The activity of voltage-gated Na\(^+\) (Na\(_v\)), Ca\(^{2+}\) (Ca\(_v\)), and K\(^+\) (K\(_v\)) channels underlies the action potential in excitable nerve, muscle, and endocrine cells. K\(_v\) channels also help set the resting membrane potential in such nonexcitable cells as human lymphocytes where inhibition of K\(_v\)1.3 channels impairs antigen-stimulated activation of T lymphocytes (Cahalan and Chandy, 2009). However, how voltage-gated channels are tuned in nonexcitable cells remains poorly understood.

Unlike soluble proteins, ion channels are solvated not only by water but also by lipids. Whereas water molecules are chemically homogenous, lipid molecules include many types that differ from each other and could therefore interact with a given channel protein differently. Variation of lipid type at a critical site of a membrane protein may markedly alter the protein’s function. These interactions between a membrane protein and lipid molecules form the physico-chemical basis of lipid-based regulation of membrane protein function. The best example of lipid-based ion channel regulators is phosphatidylinositol 4,5-bisphosphate, a lipid present in the inner leaflet of the membrane bilayer (Huang et al., 1998). Ion channels might then be regulated through metabolic turnover or enzymatic modifications of membrane lipid molecules, manifesting themselves differently in different cell types. Intuitively, such regulation seems more likely to involve relatively minor lipid species. Thus far, channel–lipid interactions in native environments remain poorly understood. For example, there is little experimental information regarding what types of lipids in the outer leaflet of plasma membranes actually interact with native channels in cells. In principle, this information could be obtained by modifying the lipids in situ.

Our group has demonstrated previously in the *Xenopus laevis* oocyte heterologous expression system that specific lipases, such as sphingomyelinase (SMase) D, can effectively modify lipids in situ (Ramu et al., 2006; Xu et al., 2008). SMase D is the active toxin in brown spider venom and is also produced by bacteria such as *Corynebacterium*.
tested a key prediction of the hypothesis that SMase D interact with lipid molecules. That makes it difficult to use the common point mutation to between sphingomyelin and the voltage sensor (Ramu et al., 2006). Such energy reduction is manifested as a shift of both the channels’ conductance–voltage (G-V) and gating charge–voltage (Q-V) relationships in the hyperpolarized direction. This effect has been hypothesized to reflect SMase D altering interactions between sphingomyelin and the voltage sensor (Ramu et al., 2006; Milescu et al., 2009).

With regard to the voltage sensor, crystallographic structures of Kv channels have revealed that the N-terminal part of the fourth transmembrane segment (NTS4) harboring voltage-sensing residues, the C-terminal part of the third transmembrane segment (CTS3 or S3b), and their linker form a helix-turn-helix motif called the “voltage-sensor paddle” (Jiang et al., 2003). Electrophysiological and biochemical studies have demonstrated that the paddle does exist in the channel’s open state where CTS3 functions as a “hydrophobic stabilizer” for NTS4, such that formation of the paddle motif helps stabilize the open state (Xu et al., 2013). It can be transferred among different channel types, and channels remain gated by membrane voltage even after deleting any consecutive residue triplets across CTS3 or NTS4 (Alabi et al., 2007; Xu et al., 2010). Therefore, the paddle motif is remarkably flexible, a feature that makes it difficult to use the common point mutation strategy to identify individual paddle residues that interact with lipid molecules.

Here, we performed the following studies. First, we tested a key prediction of the hypothesis that SMase D stimulates Kv channels by altering the interaction between sphingomyelin and the voltage sensor. Second, we examined whether SMase D also stimulates NaV and CaV channels. Third, we investigated whether sphingomyelin interacts with native voltage-gated channels in mammalian cells, including nonexcitable lymphocytes, an investigation that in turn addressed whether SMase D can stimulate voltage-gated channels in both excitable and nonexcitable cells.

**MATERIALS AND METHODS**

Cell cultures, molecular biology, and biochemistry

For oocyte expression, the cDNAs of wild-type and mutant rNaV1.2a, rNaV1.4a, hNaV1.5a, rNaV1.β1, and hNaV1.β1 were cloned in the pSP64T plasmid, and those of Kvä and the Shaker Kv channel were cloned in the pGEM-HE plasmid, where r and h denote rat and human variants, respectively (Liman et al., 1992).

The protocol for preparing frog oocytes has been described (Milescu et al., 2009). Xenopus oocytes, removed from 13- to 14-day-old Xenopus laevis ova, were dissectioned in collagenase digestion medium (from the Penn Immunology Core (Institutional Review Board lines were obtained from ATCC, whereas human T cells were trypsinized and resuspended in recording solutions. These cell lines were obtained from ATCC, whereas human T cells were from the Penn Immunology Core (Institutional Review Board protocol 705906). For expression of Kv1.3 and hNaV1.β1, CHO cells were transfected with the Fugene6 (Promega) method 24–48 h before study. Harvested Xenopus oocytes were digested in collagenase-containing solution and then stored in a gentamicin-containing solution at 18°C (Spasova and Lu, 1998).

Recombinant SMase D from Loxostes velutus (LSmase D) or C. pseudotuberculosis (PcSmase D) used in the studies was generated as described previously (Ramu et al., 2006, 2007). These two orthologues were shown previously to stimulate Kv channels similarly (Ramuz et al., 2006; Xu et al., 2008).

