Sphingomyelin, ORAI1 channels, and cellular Ca^{2+} signaling

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An immunologist, asked to connect sphingomyelin and T lymphocyte signaling, would likely think first of the sphingomyelin metabolite sphingosine-1-phosphate. That thought is understandable in light of the rapid progress that has been made in studies of sphingosine-1-phosphate signaling and its role in lymphocyte trafficking, and the new clinical avenues that these studies have opened (Spiegel and Milstien, 2011; Kunkel et al., 2013). However, sometimes there are insights to be gained by coming to a subject from a different angle. And sometimes it is possible to cast light on the functioning of immune cells by seeking to understand their dedicated adversaries, pathogenic microorganisms.

The bacterium *Corynebacterium pseudotuberculosis* is a pathogen of sheep, goats, and other livestock, and it causes sporadic cases of human lymphadenitis due to occupational exposure to infected animals (Baird and Fontaine, 2007). The bacterium can cause a chronic infection in lymph nodes, lungs, and other sites. It has been a puzzle how the bacterium, surrounded in these lesions by T cells and other immune cells, deflects immune attack. Starting with the fact that a secreted sphingomyelinase D (SMase D) is an established virulence factor of *C. pseudotuberculosis*, a recent paper in this Journal (Combs and Lu, 2015) asks whether SMase D and sphingomyelin or its metabolites can be implicated in T lymphocyte signaling. The short answer is that, indeed, SMase D inhibits T lymphocyte activation, and this inhibition is likely to contribute to virulence. In a larger context, the paper offers an unexpected insight into the control of Ca^{2+} signaling in T cells, and may have implications for Ca^{2+} signaling in a broader range of mammalian cells.

Sphingomyelin

A logical place to begin the story is with the structure of sphingomyelin (Fig. 1 A). Sphingomyelin is a 1-phosphocholine–substituted ceramide. Ceramide, in turn, is an N-acylated sphingosine. Cleavages by SMase D and SMase C that will become relevant below are indicated in Fig. 1 A. The specific examples of sphingomyelin and ceramide depicted are typically the most abundant representatives of their respective classes in mammalian cells, but the terms "sphingomyelin" and "ceramide" are used here to refer to classes of similar lipids that differ as a result of limited diversity in the chain length of the sphingosine base and somewhat more diversity in the acyl chains. This diversity affects the physical properties of individual sphingomyelins and ceramides, their association with other membrane lipids, and thereby their lateral organization in membranes, and this in turn will have consequences for cell biology. However, the diversity is not a primary consideration here.

Sphingomyelin is very abundant, amounting to perhaps 20% of plasma membrane phospholipid in mammalian cells (van Meer et al., 2008). (It is also present at other sites in the cell.) Within the plasma membrane, it is preferentially localized in the outer leaflet, with a nonnegligible fraction in the inner leaflet (Zachowski, 1993; Murate et al., 2015). Sphingomyelin is tightly anchored in the bilayer by its hydrophobic portion and, because of its polar headgroup, does not exchange readily between membrane leaflets. Thus, outer-leaflet and inner-leaflet sphingomyelin are linked only via metabolic derivatives that do exchange, such as ceramide. Diverse cellular roles have been ascribed to sphingomyelin, including as a structural element of the plasma membrane, a determinant of lateral heterogeneity within the plasma membrane, and a source of lipid-signaling messengers. The role most germane to this discussion is regulation of membrane proteins by direct interactions.

Sphingomyelin metabolism

Before considering some specific instances of regulation by sphingomyelin or its metabolites, it is useful to review sphingolipid synthesis and metabolism (Fig. 1, B and C). De novo synthesis of ceramide from serine and palmitoyl-CoA (or another acyl-CoA), and, at a later step, a second acyl-CoA, is accomplished through a multistep pathway catalyzed by enzymes at the cytoplasmic face of the ER membrane (Gault et al., 2010). For subsequent synthesis of sphingomyelin in the de novo pathway, ceramide is transported to the cytoplasmic face of the Golgi complex by the ceramide transfer protein CERT, equilibrates across the Golgi membrane, and is derivatized at the luminal face of the Golgi membrane by transfer of phosphocholine from phosphatidylcholine. Sphingomyelin reaches the plasma membrane by vesicular transport

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from the Golgi. Although ceramide is centrally positioned in the de novo synthesis of sphingomyelin and in sphingolipid metabolic pathways, it is not a particularly abundant cellular lipid, a fact that underlies its suitability for a signaling role.

