LPC was very high, ~ 8 mM (21), suggesting that LPC is not a physiological substrate; moreover, LPC hydrolysis by partially purified SMaseD preparations could well be due to contaminating lyso-PLD activity.

In the present study, we demonstrate that the spider and bacterial SMases D have intrinsic lyso-PLD activity toward albumin-bound LPC and that expression of functional LPA receptors is necessary and sufficient for at least some of the biological responses to SMaseD. Our results suggest that degradation of circulating LPC to LPA, with consequent activation of LPA receptors in cells of the circulatory and vascular systems, may significantly contribute to SMaseD-mediated pathogenicity.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were exposed to serum-free DMEM for 24 h prior to experimentation, unless indicated otherwise. All phospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) at the highest purity grade available. Fatty acid-free bovine serum albumin and monoclonal anti-phospho-MAP kinase antibody were from Sigma. Secondary antibodies (rabbit anti-mouse and swine anti-rabbit) conjugated to horseradish peroxidase were from Dako (Glostrup, Denmark). Highly purified PLD (57 kDa) from *S. chromofuscus* (24) was kindly provided by Dr. Mary F. Roberts (Boston College, Chestnut Hill, MA).

Recombinant SMaseD from *Loxosceles laeta*—Recombinant SMaseD (SMase I) from *L. laeta* was produced as described previously (7). In brief, mature *L. laeta* enzyme was expressed in *Escherichia coli* as a fusion protein, including a His₆-tag at the N terminus and a 4-amino acid linker. The cells were collected by centrifugation and the bacterial pellet was resuspended in extraction buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8.0) and disrupted by French pressure. The supernatant was loaded onto a Ni²⁺-chelating Sepharose Fast Flow column (Amersham Biosciences, Sweden, 1.0 × 6.4 cm), and the recombinant protein was eluted with buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8.0, 0.8 M imidazole). Fractions of 1 ml were collected and analyzed by SDS-PAGE. Stock solutions were prepared in PBS at 1.0 mg of protein/ml.

Recombinant SMaseD from *C. pseudotuberculosis*—Recombinant *C. pseudotuberculosis* SMaseD was expressed in *E. coli* as a fusion protein composed of the mature enzyme with a 33-amino acid N-terminal extension containing a His₆-tag (10). Recombinant enzyme was purified from the soluble fraction of cell lysates on TALON metal affinity resin (Clontech). SMaseD was eluted from the resin in 20 mM Tris-HCl, 100 mM NaCl, 100 mM imidazole at greater than 95% purity.

Choline Release Assay—SMaseD/PLD enzymatic activity was estimated by determining choline liberated from exogenously added phospholipid substrates, using a fluorimetric assay modified from Tokumura *et al.* (25). In the standard assay, the substrate was diluted in 100 μ l of DMEM or HEPES-buffered saline (HBS; 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, pH 7.4). SM and PC substrates were applied as liposomes, whereas the lysophospholipids were complexed to fatty acid-free bovine serum albumin (5 mg/ml). After SMaseD/PLD addition, the reaction was left to proceed for 20 min at 37 °C. By adding 10 μ l of a second assay mixture, the liberated free choline was oxidized (in 10 min) to betaine, and the H₂O₂ concomitantly generated was determined by fluorimetry. The second reaction mixture consisted of 1 unit/ml choline oxidase (Sigma), 0.06 unit/ml horseradish peroxidase, and 50 μ M 3-(4-hydroxy-phenyl)propionic acid in HBS. The second reaction was left to proceed for 10 min. Fluorescence of the oxidized substrate was measured at an excitation of 320 nm and emission of 405 nm using a 96-well plate reader. SMaseD-induced choline release from LPC proceeded at a constant rate for at least 1 h at 37 °C, with the rate being proportional to the enzyme concentration.

LPA₁ cDNA and Retroviral Transduction—Human LPA₁ cDNA (GenBank™ accession number U78192) was amplified by PCR using primers carrying 5' XhoI + HA/3' NotI sites. N-terminally HA-tagged and C-terminally green fluorescent protein (GFP)-fused LPA₁ receptor cDNAs were cloned into retroviral LZRS-IRES-Neo. Recombinant retrovirus produced in Phoenix packaging cells was used to infect rat B103 neuroblastoma cells or human HEK293 cells, essentially as described previously (26). After 48 h, transduced cells were selected in medium containing 1.0 mg/ml G418. Correct expression of LPA₁ was confirmed by Western blotting and immunofluorescence.