**Electrophysiological recordings**

Channel currents from whole oocytes, previously injected with the corresponding cRNA, were recorded using a two-electrode voltage-clamp amplifier (OC-725C; Warner). They were filtered at 5 kHz and sampled at 50 kHz with an analogue-to-digital converter (Digidata 1322A; Molecular Devices) interfaced to a PC. pClamp 8 (Molecular Devices) was used for amplifier control and data acquisition. The resistance of electrodes filled with 3M KCl was ~0.2 MΩ. NaV currents were isolated using a P/6 protocol. Steady-state inactivation curves were obtained using a double-pulse protocol, where a 50-ms first pulse from the 120-mV holding potential to between −85 and 10 mV was followed by a 50-ms second test pulse to −20 mV. Bath solutions contained (mM): for Shaker currents, 80 or 0 NaCl, 20 or 100 KCl, 0.3 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.60 adjusted with NaOH; for Kv1.3 currents, 80 NaCl, 20 KCl, 1 MgCl₂, and 10 HEPES, pH adjusted to 7.60 with NaOH; for NaV currents, 100 Na⁺ (90 glutamic acid and 10 Cl⁻), 3 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.60 adjusted with NaOH; for CaV currents, 10 BaCl₂, 20 NaCl, 5 KCl, 60 TEA hydroxide, 1 MgCl₂, and 5 HEPES, pH 7.60 adjusted with methanesulfonic acid. To test the effect of SMase D, 200 ng of SMase D was manually added to a 100-µl bath for a final concentration of 2 ng/µl.

Channel currents from cell lines or human T cells, which were filtered at 5 kHz and sampled at 50 kHz using a Digidata 1322 interfaced to a PC, were recorded in the whole cell configuration with a patch-clamp amplifier (Axopatch 200B). They were filtered and 50 kHz and sampled at 50 kHz using a Digidata 1322 interfaced to a PC. pClamp 8 (Molecular Devices) was used for amplifier control and data acquisition. The resistance of electrodes filled with 3M KCl was ~0.2 MΩ. NaV currents were isolated using a P/6 protocol. Steady-state inactivation curves were obtained using a double-pulse protocol, where a 50-ms first pulse from the −120-mV holding potential to between −85 and 10 mV was followed by a 50-ms second test pulse to −20 mV. Bath solutions contained (mM): for Shaker currents, 80 or 0 NaCl, 20 or 100 KCl, 0.3 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.60 adjusted with NaOH; for Kv1.3 currents, 80 NaCl, 20 KCl, 1 MgCl₂, and 10 HEPES, pH adjusted to 7.60 with NaOH; for NaV currents, 100 Na⁺ (90 glutamic acid and 10 Cl⁻), 3 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.60 adjusted with NaOH; for CaV currents, 10 BaCl₂, 20 NaCl, 5 KCl, 60 TEA hydroxide, 1 MgCl₂, and 5 HEPES, pH 7.60 adjusted with methanesulfonic acid. To test the effect of SMase D, 200 ng of SMase D was manually added to a 100-µl bath for a final concentration of 2 ng/µl.

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removing the extracellular portion of the voltage-sensor paddle eliminates the SMase D sensitivity of Shaker Kv channels

If SMase D stimulates Kv channels by altering the interaction between sphingomyelin and the voltage sensor to promote the open state, removing most of the voltage-sensor paddle sequence should attenuate or eliminate the SMase D sensitivity. To test this prediction, we used a Shaker mutant in which 43 (I325-V367) of the 49 residues in the paddle sequence were replaced by three glycine residues (Fig. 1B) (Xu et al., 2010). Like the wild type, this “deletion” mutant expresses voltage-gated NaV channels in N2A cells, 250-nA prepulses to −120 mV were used to minimize the confounding effect of potential steady-state inactivation. For Kv channel recordings, the bath solution contained (mM) 145 NaCl, 5 or 20 KCl, 0.3 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with NaOH, and the electrode solution contained (mM) 140 KCl, 10 EGTA, 1 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with KOH. For NaV channel recordings, the bath solution contained (mM) 145 NaCl, 5 KCl, 0.3 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with NaOH, and the electrode solution contained (mM) 140 CaCl2, 10 EGTA, 1 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with CsOH. For current-clamp experiments, constant currents (−3 to −30 pA) were injected to maintain a holding potential negative to −90 mV. Several series of 15–30 current pulses ranging from 100 to 1,000 pA (in 10–50-pA increments) were injected into individual cells for 2 ms to assess the action potential threshold. In these studies, the bath solution contained (mM) 145 NaCl, 5 KCl, 0.3 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with NaOH, and the electrode solution contained (mM) 110 K-gluconate, 20 KCl, 5 NaCl, 3 EGTA, 0.3 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.30 adjusted with KOH. To test the effect of SMase D, 300 ng CpSMase D was manually added to the 600-µl recording chamber to achieve a final concentration of 0.5 ng/µl.

A paired sample t test was used to determine the statistical significance of the changes both in the action potential threshold and in the amount of injected current required to depolarize the membrane potential to threshold before and after SMase D treatment.

