Published in final edited form as:

Nature. 2008 February 14; 451(7180): 826–829. doi:10.1038/nature06618.

Removal of phospho-head groups of membrane lipids immobilizes voltage sensors of K⁺ channels

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Abstract

A fundamental question regarding the gating mechanism of voltage-activated K⁺ (Kv) channels is how five positively charged voltage-sensing residues in the fourth transmembrane (TM) segment^{1, 2} are energetically stabilized, as they operate in a low-dielectric cell membrane. The simplest solution would be to pair them with negative charges³. However, too few negatively charged channel residues are positioned for such a role^{4, 5}. Recent studies suggest that some of the channel's positively charged residues are exposed to cell membrane phospholipids and interact with their head groups^{5–9}. A key question nonetheless remains: Is the phospho-head of membrane lipids necessary for proper function of the voltage sensor itself? Here, we find that a given type of Kv channel may interact with several species of phospholipid, and that enzymatic removal of their negatively charged phospho-head creates an insuperable energy barrier for the positively charged voltage sensor to move through the initial gating step(s), thus immobilizing it, and also raises the energy barrier for the downstream step(s).

Kv2.1 channels, expressed in *Xenopus* oocytes, interact with sphingomyelin⁸ present mainly in the outer leaf of plasma membranes. To investigate the importance of phospho-head groups of membrane lipids in Kv channel gating we employed bacterial sphingomyelinases C and D (SMases C and D)^{10, 11}. Both enzymes specifically hydrolyze sphingomyelin but in different ways (Fig. 1a): SMase D removes only choline and leaves the lipid ceramide-1-phosphate behind in the membrane whereas SMase C removes phosphocholine, leaving ceramide behind^{12, 13}. A comparison of the effects of these two enzymes on the channels will thus help reveal the functional significance of the phosphodiester group in voltage gating.

SMase D of *Corynebacterium pseudotuberculosis*¹¹ shifts the conductance-voltage (G-V) relation of Kv2.1 by about -30 mV⁸ (Fig. 1d), allowing channels to be activated at a negative voltage where they otherwise remain largely deactivated (Fig. 1c). The effect is maximal within 2 minutes and persists for at least 24 hours (Fig. 1c, d; cells cannot regenerate sphingomyelin from ceramide-1-phosphate). It does not require direct exposure of channels to SMase D as it also occurred in Kv2.1-expressing oocytes that had been treated with SMase D and then thoroughly washed prior to injection with Kv2.1 cRNA (Fig. 1e).

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Additional exposure of such oocytes to SMase D, as expected, caused no further shift. Thus, SMase D acts through its lipase activity, not by direct binding to the channel. Our previous study⁸ supports an electrostatic mechanism whereby removal of the positively charged choline favors the activated state of the positively charged voltage sensors.

Unlike SMase D, SMase C removes the negatively charged phosphodiester group as well as choline. SMase C of *Bacillus anthracis*¹⁴ (*Ba*SMase C) reduces Kv2.1 current ~90% (Fig. 2a–c) and the reduction is voltage independent for both partial and complete enzymatic treatment (Fig. 2d). SMase C of *Staphylococcus aureus*¹⁵ (*Sa*SMase C) caused a comparable level of inhibition but with faster kinetics, and only the kinetics – not the extent – of inhibition are enzyme-concentration dependent (Fig. 2b). Thus, SMase C inhibits the channels via its enzymatic activity rather than by direct binding (more evidence below).

Sphingomyelin and cholesterol may form membrane domains called “lipid rafts”. We used the cholesterol-extracting agent methyl- β -cyclodextrin (M β CD)¹⁶ to test whether disruption of sphingomyelin-cholesterol interactions underlies the SMase C effect. A 2-hour exposure of oocytes to 5 mM M β CD (a common regimen) affected little the G–V curves obtained before or after SMase C treatment (Fig. 2e). Thus, the channel-inhibitory effect of SMase C does not appear to simply reflect disruption of sphingomyelin-cholesterol interactions.