Sphingomyelin can be broken down to ceramide at various sites in the cell, and further processed there to other metabolites or converted back to sphingomyelin (Fig. 1 C) (Gault et al., 2010). The subcellular localization of sphingomyelin metabolism can be important, as can the side of a cellular membrane where a processing step is performed. For example, an inner-leaflet pool of plasma membrane sphingomyelin may be used selectively in responding to TNF and certain other signals (Linardic

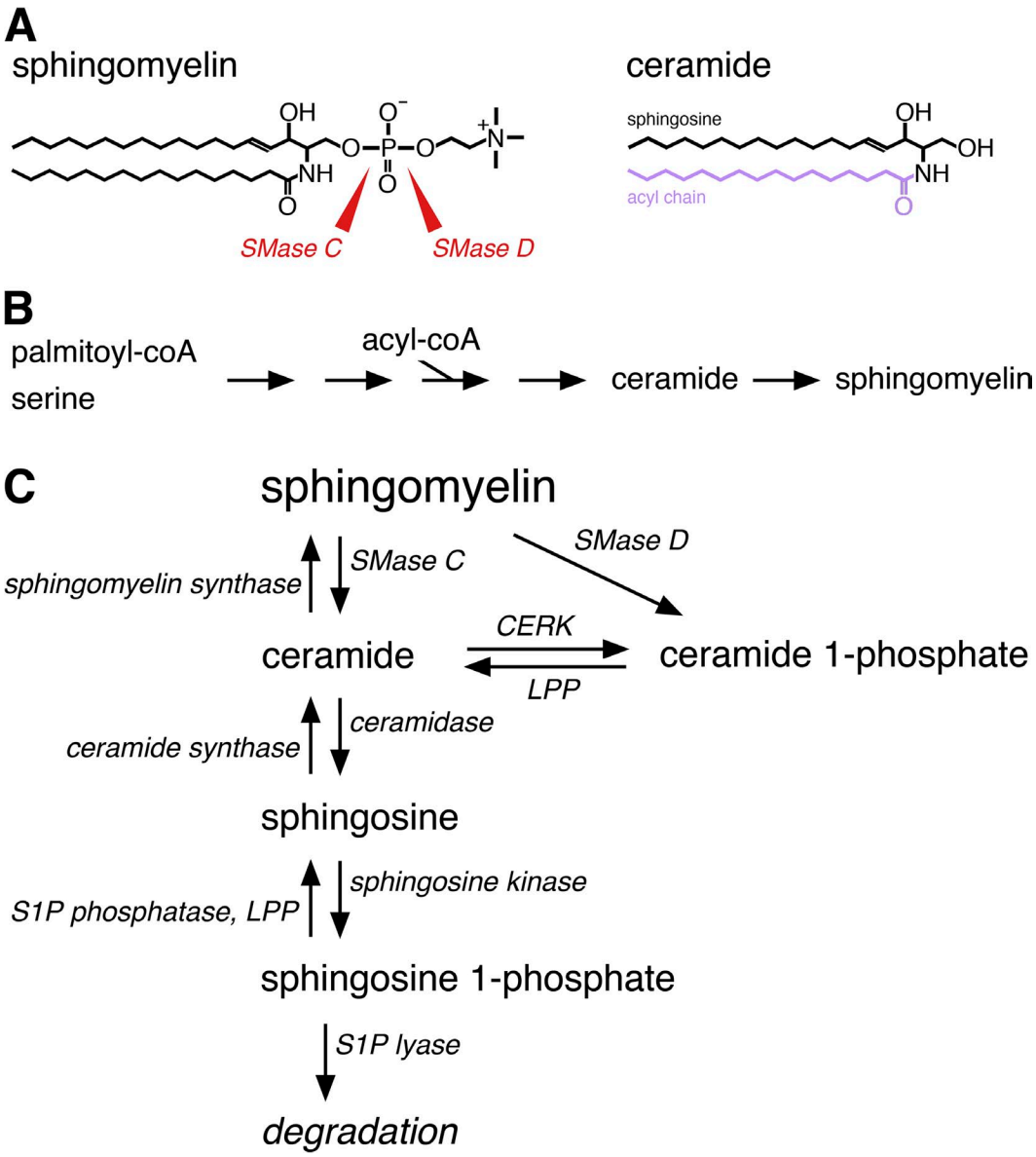

Figure 1. Some sphingolipid fundamentals. (A) Depictions of sphingomyelin and ceramide. The bonds cleaved by SMase C and SMase D are indicated. (B) De novo synthesis of sphingomyelin. The first four enzymatic steps of the de novo pathway take place at the cytoplasmic face of the ER, the last step at the cytoplasmic face of the Golgi complex. Breakdown of cellular sphingomyelin and resynthesis of sphingomyelin from ceramide also occur at other locations in the cell. Separate branches of the de novo synthetic pathway (not depicted) lead from ceramide to the two major classes of glycosphingolipids. (C) Sphingomyelin metabolism. Mammalian cellular enzymes are able to catalyze all the steps indicated, except the conversion of sphingomyelin to ceramide-1-phosphate. Specific SMase D enzymes that catalyze this step have been isolated from *C. pseudotuberculosis* and from spider venoms. The spider SMase D product is ceramide 1,3-cyclic phosphate rather than ceramide-1-phosphate (Lajoie et al., 2013), adding a further nuance, since it is not clear whether the cyclic phosphate is readily metabolized by mammalian cellular enzymes. Although the mapped metabolic pathways provide guidance, it is still necessary to determine the actual extent and pattern of sphingomyelin metabolism for any specific cell type, localization in the cell, and experimental condition. CERK, ceramide kinase; LPP, lipid phosphate phosphatase; S1P phosphatase, sphingosine-1-phosphate phosphatase; S1P lyase, sphingosine-1-phosphate lyase. See Gault et al. (2010) for a complete discussion of sphingolipid synthesis and degradation.

and Hannun, 1994; Andrieu et al., 1996; Philipp et al., 2010). Each metabolic step depicted in Fig. 1 C, except for sphingomyelin cleavage by SMase D, can potentially occur at the plasma membrane in a mammalian cell, catalyzed by endogenous cellular enzymes (Gault et al., 2010; Milhas et al., 2010). However, the presence and activity of specific enzymes, and therefore whether an individual step occurs at a meaningful rate, may vary depending on the particular cell and conditions.

Sphingolipid signaling messengers