RESULTS

Removal of the extracellular portion of the voltage-sensor paddle eliminates the SMase D sensitivity of Shaker Kv channels

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current (Fig. 1, C and E). The midpoint of its G-V curve (constructed from the tail currents) is comparable to that of the wild type, but the slope is reduced because it has fewer voltage-sensing residues (Fig. 1, G and H). As expected, before SMase D treatment, stepping the membrane potential from the holding potential to −40 mV elicited essentially no visible wild-type Shaker current, whereas after SMase D treatment, the same voltage step elicited sizable current (Fig. 1, C and D). In contrast, the deletion mutant conducted comparably small currents at −40 mV before or after the SMase D treatment (Fig. 1, E and F). Effectively, SMase D shifted the G-V curve of the wild-type channels in the hyperpolarized direction but had little effect on that of the deletion mutant (Fig. 1, G and H). These results demonstrate the dependence of SMase D sensitivity on the paddle motif.

SMase D stimulates both heterologously expressed \( K_{v1.3} \) channels in CHO cells and native \( K_{v1.3} \) channels in human T lymphocytes

A major goal of this study is to use SMase D as a tool to determine whether sphingomyelin interacts with native \( K_{v1.3} \) channels in human T lymphocytes and whether chemical modification of sphingomyelin’s phosphorylcholine group by SMase D stimulates these channels. These cells are known to express a single type of \( K_{v} \) channel, \( K_{v1.3} \) (Cahalan and Chandy, 2009), which we have found to be sensitive to SMase D in the oocyte expression system (Fig. 2 A). A direct demonstration of SMase D sensitivity of \( K_{v1.3} \) in human lymphocytes would in turn offer a clue to the question of how these voltage-gated ion channels may be tuned in nonexcitable cells.

Sphingomyelin may make up as much as 25% of total phospholipids in oocyte membranes as opposed to <15% in many mammalian cells (Stith et al., 2000; Hermansson et al., 2005; Hill et al., 2005; Pike et al., 2005). This high sphingomyelin content of oocyte membranes could create artificial sphingomyelin–channel interactions absent in some mammalian cells. To address this issue, we first tested SMase D on \( K_{v1.3} \) channels heterologously expressed in CHO cells where sphingomyelin has been determined to be only 12–14% of total plasma membrane phospholipids (Hermansson et al., 2005; Pike et al., 2005). As shown in the top panel of Fig. 2 B, a voltage step to −40 mV caused \( K_{v1.3} \) channels to conduct no visible current, and current gradually appeared after the addition of SMase D; the time course is presented in the bottom panel. Examples of \( K_{v1.3} \) current responding to a series of voltage steps before and after SMase D treatment are shown in Fig. 2 (C and D). SMase D treatment shifted the G-V curve by about −15 mV (Fig. 2 E).
This result shows that SMase D sensitivity is not merely a consequence of the high sphingomyelin content of oocytes. Additionally, SMase D also affects the kinetics of channel gating (Fig. 2, C and D). We estimated the rate of both current onset upon depolarization and current decay upon hyperpolarization from single-exponential fits, and plotted the τ values against membrane voltage (Fig. 2 F). SMase D left-shifts these kinetics plots by amounts comparable to that of the steady-state G-V curve. All of these findings can be explained by the scenario in which the removal of the choline group by SMase D creates a local negative offset in membrane voltage.

In principle, the apparent interactions between sphingomyelin molecules and K_v1.3 channels heterologously expressed in oocytes and CHO cells may be the consequence of overexpression that drives the channels into some membrane domains differing from the native membrane environment of K_v1.3 channels in lymphocytes. To rule out this possibility, we directly tested SMase D against native K_v1.3 channels in human peripheral blood T lymphocytes, whose plasma membrane contains 11–12% sphingomyelin (Leidl et al., 2008). Fig. 3 (A–E) shows that after the addition of SMase D, K_v1.3 current at −40 mV markedly increased and the channels’ G-V curve shifted by about −15 mV. However, in the absence of SMase D treatment, T cells exhibited a small “spontaneous,” time-dependent shift (Fig. 3 F), a known phenomenon of this particular cell type (Cahalan et al., 1985). This spontaneous shift itself did not result in measurable increases in K_v1.3 activity at −50 mV, compared with an increase from near zero to ~20% of maximum after SMase D treatment. Nonetheless, to rule out the possibility that the SMase D effect is merely an amplification of the time-dependent shift, we repeated the experiment on endogenous K_v1.3 channels in Jurkat T cells, a leukemic cell line that lacks certain proteins (Abraham and Weiss, 2004) and exhibits no time-dependent G-V shift under comparable conditions (Fig. 3 H). Fig. 3 G shows that SMase D also shifted the G-V relationship of K_v1.3 channels in Jurkat cells in the hyperpolarized direction. Thus, sphingomyelin apparently interacts with K_v1.3 channels in human T lymphocytes, and removal of its choline group by SMase D indeed stimulates K_v1.3 in these cells at −50 mV, the typical