Inhibition of Kv2.1 function by removal of the phospho-head of sphingomyelin is consistent with the observation that the voltage-gated (6TM) bacterial channel KvAP, unlike the non-voltage-gated (2TM) bacterial channel MthK, is non-functional when reconstituted in a non-phospholipid bilayer⁹, an observation suggesting that voltage sensors, not the ion conduction pore, require phospholipids for proper functioning. Pursuing that reasoning, we tested the effect of SMase C on two other non-voltage-gated (2TM) K⁺ channels: Kir1.1 and a KcsA pore with the Kir2.1 cytoplasmic termini attached¹⁷. Our finding that SMase C profoundly inhibits K⁺ conduction in both channels (Fig. 2k, l) indicates that sphingomyelin head groups are in fact critical for the proper function of these 2TM channels that lack voltage sensors. More direct approaches are thus needed to assess how SMase C inhibits voltage-gated Kv2.1 channels.

Looking for evidence that the observed SMase C inhibition of Kv2.1 results from removal of sphingomyelin phospho-heads around voltage sensors, we tested whether hanatoxin (HaTx), known to bind to voltage sensors^{18, 19}, prevents SMase C from reaching the lipids and thus mitigates its effect (kinetics of HaTx inhibition and recovery¹⁸ are slow compared to the rate of SMase C-caused inhibition). Exposure to 3 μ M HaTx inhibits Kv2.1 current ~70% at 30 mV and ~30% at 60 mV (Fig. 2f). (Extent of current reduction does not reflect extent of HaTx binding: this is because a channel bound with HaTx can still be activated by stronger-than-usual depolarization^{19, 20}.) In the absence of HaTx, SMase C reduces current by 90% at all voltages but, in its presence, the observed current reduction is much smaller (Fig. 2f). Thus, HaTx binding protects a large fraction of channels against SMase C, in support of the notion that SMase C inhibits the channels by removing sphingomyelin phospho-heads around voltage sensors.

We next examined whether SMase C affects gating current, a capacitive current arising from voltage sensor movement²¹. Unfortunately, measuring Kv2.1's gating current is technically challenging due to the lack of high-expression mutants that produce large gating currents but little ionic current. To circumvent this limitation, we employed the well-studied Shaker(-IR²²) Kv channel for ionic current, and its non-conducting V478W mutant²³ for gating current measurements. SMase C treatment reduces both ionic and gating currents by about half (Fig. 3a–d). Such a proportional reduction could be a reflection of the fact that about half the channels interact with sphingomyelin in such a manner that removal of the negatively charged phosphate groups creates an insuperable energy barrier for the positive gating charges to move during early gating transitions, effectively precluding activation by experimentally accessible depolarizations. Alternatively, the proportional reduction might be a coincidence peculiar to this particular Kv subtype, where half of the gating charges in individual channels remain functional and these channels, hence, susceptible to partial activation. However, as shown below, we similarly observed an approximately proportional reduction of ionic and gating currents in Kv1.3 channels. Additionally, SMase C does not reduce the slope of the Q-V curve (Fig. 3f), a parameter related to the effective number of gating charges. It is thus more probable that in about half the Shaker channels (more than half in the case of Kv1.3 and Kv2.1), sphingomyelin phospho-heads play an essential role in allowing voltage sensors to undergo early transitions involving the bulk of gating-charge movement. Phospholipids other than sphingomyelin presumably fulfill that role in the remaining channels. The two channel populations may exist in membrane domains that differ in their sphingomyelin content.