LPA Receptor Internalization—HEK293 cells stably transfected with GFP-LPA₁ receptor cDNA were fixed in 3.7% formaldehyde in PBS.

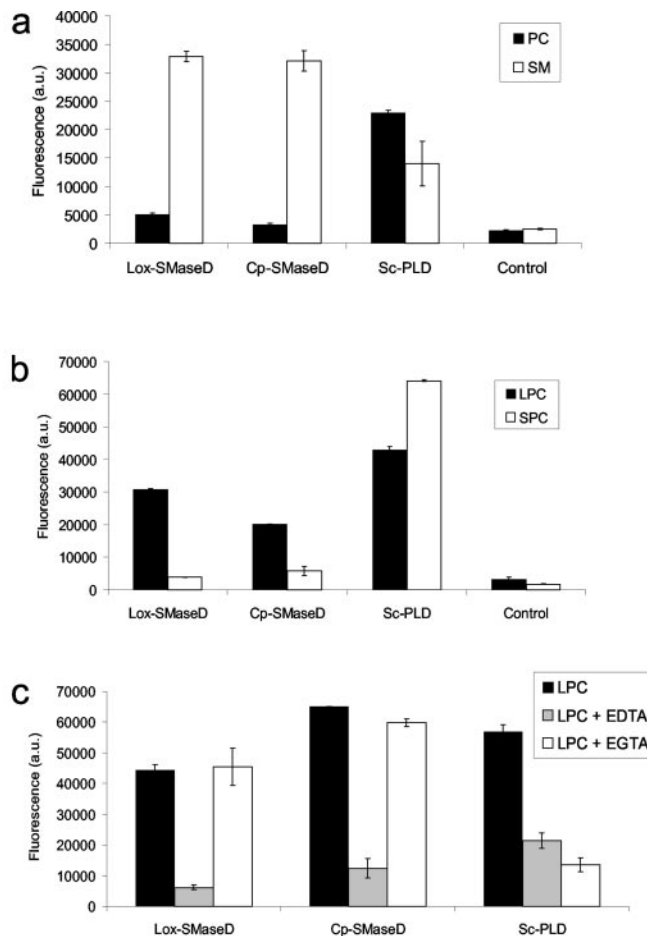


FIG. 1. Choline release from phospholipids induced by recombinant SMaseD from *L. laeta* and *C. pseudotuberculosis*. Choline release was determined fluorimetrically in HEPES-buffered DMEM, pH 7.4, at 37 °C (see "Experimental Procedures"). Lox-SMaseD and Cp-SMaseD were used at 10 nM. The nonspecific PLD from *S. chromofuscus* (Sc-PLD; 10 nM) was used as a positive control. Qualitatively similar results were obtained with enzyme concentrations up to 100 nM. Control denotes no enzyme addition. *a*, hydrolysis of PC and SM, added at 50 μ M as multilamellar vesicles (liposomes). *b*, hydrolysis of LPC (1-oleoyl) and SPC, added at 50 μ M and complexed to fatty acid-free albumin (5 mg/ml). *c*, dependence of LPC hydrolysis on the presence of EDTA or EGTA (4 mM). Error bars represent S.E. of the mean ($n = 5$).

After treatment with agonist, cells were washed and coverslips were mounted with vectashield (Vector Laboratories Inc., Burlingame, CA). GFP-LPA₁ receptors were visualized by confocal microscopy.

RESULTS AND DISCUSSION

The secreted SMases D from *L. laeta* (Lox-SMaseD) (7) and *C. pseudotuberculosis* (Cp-SMaseD) (10) have the same molecular mass (31–32 kDa) and share 32% sequence similarity and 20% identity, including a conserved N-terminal histidine residue required for the enzymatic activity of Cp-SMaseD (see Supplemental Fig. 1, for sequence alignment, and Ref. 27). Despite being *de facto* PLDs, both SMases D are unique in that they show no homology to other phospholipid-metabolizing enzymes, and along with the broad specificity PLD from *S. chromofuscus* (28), they lack the conserved HKD sequence motif that characterizes the PLD superfamily (29).