Figure 3. SMase D activates native K_v1.3 channels in human T cells. (A) K_v1.3 currents of a T cell recorded in the presence of 5 mM [K^+]_ext elicited by stepping the voltage every 15 s from the −100-mV holding potential to the −40-mV test potential. After the addition of SMase D, the current gradually increased. (B) Time course of K_v1.3 current increase after the addition of SMase D. (C and D) K_v1.3 currents ([K^+]_ext = 20 mM) elicited by stepping the membrane voltage from the −100-mV holding potential to between −80 and 10 mV in 10-mV increments at 15-s intervals before (C) and after (D) the addition of SMase D to the bath solution, where the current traces for −30 mV are colored maroon. Dashed lines in A, C, and D indicate zero current levels. (E–H) G-V curves of K_v1.3 channels in T cells (E and F) and Jurkat cells (G and H) before (open squares) and after (closed circles) either a 3-min treatment with SMase D (E and G) or a 3-min control period in the absence of SMase D (F and H), where all data are presented as means ± SEM (n = 5). The curves are fits of Boltzmann functions, yielding V_1/2 = −26.1 ± 0.4 mV and Z = 3.7 ± 0.2 (open squares), or V_1/2 = −42.4 ± 0.6 mV and Z = 4.0 ± 0.3 (closed circles) for E; V_1/2 = −27.0 ± 0.4 mV and Z = 3.8 ± 0.2 (open squares) or V_1/2 = −33.2 ± 0.5 mV and Z = 4.1 ± 0.3 (closed circles) for F; V_1/2 = −28.8 ± 0.4 mV and Z = 4.3 ± 0.2 (open squares) or V_1/2 = −38.6 ± 0.5 mV and Z = 4.5 ± 0.2 (closed circles) for G; and V_1/2 = −30.6 ± 0.5 mV and Z = 3.5 ± 0.2 (open squares) or V_1/2 = −32.1 ± 0.5 mV and Z = 3.6 ± 0.2 (closed circles) for H.
resting membrane potential of human T cells (Cahalan and Chandy, 2009).

SMase D stimulates NaV and CaV channels
Because Kv, NaV, and CaV channels all have similar voltage sensors (Swartz, 2008), we wondered whether NaV and CaV channels also interact with sphingomyelin such that they are sensitive to SMase D. For technical simplicity, we started by examining skeletal muscle rNaV1.4 (a type) (Mori et al., 1991) and CaV2.2 channels expressed in Xenopus oocytes. These channels normally exhibit little current at −50 mV, but after exposure to SMase D, inward current increased dramatically (Fig. 4 A). SMase D activated the channels by shifting their G-V relation in the hyperpolarized direction (Fig. 4 B). In addition, SMase D also shifted the G-V curves of CaV channels CaV2.1α (P/Q type) (Mori et al., 1991) and CaV2.2α (N type) (Williams et al., 1992), coexpressed with β1α and α2δ subunits in oocytes (Fig. 4, C and D). This SMase D effect on CaV channels might be lessened by the use of a high concentration of extracellular Ba2+ as the charge carrier. In any case, SMase D can stimulate NaV and CaV channels, as well as Kv channels.

SMase D slows fast inactivation of NaV channels in Xenopus oocytes
When examining wild-type NaV channels coexpressed with the NaVβ1 subunit in Xenopus oocytes, we surprisingly found that SMase D had a strong impact on the kinetics of channel inactivation. Here, we mainly discuss the data obtained from rNaV1.4 channels. However, as we will later examine native NaV1.2 channels in mammalian cells, we illustrate here as a control that in oocytes, heterologously expressed rNaV1.2 and rNaV1.4 channels exhibited similar responses to SMase D (Fig. 5, A–D). SMase D markedly slowed the inactivation of rNaV1.4α + β1 with a small alteration of the steady-state inactivation curve (Fig. 5, C–E). The current-voltage (IV) curves constructed from NaV peak currents (Fig. 5 F) show that, as with rNaV1.4-IR, SMase D clearly induced inward Na+ current at membrane potentials as negative as −50 mV. As discussed above, this phenomenon reflects a leftward shift of the G-V relation and therefore of the I-V relation. Given that increasing the extracellular Mg2+ concentration attenuates the SMase D effect on activation gating (Ramu et al., 2006), this maneuver should also attenuate its effect on the inactivation kinetics if SMase D affects both gating properties by the same mechanism. Fig. 6 A shows that in the presence of a high extracellular concentration (10 mM) of Mg2+, the application of SMase D resulted in little shift of the I-V curve of rNaV1.4α + β1. It nonetheless still slows inactivation (Fig. 6 B). These results not only confirm that under this high Mg2+ condition SMase D remains active but also show that SMase D does not affect activation energetic and inactivation kinetics by the same mechanism.

We next investigated whether the SMase D effect on inactivation kinetics depends on the NaVβ1 subunit. For this particular study, we switched to hNaV1.4 for two reasons. First, rNaV1.4 and hNaV1.4 behave similarly (Trimmer et al., 1989; George et al., 1992). Second, we realized that we would need to perform a comparative study between NaV1.4 and NaV1.5 and had already obtained the human versions of the two channels. When expressed alone in oocytes, NaV1.4α is known to exhibit slow (~8-ms time constant τ) inactivation kinetics (Trimmer et al., 1989). Inactivation of hNaV1.4α was barely affected by SMase D treatment (Fig. 6 C). Coexpression of the NaVβ1 subunit is known to markedly accelerate the inactivation rate (τ of ~1 ms; Fig. 6 D) (Isom et al., 1992; Patton et al., 1994). Under this condition, SMase D treatment slowed inactivation (Fig. 6 D), causing it to follow a double- rather than a single-exponential time course in most cases (Table 1). The τ values for the fast and slow components are ~1 and ~7 ms, comparable to