The above inference that the head groups of other phospholipids besides sphingomyelin enable early gating transitions in about half of Shaker channels expressed in *Xenopus* oocytes does not exclude the possibility that voltage sensors in these channels still interact with sphingomyelin, but in a manner important for late (downstream) transitions only. Rather, the very existence of channels whose early transitions do not require sphingomyelin permits investigation of the impact of sphingomyelin phospho-head groups on late transitions. We found that the kinetics of ionic currents that remain after SMase C treatment are markedly slowed (Fig. 3e) whereas those of the remaining gating currents are barely affected (Fig. 3g, h). Removal of phospho-head groups of sphingomyelin therefore must also raise (but not insuperably) the energy barrier for one or more gating transitions downstream from the bulk of the gating charge movement. This modest increase in the transition energy for one or more late rate-limiting steps is consistent with only 5–10% of total gating charge movement occurring late in the gating sequence^{24, 25}. The resulting small change in the shallow part of the normalized Q-V curve at more depolarized potentials (Fig. 3f) is reminiscent of a S4 mutant (L370V) whose late transition is altered²⁴.

Consistent with our early conclusion that SMase C acts by means of its lipase activity, gating current did not recover after SMase C washout (Fig. 3i). For further confirmation, we exploited the fact that SMase C acts only on sphingomyelin, not on ceramide-1-phosphate. As expected, conversion of sphingomyelin to ceramide-1-phosphate with SMase D shifts the activation curve to the left (Fig. 3l), i.e., allows mobilization of gating charges at more negative voltages (Fig. 3j). Indeed, pre-treatment with SMase D prevents subsequently added

SMase C from causing current inhibition (Fig 3j, l). The differing effects of SMases C and D indicate that the lipid product of at least one enzyme, perhaps both, continues to interact with the channel protein for the duration of the experiment. The persistence probably reflects strong channel-lipid interactions and/or confinement of relevant lipid molecules to microdomains. Consistent with this view, channel current was not significantly affected by acute addition of products of sphingomyelin hydrolysis catalyzed by either enzyme (Fig. 2g–j).

To rule out the possibility that the observed SMase C effect results from non-specific hydrolysis of the predominant outer-leaf phospholipid phosphatidylcholine (PC), we tested a PC-specific phospholipase (PC-PLC) (Fig. 1b) from *Bacillus cereus* directly against the channels, and found only modest ($11.2 \pm 2.8\%$) gating current depression, compared to ~50% for SMase C (Fig. 3k). SMase C, therefore, does not cause channel inhibition by hydrolyzing PC. The small inhibition caused by PC-PLC is, again, consistent with some channels interacting with phospholipids other than sphingomyelin.

SMase C was originally termed β -hemolysin after its *in vitro* hemolytic effect¹⁰, yet its role in pathogenesis remains largely unknown. The fact that inhibition of Kv1.3 in lymphocytes^{26, 27} is immuno-suppressive²⁸ motivated us to test ^{Sa}SMase C against human Kv1.3. We found that ^{Sa}SMase C eliminates well over half of the ionic current of Kv1.3 and of the gating current of its non-conducting W384F mutant²⁹ (Fig. 4), in an approximately proportional manner as in the case of Shaker channels. This finding raises the intriguing possibility that the SMase C action against Kv1.3 helps *S. aureus* to neutralize host defenses.

In summary, the voltage sensor of Kv channels may interact strongly with multiple molecules of several kinds of phospholipid. In the present experiments on *Xenopus* oocytes, sphingomyelin appears to be preferred over PC (these lipids differ in hydrogen-bonding characteristics). The apparent preference probably reflects both inherent channel-lipid affinity and/or relative abundance in the channels' lipid micro-environments. Some interactions between voltage-sensors and phospholipids are important for the early gating transitions, and others for late transitions. These findings strongly support the hypothesis that phospho-head groups of membrane lipids, together with certain acidic channel residues^{4, 5}, provide the necessary countercharges for the positively charged voltage-sensing residues during individual steps of the voltage-sensor movement. This charge-neutralizing action lowers the free energy of the overall gating process as well as the energy barrier for individual transitions, so that a modest voltage can drive the charged sensors through a series of conformational steps in a low-dielectric cell membrane to open the channel gate.