We confirmed that the recombinant Lox- and Cp-SMases D catalyze the release of choline from SM but not PC, whereas the unrelated PLD from *S. chromofuscus* (Sc-PLD) cleaves both SM and PC (Fig. 1A). The intrinsic lyso-PLD activity of the recombinant SMases D was then assessed using 1-oleoyl-LPC and sphingosylphosphorylcholine (SPC or lyso-SM) as substrates; SPC is a normal plasma constituent (30) that would yield the

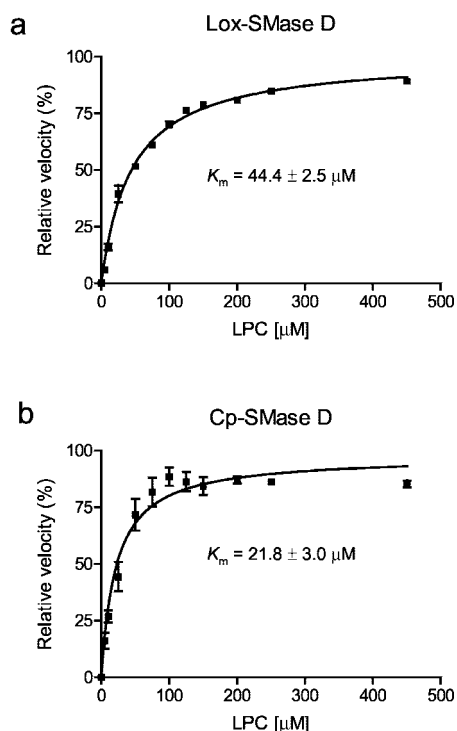


FIG. 2. Saturation kinetics of SMaseD from *L. laeta* (a) and *C. pseudotuberculosis* (b). Rates of choline release from 1-oleoyl-LPC are plotted against increasing concentration of 1-oleoyl-LPC. Assays were carried out in HEPES-buffered DMEM (pH 7.4) at 37 °C (see “Experimental Procedures”). Data were fitted to the Michaelis-Menten equation, yielding the indicated apparent K_m values. The V_{max} values were 212 ± 6 nmol/min/mg for Lox-SMaseD and 66 ± 2 nmol/min/mg for Cp-SMaseD. Data points are the mean of three independent experiments each performed in triplicate. Error bars represent S.E. values.

potent lipid mediator sphingosine 1-phosphate following choline release. As shown in Fig. 1 (B and C), the spider and bacterial SMases D were both capable of liberating choline from albumin-bound LPC in physiological medium, thereby producing LPA (as confirmed by thin layer chromatography analysis). Surprisingly, SPC did not serve as a substrate for either SMaseD, even when SPC was applied at supra-physiological concentrations ($50 \mu\text{M}$, *i.e.* 3 orders of magnitude above normal plasma levels (30)) (Fig. 1B; only at still higher concentrations, some hydrolysis of SPC by Cp-SMaseD was observed, ~10% of that for LPC; data not shown). We further observed that both SMases D were capable of hydrolyzing naturally occurring ether-linked LPC (1-*O*-hexadecylglycero-3-phosphocholine or “lyso-platelet-activating factor”) thereby producing alkyl-LPA, the most potent platelet-activating form of LPA (31); the unnatural alkyl-lysophospholipid Et-18-OCH(3) (1-octadecyl-2-methylglycero-3-phosphocholine; $50 \mu\text{M}$) was not hydrolyzed by either SMaseD nor did it act as an inhibitor (data not shown). Many secreted phospholipases, including Sc-PLD and mammalian lyso-PLD (25), are dependent on calcium for their activity. In contrast, SMaseD-induced LPC hydrolysis has an absolute requirement for magnesium rather than calcium, since enzyme activity was abolished in the presence of EDTA but not EGTA, when assayed in buffer containing Ca^{2+} and Mg^{2+} as the only divalent metal ions (Fig. 1C).