![Figure 4](image-url)
SMase D stimulates activation but does not slow inactivation of native NaV channels in tested mammalian cells

To test whether NaV channels in mammalian cells exhibit the same types of SMase D sensitivity observed in Xenopus oocytes, we continued to use CHO cells for the following reasons. These cells endogenously express NaV1.2 channels with fast inactivation and may not express endogenous NaVβ1 subunits (West et al., 1992b; Lalik et al., 1993; Isom et al., 1995). Recall that in oocytes, when coexpressed with the NaVβ1 subunit, rNaV1.2 exhibited fast inactivation kinetics that were sensitive to SMase D (Fig. 5, A and B). Fig. 7 (A and B) shows two series of NaV currents recorded at various membrane potentials from a CHO cell before or after SMase D treatment. For better comparison, the current traces at −20 mV, recorded before and after SMase D treatment, are superimposed in Fig. 7C. To our surprise, unlike what was observed in oocytes, SMase D did not slow the inactivation and, if anything, slightly accelerated it because of an expected left-shift of the voltage dependence (Fig. 7, C and F). However, like what was observed in oocytes, SMase D did activate NaV channels in CHO cells at more hyperpolarized potentials, causing a leftward shift of the descending limb of the I-V curve; peak current also increased (Fig. 7D). In the ascending limb, which primarily reflects those with and without coexpression of the NaVβ1 subunit, respectively (Table 1). In most oocyte batches, the relative amplitude of the slow phase is ~60%, although in some batches it reaches practically 100%. Therefore, in a variable fraction (60–100%) of channels, SMase D eliminates the NaVβ1 subunit’s apparent acceleration of the inactivation.

For comparison, we also studied cardiac hNaV1.5 channels. Inactivation kinetics of cardiac hNaV1.5 channels are comparably fast (τ of ~1 ms) with or without the NaVβ1 subunit (Fig. 6, E and F, and Table 1) (Makita et al., 1994). Under either condition, SMase D treatment slows the inactivation kinetics of a large fraction of hNaV1.5 channels (Fig. 6, E and F, and Table 1). The S5–S6 linker of the α subunit underlies the NaVβ1-subunit effect on inactivation kinetics (Qu et al., 1999). An hNaV1.4 mutant, whose S5–S6 linkers are all replaced by the corresponding ones from hNaV1.5, acquires fast inactivation kinetics with and without coexpression of the NaVβ1 subunit (Vilin et al., 1999). We therefore tested whether the inactivation kinetics of this mutant also become sensitive to SMase D, even without coexpression of the NaVβ1 subunit. This was in fact the case (Fig. 6G and Table 1). Thus, transferring the S5–S6 linkers of hNaV1.5α channels to hNaV1.4α channels confers both the fast inactivation kinetics and the SMase D sensitivity of hNaV1.5α upon hNaV1.4α channels.

**Figure 5.** SMase D stimulates NaVα + β1 channels in oocytes and slows their inactivation. (A–D) rNaV1.2α + β1 (A and B) or rNaV1.4α + β1 (C and D) currents from an oocyte, before (A and C) and after (B and D) SMase D treatment, by stepping the membrane voltage from the −120-mV holding voltage to various test potentials between −90 and 10 mV. (E and F) Steady-state inactivation curves (E) and I-V relations (F) before (open squares) and after (closed circles) SMase D treatment; all data are presented as means ± SEM (n = 3–6). Curves in E are fits to single Boltzmann functions, yielding $V_{1/2} = -52.0 ± 0.2$ mV and $Z = 4.7 ± 0.2$ (open squares), and $V_{1/2} = -56.1 ± 0.8$ mV and $Z = 3.6 ± 0.2$ (closed circles).
decreasing driving force, the two curves tended to merge as the open probability of channels approached its maximum. As expected for an effect on voltage-sensor activation, SMase D also left-shifted both the steady-state inactivation curve (Fig. 7 E) and the voltage dependence of the fast inactivation kinetics in the hyperpolarized direction (Fig. 7 F). Thus, as opposed to what is observed in Xenopus oocytes, SMase D does not slow inactivation of Na\textsubscript{v} channels in mammalian CHO cells. However, as in oocytes, it does promote channel activation in these

| Parameters | hNa\textsubscript{v}1.4α | hNa\textsubscript{v}1.4α + β1 | hNa\textsubscript{v}1.5α | hNa\textsubscript{v}1.5α + β1 | mUT\textsubscript{hNa\textsubscript{v}1.4α} |
|------------|------------------|------------------|------------------|------------------|------------------|
| Control    |                  |                  |                  |                  |                  |
| \(\tau\)   | 7.8 ± 0.2 ms\(^d\) | 7.8 ± 0.2 ms\(^d\) | 7.8 ± 0.2 ms\(^d\) | 7.8 ± 0.2 ms\(^d\) | 7.8 ± 0.2 ms\(^d\) |
| \(\tau_1\) | 9.5 ± 0.2 ms\(^d\) |                  |                  |                  |                  |
| \(\tau_2\) |                  |                  |                  |                  |                  |
| \(A_1/(A_1 + A_2)\) | 0.32 ± 0.01\(^d\) | 0.34 ± 0.01\(^d\) | 0.34 ± 0.01\(^d\) | 0.34 ± 0.01\(^d\) | 0.34 ± 0.01\(^d\) |
| \(A_2/(A_1 + A_2)\) | 0.68 ± 0.01\(^d\) | 0.66 ± 0.01\(^d\) | 0.66 ± 0.01\(^d\) | 0.66 ± 0.01\(^d\) | 0.66 ± 0.01\(^d\) |