Methods

The cDNAs of Kv2.1, Shaker-IR, Kir1.1 and the KcsA-Kir2.1 chimera were cloned in the pGEMHess vector, and Kv1.3 in the pSP64 vector. The mutant channel cDNAs were produced through PCR-based mutagenesis and conformed with DNA sequencing. The cRNAs were synthesized with T7 or SP6 polymerases using the corresponding linearized cDNAs as templates. Channel currents were recorded from whole oocytes (previously injected with cRNA encoding relevant channels) using a two-electrode voltage clamp

amplifier (Warner OC-725C). The resistance of electrodes filled with 3 M KCl was 0.2 - 0.3 MΩ. Unless specified otherwise, the bath solution contained (in mM): 95/5 NaCl/KCl (or 80/20 for tail current measurements), 0.3 CaCl₂, 1 MgCl₂ and 10 HEPES; pH was adjusted to 7.6 with NaOH. Chemical reagent and lipase stock solutions (2 μl) were manually added to the 100 μl recording chamber. Ceramide-C₈ (Cayman Chemicals) was pre-dissolved in ethanol, and ceramide-1-phosphate-C₁₂ (Avanti Polar lipids) in 1: 49 dodecane/methanol. Unless specified otherwise, the final concentrations of *Ba*SMase C, *Sa*SMase C, SMase D, and PC-PLC were 40, 14, 4, and 50 ng/μl, respectively. PC-PLC from *B. cereus* was purchased from Sigma-Aldrich. To produce recombinant SMases C and D³⁰, *E. coli* BL21 (DE3) cells were transformed with the respective cDNAs cloned into pET30 vector, grown in LB medium to ~0.6 OD at 600 nm, and induced with 1 mM IPTG for 2 hours. The bacteria were harvested, resuspended, and sonicated. The resulting samples were loaded onto a cobalt affinity column and eluted by stepping the imidazole concentration from 50 mM to 500 mM (all SMase proteins contain N- and C-terminal His tags). The imidazole was later removed by dialysis. HaTx was purified as previously described³¹. Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich/Fluka.

Acknowledgments

We thank S. Billington for sharing SMase D cDNA, C. Deutsch for Kv1.3 cDNA, K. Ho and S. Hebert for Kir1.1, R. Joho for Kv2.1 cDNA, K. Swartz for Shaker-V478W cDNA, C. Armstrong for comments on our manuscript, and P. De Weer for review and discussion of our manuscript. This study was supported by a grant from NIGMS to Zhe Lu.

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Figure 1.

Reaction schemes of lipid hydrolysis and the SMase D effect on Kv2.1 channels. **a, b**, Hydrolysis of sphingomyelin by SMases C or D (**a**) and of phosphatidylcholine by PC-PLC (**b**). R and R' represent acyl chains. **c**, Amplitude of Kv2.1 current, repeatedly elicited by stepping membrane voltage from -80 to -40 mV, where the arrow indicates addition of recombinant SMase D to the bath. **d**, G - V relations obtained before SMase D treatment (squares), and 30 min (circles) or 24 h (triangles) after a 10-min exposure of oocytes to SMase D. **e**, Oocytes were exposed to SMase D for 30 min and then washed with enzyme-free solution before being injected with Kv2.1 cRNA. Data were collected from each oocyte 18 hr after the injection (circles), and again after an additional 15-min SMase D treatment (triangles). The control G - V relation was obtained without SMase D treatment (squares). All data in **d** and **e** are presented as mean $\pm$ s.e.m. ($n = 5$), and the controls were pooled.

Figure 2.