Ceramide and sphingosine-1-phosphate have attracted considerable attention as sphingolipid signaling messengers. A key turning point in ceramide research came in 1993, when ceramide was implicated in TNF-induced apoptosis (Obeid et al., 1993). This finding motivated a focus on the connections among ceramide, apoptosis, and cancer biology. For sphingosine-1-phosphate, the discovery that a phosphorylated metabolite of the synthetic immunomodulator FTY720 acts on sphingosine-1-phosphate receptors, and thereby causes lymphocyte sequestration in secondary lymphoid organs (Brinkmann et al., 2002; Mandala et al., 2002), led to intensive research on the sphingolipid signaling mechanisms that control trafficking of T cells and other immune cells. Not surprisingly, there have been sustained efforts to develop therapeutic compounds targeting sphingolipid signaling for treatment of cancer and autoimmune diseases. Both ceramide and sphingosine-1-phosphate have numerous biological effects in addition to their effects on cell proliferation, apoptosis, and immune cell trafficking, and there has been increasing awareness that the tissue location, subcellular site, and context in which these signaling lipids are produced all factor into the biological response. The work on ceramide and sphingosine-1-phosphate has been covered in many informative reviews, including Spiegel and Milstien (2011), Kunkel et al. (2013), Morad and Cabot (2013), and Mendelson et al. (2014).

Sphingomyelin and K^+ channels

Sphingomyelin itself has direct effects on certain channels, which parallel the documented direct effects of the lipids phosphatidylinositol 4,5-bisphosphate (Suh and Hille, 2008; Hansen, 2015; Rohacs, 2015) and phosphatidic acid (Hite et al., 2014) on various ion channels. This modulation by sphingomyelin has been particularly well worked out for specific K^+ channels (Ramu et al., 2006; Xu et al., 2008; Milescu et al., 2009; Combs et al., 2013), in substantial part by the Lu laboratory.

It was first shown for Kv2.1 and Shaker Kv channels that spider SMase D shifts the G-V curve to more negative membrane potentials (Ramu et al., 2006). The finding was subsequently extended to Kv1.3 channels and to some representative voltage-dependent Na^+ and Ca^{2+} channels (Combs et al., 2013). Correspondingly, SMase D shifts the gating Q-V curve of Shaker channels (Ramu et al., 2006). SMase also causes a shift in the Q-V curve of the *Ciona intestinalis* voltage-sensitive phosphatase, indicating that the voltage-sensing domain is the target sensitive to sphingomyelin cleavage. More specifically, it is the S3b–S4 paddle region, as shown by the differing sensitivities to SMase D of chimeric channels in which the four Nav1.4 paddle motifs were grafted individually into the Kv2.1 channel, and by a series of experiments using tarantula toxins to probe lipid–paddle interactions (Milescu et al., 2009). A Shaker channel with most of the paddle deleted retains voltage-dependent gating but lacks sensitivity to SMase D (Combs et al., 2013).