By varying the LPC concentration, we determined that the spider and bacterial SMases D have an apparent K_m value for 1-oleoyl-LPC of $44.4 \pm 2.5 \mu\text{M}$ and $21.8 \pm 3.0 \mu\text{M}$, respectively (Fig. 2). Of note, these K_m values are severalfold lower than the physiological LPC levels in human plasma (approximately $150 \mu\text{M}$; (32, 33)), implying that LPC serves as a *bona fide* physio-

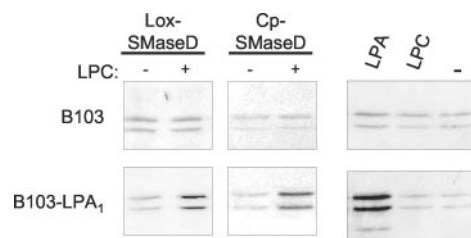


FIG. 3. LPA₁ receptor expression confers cellular susceptibility to SMaseD. Detection of activated MAP kinase (ERK1/2) in LPA receptor-deficient B103 neuroblastoma cells and LPA₁ receptor-expressing B103-LPA₁ cells (26), as determined by using phospho-specific antibodies. Cells in serum-free DMEM were treated for 5 min with the indicated enzymes (20 nM) or 1-oleoyl-LPA (1 μM), in the presence or absence of 1-oleoyl-LPC (10 μM ; complexed to 5 mg/ml fatty acid-free albumin).

logical substrate. For Lox-SMaseD, the apparent V_{max} for LPC was 212 ± 6 nmol/min/mg of protein; for Cp-SMaseD, the V_{max} value was 68 ± 2 nmol/min/mg of protein (Fig. 2). Taken together, these results indicate that the spider and bacterial SMases D possess Mg^{2+} -dependent lyso-PLD activity toward acyl- and alkyl-LPC, but not toward SPC, with both enzymes showing similar catalytic efficiency (V_{max}/K_m , if V_{max} is considered an approximation of k_{cat}). For comparison, the estimated efficiency of SMaseD for LPC hydrolysis is approximately 1 order of magnitude lower than that of mammalian lyso-PLD or “autotaxin” ($K_m = 250 \mu\text{M}$ and $V_{max} = 9$ nmol/min/mg (34)).

We next examined the responsiveness of mammalian cells to SMaseD. While the nonspecific Sc-PLD can trigger diverse signaling events (35, 36), the SMases D failed to induce cellular responses under the same conditions. When preincubated with albumin-LPC complexes, however, the SMases D exhibited biological activity as evidenced by the induction of MAP kinase activity and cellular shape changes, consistent with SMaseD hydrolyzing extracellular LPC to receptor-active LPA. To prove this directly, we used rat B103 neuroblastoma cells that lack endogenous LPA receptors. Fig. 3 shows that SMaseD failed to activate MAP kinase (ERK1/2) in native B103 cells, either in the presence or absence of albumin-LPC. After forced expression of the prototypic LPA₁ receptor in these cells, both SMases D mimicked LPA in activating MAP kinase, but only when preincubated with albumin-LPC (Fig. 3). Likewise, SMaseD mimicked LPA in inducing rapid cytoskeletal contraction in N1E-115 neuroblastoma cells, a typical RhoA-mediated response that also underlies LPA-induced endothelial barrier dysfunction (22, 37); but again, SMaseD acted only in the presence of albumin-LPC (see Supplemental Fig. 2).

Ligand activation of transmembrane receptors causes their internalization from the cell surface. As illustrated in Fig. 4, LPA triggers rapid internalization of its cognate receptors in stably transfected HEK293 cells; at 20–30 min following LPA addition, nearly all receptors were found intracellularly. In contrast, SMaseD alone did not affect LPA receptor localization in LPC-free medium. Upon incubation in albumin/LPC-containing medium, however, SMaseD induced efficient LPA receptor internalization albeit with somewhat slower kinetics than those observed with LPA (Fig. 4). Collectively, these results indicate that (i) SMaseD-mediated hydrolysis of albumin-bound LPC yields receptor-active LPA; and (ii) LPA receptor expression is necessary and sufficient for cellular susceptibility to SMaseD, provided that the cellular microenvironment contains albumin-LPC.