\(^a\)Data is presented as mean ± SEM (\(n = 4 – 8\)).
\(^b\)Contains the S5–S6 linkers of hNa\textsubscript{v}1.5α.
\(^c\)Inactivation time courses were fit to \(I(t) = A_1 e^{-t/\tau} + A_2 e^{-t/\tau_2}\).
cells. We also tested the effect of SMase D on Na\textsubscript{V} channels in a second commonly used cell line, mouse neuroblastoma N2A cells, and observed qualitatively the same effects (Fig. 8).

To test whether the failure of SMase D to slow Na\textsubscript{V} inactivation in CHO cells was caused by the apparent absence of the Na\textsubscript{V}β1 subunit, we expressed the Na\textsubscript{V}β1 subunit in CHO cells. As reported previously, expression of the Na\textsubscript{V}β1 subunit shifted the voltage dependence of steady-state inactivation (Fig. 7, E vs. G) and inactivation kinetics (Fig. 7, F vs. H) in the hyperpolarized direction (Isom et al., 1995). These results argue that native Na\textsubscript{V} channels are under the influence of the expressed Na\textsubscript{V}β1 subunit. Despite the functional expression of the Na\textsubscript{V}β1 subunit in CHO cells, just as without this expression, SMase D only slightly accelerated Na\textsubscript{V} inactivation instead of slowing it as in oocytes. Thus, the insensitivity of Na\textsubscript{V} inactivation kinetics to SMase D in CHO cells is not simply caused by a lack of the Na\textsubscript{V}β1 subunit.

**Figure 7.** Effect of SMase D on native Na\textsubscript{V} channels in CHO cells. (A–C) Na\textsubscript{V} currents from a CHO cell before (A) and after (B) the addition of SMase D, elicited by stepping the membrane voltage from the −100-mV holding voltage to various test potentials between −80 and 40 mV in 10-mV increments. The current traces at −20 mV from A (black) and B (maroon) are superimposed in C. Dashed lines indicate zero current levels. (D) I-V relations before (open squares) and after (closed circles) SMase D treatment; all data are presented as means ± SEM (n = 5). (E–H) Steady-state inactivation curves (E and G) and voltage dependence of inactivation time constants (F and H) from cells expressing (G and H) or not expressing (E and F) hNa\textsubscript{V}β1 before (open squares) and after (closed circles) SMase D treatment; all data are presented as means ± SEM (n = 5). Curves are fits to single Boltzmann functions, yielding $V_{1/2} = -26.8 \pm 0.4$ mV and $Z = 4.7 \pm 0.3$ (open squares), and $V_{1/2} = -36.8 \pm 0.3$ mV and $Z = 4.6 \pm 0.2$ (closed circles) in E; and $V_{1/2} = -40.9 \pm 0.4$ mV and $Z = 3.5 \pm 0.2$ (open squares), and $V_{1/2} = -53.9 \pm 0.7$ mV and $Z = 3.6 \pm 0.3$ (closed circles) in G.

SMase D shifts excitable cells’ action potential threshold in the hyperpolarized direction

In principle, a left-shift of the G-V curve of Na\textsubscript{V} channels may result in an increase of cellular excitability. In fact, shifts as small as 5 mV can have a profound functional impact, including causing human genetic diseases (Dib-Hajj et al., 2010). We thus investigated whether SMase D could tune cellular excitability. We chose to use N2A cells because their spherical shape and modest density of Na\textsubscript{V} channels would allow better electrophysiological control compared with primary neurons. As shown in Fig. 8 D, SMase D treatment shifted the I-V curve of Na\textsubscript{V} channels in N2A cells by about −10 mV. This left-shift of the Na\textsubscript{V} I-V curve caused by SMase D is expected to result in a shift of the action potential threshold in the hyperpolarized direction. We tested this prediction by examining action potentials in the current-clamp mode. We injected a small constant negative current, hyperpolarizing the resting membrane potential to around −100 mV to minimize the confounding factors.
DISCUSSION

Voltage-gated ion channels are embedded in membrane lipids. Questions remain as to what types of phospholipids, including relatively minor species, actually interact with these integral membrane proteins in native membrane environments, and how such interactions may impact channel function. As a step toward answering these important questions, we have used SMase D as a tool to investigate sphingomyelin’s functional impact on voltage-gated ion channels in native environments.