A difference between the effects of SMase D and SMase C is informative. Bacterial SMase C decreases Kv2.1 channel and Shaker channel conductances, and diminishes Shaker channel gating charge movements correspondingly, without shifting the G-V and Q-V curves along the voltage axis (Xu et al., 2008). Yet the part of the channels targeted by sphingolipid is again the voltage sensor, since hanatoxin, which binds to the Kv2.1 voltage sensor, substantially protects the channels from the effect of SMase C (Xu et al., 2008). The unifying interpretation of these divergent effects of SMase D and SMase C has been that at least one, and possibly both, of the distinct sphingomyelin cleavage products can still bind to the channel and influence gating (Xu et al., 2008; Milescu et al., 2009).

Sphingomyelin and the ORAI1 channel

The recent paper by Combs and Lu (2015) extends the class of plasma membrane channels governed directly by outer-leaflet sphingomyelin to include the ORAI1 Ca^{2+} channel. The ORAI1 channel is a highly selective Ca^{2+} channel that is activated in response to the release of Ca^{2+} from ER stores during physiological signaling (Hogan and Rao, 2015). STIM proteins in the ER membrane sense the decrease in free Ca^{2+} in the ER lumen, undergo a conformational change, and collect at sites of close ER–plasma membrane apposition. The cytoplasmic C-terminal domain of STIM bridges the gap between ER and plasma membrane at these sites to recruit and gate ORAI1 channels. ORAI1 channel currents were first characterized in T cells and mast cells, and they have been extensively studied in those cells, but they contribute to physiological Ca^{2+} signaling in most mammalian cells.

Several key observations from the Combs and Lu paper establish that ORAI1 function is sensitive to the application of sphingomyelinases. T cell Ca^{2+} entry after ER store depletion—determined with the Ca^{2+}-sensitive dyes Fura-2 or Indo-1—is decreased by the application of SMase D in the external medium, even though release of Ca^{2+} from ER stores is normal. Recombinant SMase C has a similar effect on Ca^{2+} entry, a point of divergence from the findings with the Kv2.1 channel, and a first hint that sphingomyelin itself, rather than one of its metabolites, might be the key player. The definitive experiments

are electrophysiological. Whole-cell ORAI1 currents elicited by ER Ca^{2+} store depletion in CHO cells overexpressing human STIM1 and ORAI1 are dramatically reduced within 30 s of applying a low concentration of recombinant SMase D. Finally, the classical Ca^{2+} release–activated Ca^{2+} current of human Jurkat T cells, which is carried by ORAI1 channels, is also sharply reduced by SMase D treatment.

The analysis for ORAI channels has necessarily been less detailed than that for K^+ channels. The kinetics of physiological ORAI channel activation in cells are dominated by the movement of STIM and ORAI to ER–plasma membrane junctions, which takes place over tens of seconds. Moreover, neither the very small ORAI1 single-channel currents nor any ORAI1 gating currents have been experimentally resolved. These limitations make it impossible, so far, to pinpoint a particular activation step that is affected, for example, a channel-gating movement, rather than the initial interaction with STIM or ORAI1 channel residence in a permissive lipid microdomain. Nonetheless, the basic conclusion that exposure to SMase D reduces ORAI1 currents is clear.

A first concern in interpreting the new data might have been that the abundant sphingomyelin in the plasma membrane will be converted by SMase D treatment into massive amounts of ceramide-1-phosphate, or conceivably into ceramide 1,3-cyclic phosphate, the product obtained with the distantly related SMase D of spider venom (Cordes and Binford, 2006; Lajoie et al., 2013). ORAI1 channel function might be impaired if ceramide-1-phosphate itself were an inhibitor or if even a minor fraction of sphingomyelin were further processed to an inhibitory metabolite. The authors have dealt with this issue by applying ceramide-1-phosphate to cells and monitoring ORAI1-dependent Ca^{2+} entry. In a carefully designed experiment, the physiological lipid *N*-palmitoyl-ceramide-1-phosphate was delivered to human T lymphocytes using BSA as carrier. Exogenously added ceramide-1-phosphate enhanced the cytoplasmic Ca^{2+} signal observed upon treatment with the SERCA inhibitor thapsigargin—which is determined by the balance between Ca^{2+} leakage from ER stores and Ca^{2+} buffering, sequestration, and extrusion—implying that ceramide-1-phosphate or a metabolite reached responsive sites in the cells. (The site and mechanism were not further defined.) In contrast, ceramide-1-phosphate did not suppress the peak store–dependent Ca^{2+} entry, an indirect measure of ORAI1 channel function, making it unlikely that ceramide-1-phosphate or its metabolites account for the observed effects of SMase D treatment on ORAI1 channels.