Thus, when SMaseD comes into contact with blood, it will provoke the hydrolysis of albumin-bound LPC to generate LPA. Through activation of its cognate G protein-coupled receptors, LPA evokes a host of biological and pathophysiological responses in numerous cell types, including those of the circulatory and vascular systems (22, 23, 38). LPA levels in whole

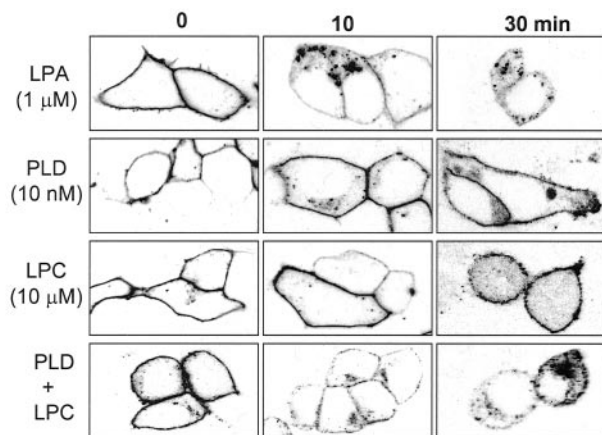


FIG. 4. **LPA₁ receptor internalization induced by SMaseD.** Subcellular localization of LPA₁-GFP receptors stably expressed in HEK293 cells. GFP fluorescence is represented in gray-black for better contrast. Note complete LPA₁ receptor internalization induced by 1-o-leoyl-LPA (1 μ M) after 10 min. Bacterial SMaseD/PLD induces receptor internalization only when preincubated with albumin-LPC (10 μ M). Similar results were obtained with Lox-SMaseD.

blood are normally very low, but the lipid accumulates during blood clotting to promote wound healing processes (39, 40). However, aberrant accumulation of LPA in blood may have deleterious effects, including endothelial barrier dysfunction (37), intravascular coagulation, and infiltration of inflammatory cells such as neutrophils (31, 38, 41, 42), responses that are strongly reminiscent of those to SMaseD *in vivo*. It is also of note that LPA stimulates Ca²⁺ entry into human erythrocytes (43) with subsequent exposure of phosphatidylserine (PS) on the cell surface.² SMaseD likewise induces PS exposure in erythrocytes and this apparent loss of plasma membrane asymmetry is thought to contribute to complement-dependent hemolysis (16). The potential role of lyso-PLD activity in the hemolytic action of SMaseD obviously warrants further study.

Unlike envenomation by a *Loxosceles* spider bite, infections such as lymphadenitis caused by *C. pseudotuberculosis* result not only from the toxic effects of SMaseD *per se* but also from bacterial dissemination to host tissues such as the lymph nodes. A SMaseD-deficient mutant of *C. pseudotuberculosis* is unable to disseminate from the site of inoculation (13), and a popular theory is that vascular hyperpermeability caused by SMaseD, shown here to be likely a result of LPA production, aids in the escape of bacteria into the lymphatic system and subsequent spread to regional lymph nodes (44).

In conclusion, by building on early but largely overlooked evidence (21), we have demonstrated here that spider and bacterial SMases D have intrinsic lyso-PLD activity toward albumin-bound LPC and thereby generate bioactive LPA. To be consistent with phospholipase terminology, SMaseD should therefore be renamed "SM- and LPC-specific PLD." Our data thus provide, at least in part, a mechanistic explanation for the multiple biological responses to SMaseD observed *in vivo*. To what extent the hydrolysis of SM to ceramide 1-phosphate in the plasma membrane of target cells may cooperate with the concurrent generation of extracellular LPA to mediate pathogenicity remains an open question, although a recent report suggests that ceramide 1-phosphate may indirectly activate cytosolic phospholipase A₂ leading to arachidonic acid release (45). Whatever the precise role of ceramide 1-phosphate, the finding that LPA receptor deficiency protects (nucleated) cells against SMaseD suggests that LPA receptors are potential

targets in the treatment of *Loxosceles* envenomation as well as certain corynebacterial infections.

Acknowledgments—We thank Paula Ruurs, Rafael Bernad, and Trudi Hengeveld for excellent technical assistance and Dr. Mary F. Roberts for providing Sc-PLD.

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