Previously, our group discovered that in Xenopus oocytes, SMase D stimulates the Shaker and Kv2.1 channels (Ramu et al., 2006). Here, we show that SMase D promotes activation of all three classes of classical voltage-gated channels, and that removing most of the Shaker channel’s paddle sequence virtually eliminates the channel’s SMase D sensitivity (Figs. 1 and 4). These results, together with the following previous findings, support the hypothesis that SMase D produces this effect by altering interactions between voltage sensors and sphingomyelin molecules. First, an elevated extracellular divalent cation concentration antagonizes SMase D’s action (Ramu et al., 2006). Increasing extracellular divalent cation concentrations is known to reduce the effect of membrane surface charges on voltage sensors and inhibits activation of voltage-gated channels (Frankenhaeuser effect of steady-state inactivation (Fig. 8 E)). Fig. 9 (A and B) shows action potentials recorded from a cell, elicited before or after SMase D treatment by a series of brief and incremental depolarizing current pulses. The SMase D–caused shift of the action potential threshold in the hyperpolarized direction can be more clearly seen in Fig. 9 C. Before SMase D treatment, action potentials were triggered at about −40 mV, whereas afterward, the threshold dropped to about −50 mV. To further illustrate this point, we plotted the peak voltage for both types of traces against the membrane voltage at the end of the brief current pulse (Fig. 9 D). As expected, passive voltage responses initially rose linearly, but once action potential threshold was exceeded, the voltage rose steeply. SMase D creates a leftward shift of this plot. On average, the action potential threshold was shifted by −13 mV after SMase D treatment, and a smaller amount of injected current was required to depolarize the membrane potential to this action potential threshold (Fig. 10, A and B). As a control, we show that neither the action potential threshold nor the amount of the injected current exhibited any significant spontaneous change without the application of SMase D during the same period of time (Figs. 9, E–H, and 10, A and B). Thus, SMase D increases the N2A cell’s excitability by making the action potential threshold more negative.

Figure 8. SMase D stimulates native Na⁺ channels in N2A cells. (A–C) Na⁺ currents from an N2A cell before (A) and after (B) the addition of SMase D, elicited by stepping the membrane voltage from the −100-mV holding voltage to a −120-mV prepulse and then to various test potentials between −80 and 40 mV in 10-mV increments. The current traces at −20 mV from A (black) and B (maroon) are superimposed in C. Dashed lines indicate zero current levels. (D–F) I–V relations (D), steady-state inactivation curves (E), and voltage dependence of inactivation time constants (F) before (open squares) and after (closed circles) SMase D treatment; all data are presented as means ± SEM (n = 5). Curves in E are fits to single Boltzmann functions, yielding V1/2 = −67.2 ± 0.4 mV and Z = 3.1 ± 0.1 (open squares), and V1/2 = −76.0 ± 0.3 mV and Z = 3.0 ± 0.1 (closed circles).
channel and a K⁺ channel gated by both voltage and Ca²⁺ appear to be insensitive to SMase D, even when heterologously expressed in the oocytes (Ramu et al., 2006). Second, a mutant cycle analysis of the interaction between a gating-modifier toxin and the voltage-sensor paddle with and without SMase D treatment suggests that sphingomyelin interacts with the paddle motif (Milescu et al., 2009). Third, SMase D also causes a hyperpolarizing shift of the Q-V curve of a bacterial voltage-sensitive phosphatase, a protein quite unlike a channel apart from its voltage sensor (Milescu et al., 2009). Additionally, structural and spectroscopic studies have shown that the outermost extracellular voltage-sensing residues of Kv channels are indeed poised to interact with membrane lipids (Cuello et al., 2004; Long et al., 2007; Krepkiy et al., 2012). Moreover, electrophysiological studies indicate that the phosphoryl group of membrane lipids is critical for the proper function of the channels’ voltage sensors (Schmidt et al., 2006; Xu et al., 2008; Zheng et al., 2011). Therefore, it is reasonable to conclude that the channels’ voltage sensors interact with sphingomyelin so that SMase D modification of sphingomyelin’s head group promotes voltage-sensor activation.

The generality of the channel-stimulating effect of SMase D has been called into question by the following findings. First, under the reported conditions, the Kv10.1 channel and a K⁺ channel gated by both voltage and Ca²⁺ appear to be insensitive to SMase D, even when heterologously expressed in the oocytes (Ramu et al., 2006).
This observation raises the question of whether the observed SMase D effect on voltage gating occurs only in a small subset of Kv channels. To address this question, we have tested other Kv channels, such as Kv1.3 as well as NaV and CaV channels in oocytes, and found that all of these tested channels are stimulated by SMase D (Figs. 2 A, 4, and 5). Thus, this SMase D effect seems to be a widespread phenomenon across the voltage-gated channel family. Second, the suggestion that the Xenopus oocyte membrane has much more sphingomyelin than that of many mammalian cell types, including CHO cells and lymphocytes, raises the question of whether the SMase D effect only occurs in cell membranes with very high sphingomyelin content (Stith et al., 2000; Hermansson et al., 2005; Hill et al., 2005; Pike et al., 2005; Leidl et al., 2008). To address this second question, we have tested heterologously expressed Kv1.3 channels and native NaV1.2 channels in CHO cells and have found that SMase D does promote channel activation in both cases (Figs. 2, B–F, and 7). These results indicate that the voltage sensors of these voltage-gated channels do interact with sphingomyelin, even in cell membranes where sphingomyelin is a relatively minor phospholipid species. Thus, either the channels’ voltage sensors tend to interact with sphingomyelin over some other lipid types or the channels tend to be trafficked to sphingomyelin-rich domains.