A pair of plausible mechanisms

It is reasonable, then, to conclude that sphingomyelin in the plasma membrane supports ORAI1 channel function. One appealing mechanism, favored by Combs and Lu (2015), is that the modulatory role of sphingomyelin reflects direct sphingomyelin–ORAI1 channel binding (Fig. 2 A), similar to the binding of sphingomyelin and its sphingomyelinase products to K^+ channels. A decisive part of the argument for direct sphingomyelin–channel binding in the case of K^+ channels was evidence mapping an interaction specifically to the voltage-sensor paddle (Milescu et al., 2009; Combs et al., 2013). Comparable evidence of sphingomyelin binding to ORAI1 channels will be required before this mechanism can be considered established.

An alternative mechanism consistent with the data is that SMase D alters the organization of plasma membrane lipid microdomains within the ER–plasma membrane junction, resulting in a local environment less conducive to STIM–ORAI interaction or less conducive to ORAI channel gating (Fig. 2 B). This explanation also has appeal in light of recent findings revealing striking rearrangements of lipid and protein within ER–plasma membrane junctions during STIM–ORAI signaling (Sharma et al., 2013; Maléth et al., 2014). Although many

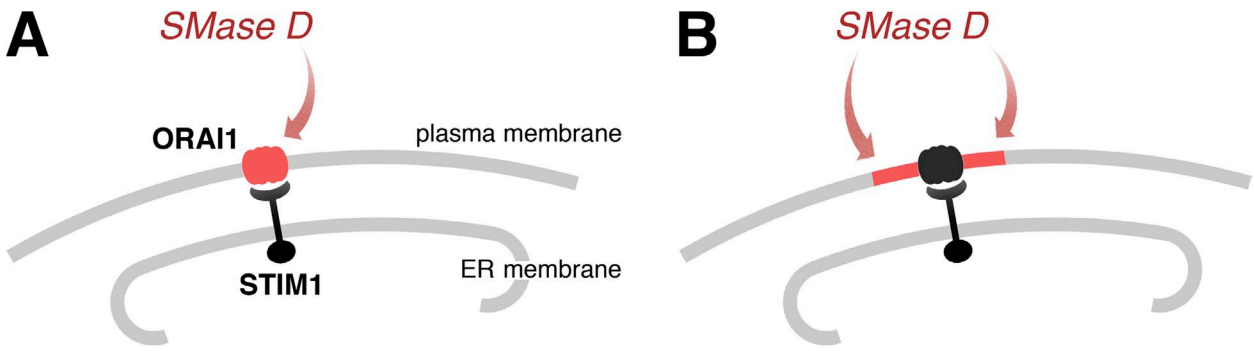

Figure 2. Two possible mechanisms. (A) Cartoon of STIM–ORAI interaction at an ER–plasma membrane junction. The ORAI1 channel itself is highlighted in red as the target of SMase D, representing a possible mechanism in which SMase D acts on sphingomyelin tightly bound to the channel. This mechanism would parallel the action of SMase D on K^+ channels. (B) In an alternative—or additional—mechanism, SMase D might suppress channel function by altering the lipid microdomain surrounding the channel. The relevant changes could be in a relatively localized lipid microdomain, as portrayed with the red highlight, or could involve the plasma membrane of the entire ER–plasma membrane junction.

details remain to be worked out, the gating of ORAI1 by STIM1 is in fact sensitive to local lipid microdomains at the ER–plasma membrane junction (Maléth et al., 2014). Further work will parse the contributions of these two possible mechanisms.

A question for the future

In the long term, the most influential message of the paper by Combs and Lu (2015) might derive from the authors' observation that recombinant SMase C has effects similar to those of SMase D on ORAI1-mediated Ca^{2+} entry. Although mammalian cells lack SMase D, they can signal via secreted SMase C, a differentially processed and trafficked protein product of the *SMPD1* gene that encodes lysosomal "acid SMase C" (Smith and Schuchman, 2008; Jenkins et al., 2009). Thus, the *Corynebacterium* enzyme may exploit a natural negative regulatory mechanism that controls ORAI1 channels under certain conditions and that also modulates other plasma membrane proteins. Although the analogy is speculative, and could prove to be a false lead, it will be worth some effort to determine whether secreted cellular SMase C targets ORAI1 channels.

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