Effects of SMase C on Kv2.1, Kir1.1 and a KcsA-Kir2.1 chimera. **a**, Kv2.1 currents elicited at 5 second intervals with the voltage protocol shown, gradually declining following addition of recombinant *Ba*SMase C to the bath. Dotted line indicates zero current level. **b**, Time course of current, where the arrow indicates addition of 14 ng/μl (open circles, left) or 1.4 ng/μl (open triangles, middle) *Sc*SMase C, or 40 ng/μl *Ba*SMase C (closed squares, right). **c**, G–V curves of Kv2.1 before (squares) and after (dots) SMase C treatment. **d**, Fraction of current remaining at various times after addition of *Sc*SMase C, plotted against voltage: top three data sets correspond to different levels of partial enzymatic treatment and the bottom set to complete treatment. The currents were normalized to those before addition of SMase C at the corresponding voltages. **e**, G–V curves without (open symbols) or with (closed symbols) 2 h incubation in a 5 mM MβCD-containing solution, and before (squares) or after (circles) *Sc*SMase C treatment. **f**, Fraction of current remaining after addition of 3 μM HaTx (squares, top) and subsequent treatment with *Sc*SMase C (circles, middle), or after *Sc*SMase

C treatment alone (triangles, bottom). **g–j**, Kv2.1 currents plotted against time where the arrows labeled 1 indicate addition of 10 mM phosphocholine (**g**), 0.25 mg/ml ceramide (C₈) pre-dissolved in ethanol (**h**), 10 mM choline (**i**), or 0.25 mg/ml ceramide-1-phosphate (C-1-P; C₁₂) pre-dissolved in 1: 49 dodecane/methanol (**j**). SMase C (**g, h**) or SMase D (**i, j**) were used as positive controls (arrow labeled 2). Similar results were observed in at least three experiments for each case. **k**, I–V curves of Kir1.1 before (squares) or after (circles) SMase C treatment, and following subsequent application of 0.5 μM pore-blocking tertiapin-Q (triangles). **l**, I–V curves of a KcsA-Kir2.1 chimera before (squares) and after (circles) SMase C treatment. All data in **c–f**, **k** and **l** are presented as mean ± s.e.m. (n = 5 - 8).

Figure 3.

Effect of SMase C and PC-PLC on ionic and gating currents of Shaker channels. **a, b**, Ionic currents of Shaker(-IR) (**a**) and gating currents of the V478W Shaker mutant (**b**) elicited with the protocols shown, gradually decreasing on addition of recombinant *Ba*SMase C. **c, d**, $G-V$ (**c**) and $Q_{on}-V$ (**d**) relations before (squares) or after (filled circles) SMase C treatment. The data are presented as mean $\pm$ s.e.m. ($n = 6-11$); the error bars are generally smaller than the symbols. **e**, Ionic current trace after SMase C treatment normalized in amplitude to that before treatment in **a**. **f**, Normalized $Q-V$ curves before (squares) and after (filled circles) SMase C treatment in **d**. **g, h**, Integrals of on (**g**) and off (**h**) gating charges (before or after SMase C) versus time; all traces are normalized in amplitude. **i-k**, On-gating currents of

Shaker's V478W mutant versus time (similar results were observed in at least three experiments for each case). Gating currents were collected by stepping voltage from -100 mV to 0 mV (**i**, **k**) or -60 mV (**j**). The arrows indicate the addition and washout of SMase C (**i**); addition and washout of SMase D and subsequent addition of SMase C (**j**); addition and washout of purified native PC-PLC (**k**). The current was on average reduced $11.2 \pm 2.8\%$ following addition of PC-PLC (mean $\pm$ s.e.m., $n=3$). **l**, Q-V curves of control (closed squares) or after SMase D (open triangles) and subsequent SMase C (open circles) treatment (mean $\pm$ s.e.m, $n=5$).

Figure 4.

SMase C inhibition of Kv1.3. **a, b**, Ionic currents of wild-type Kv1.3 (**a**) and gating currents of the W384F mutant (**b**) elicited at 5 second intervals with the voltage protocol shown, gradually declining following addition of recombinant *Sa*SMase C (the off-gating current is largely immobilized). **c, d**, G-V (**c**) and Q_{on} -V (**d**) relations before (squares) and after (dots) treatment with *Sa*SMase C. The data are presented as mean $\pm$ s.e.m. (n = 9–11).