The stimulatory effect of SMase D on voltage-gated channels implies that SMase D can increase cellular excitability. It may even be able to tune the activity of voltage-gated channels in nonexcitable cells. We thus directly tested SMase D against native voltage-gated channels in relevant types of mammalian cells. In a series of studies, we have demonstrated that SMase D can indeed effectively stimulate NaV channels in excitable cells, boosting their excitability (Figs. 8–10). SMase D does so by a mechanism different from that of canonical excitatory neurotransmitters, which generally increase excitability by depolarizing the resting membrane potential toward the action potential threshold. It is noteworthy that although both types of mechanisms can effectively enhance cellular excitability by narrowing the gap between the resting membrane potential and the action potential threshold, they differ fundamentally in that depolarizing the membrane potential, unlike shifting the action potential threshold in the hyperpolarized direction, directly affects the driving force for permeant ions, e.g., Ca^2+. Additionally, we have found that SMase D robustly stimulates endogenous Kv1.3 channels in nonexcitable human T lymphocytes (Fig. 3), an experimental demonstration of how voltage-gated ion channels may be tuned in these nonexcitable cells.

One important and unexpected SMase D effect is that it markedly slows inactivation of certain NaV channels under specific expression conditions. In oocytes, SMase D slows the inactivation of Na_{1.4α} channels only when they are coexpressed with the Na_{1β} subunit and that of Na_{1.5α} channels, even when they are not coexpressed with the Na_{1β} subunit (Figs. 5 and 6). In stark contrast, SMase D does not slow the inactivation kinetics of native NaV channels in two tested mammalian cell lines (Figs. 7 and 8), and this is true even when the Na_{1β} subunit is functionally overexpressed (Fig. 7 H). These findings offer new insight into how the Na_{1β} subunit and phospholipids may affect NaV inactivation kinetics.

First, the appearance that in Xenopus oocytes SMase D eliminates the Na_{1β} acceleration of Na_{1.4α} inactivation may be explained by the scenario in which sphingomyelin couples the Na_{1αβ} subunit interaction. However, this scenario cannot straightforwardly explain why in oocytes Na_{1.5α} itself exhibits fast inactivation and its inactivation kinetics can likewise be slowed by SMase D. An alternative explanation is that the Na_{1β} subunit helps optimize the sphingomyelin–NaV_{1.4α} interactions that enable fast inactivation, whereas Na_{1.5α} can achieve functionally comparable interactions with sphingomyelin even without the Na_{1β} subunit.

Second, in mammalian cells, NaV’s activation energetics but not its inactivation kinetics are sensitive to SMase D (Figs. 7 and 8). Thus, sphingomyelin has functionally impactful interactions with the channel protein at the activation-related site(s) but not at the site(s) related to inactivation kinetics. The idea of functionally separate channel–sphingomyelin interactions is supported by the following findings. A high concentration of the extracellular divalent cation Mg^2+ apparently counteracts the SMase D effect on the channels’ activation energetics but not on their inactivation kinetics (Fig. 6, A and B). Furthermore, SMase D sensitivity of activation energetics depends on the paddle motif, whereas SMase D sensitivity of inactivation kinetics can be conferred upon insensitive channels by transferring the S5–S6 linkers from sensitive channels (Figs. 1 and 6). The observation that in mammalian cells NaV channels exhibit SMase D–insensitive fast-inactivation implies that the site related to inactivation kinetics is occupied by lipids other than sphingomyelin. In oocytes, sphingomyelin occupancy at this site may then be a consequence of the greater sphingomyelin abundance of oocyte membranes. On the other hand, if in CHO cells sphingomyelin truly does not occupy the inactivation kinetics–related site, its occupancy at the activation-related site then implies that sphingomyelin has a lower “affinity” (relative to competing lipids) for the former site than the latter one.

In summary, enzymatic removal of sphingomyelin’s choline group can activate certain members of all three classes of voltage-gated channels at near-resting membrane potentials by shifting their G–V curves in the hyperpolarized direction. SMase D stimulation of native Kv1.5 channels in human T lymphocytes provides a clue
to the extant question of how voltage-gated ion channels in nonexcitable cells may be tuned. On the other hand, the effect of SMase D on the action potential threshold exemplifies how an extracellular lipase may tune cellular excitability by modulating activation gating of NaV channels. Thus, these proof-of-concept studies demonstrate the potential for the voltage-gated channel activity of nonexcitable cells and the membrane electrical excitability of excitable cells to be pharmacologically or physiologically tuned by enzymatically modifying their head-group chemistry or regulating the relative abundance of minor lipid species. Given that sphingomyelin is a relatively minor species in some mammalian cell membranes, the observed SMase D effect on activation gating of these voltage-gated channels implies that the channels’ voltage sensors have some preference for sphingomyelin over some other lipid types, or that the channels are preferentially trafficked to sphingomyelin-rich domains. In addition, SMase D slows inactivation of NaV channels heterologously expressed in oocytes but not those of native NaV channels in tested mammalian cells. This different, NaVβ1-related SMase D sensitivity of NaV inactivation kinetics underscores the impact of cell membrane lipid environments on channel function, which are set by characteristic cellular lipid metabolism. Thus, SMase D has helped to reveal the potentially profound impact of modification and metabolism of membrane lipids on ion channel function.

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