Vol 451|14 February 2008|doi:10.1038/nature06618

LETTERS

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

Yanping Xu[1], Yajamana Ramu[1] & Zhe Lu[1]

A fundamental question about the gating mechanism of voltage-activated K^+ (Kv) channels is how five positively charged voltage-sensing residues[1,2] in the fourth transmembrane segment are energetically stabilized, because they operate in a low-dielectric cell membrane. The simplest solution would be to pair them with negative charges[3]. 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 nevertheless remains: is the phospho-head of membrane lipids necessary for the proper function of the voltage sensor itself? Here we show that a given type of Kv channel may interact with several species of phospholipid and that enzymatic removal of their 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 sphingomyelin[8] 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 hydrolyse 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 therefore help to 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 mV (ref. 8) (Fig. 1d), allowing channels to be activated at a negative voltage at which they otherwise remain largely deactivated (Fig. 1c). The effect is maximal within 2 min and persists for at least 24 h (Fig. 1c, d; cells cannot regenerate sphingomyelin from ceramide-1-phosphate). It does not require direct exposure of channels to SMase D, because it also occurred in Kv2.1-expressing oocytes that had been treated with SMase D and then thoroughly washed before injection with Kv2.1 complementary RNA (cRNA; Fig. 1e). Additional exposure of such oocytes to SMase D, as expected, caused no further shift. SMase D therefore acts through its lipase activity rather than by direct binding to the channel. Our previous study[8] supports an electrostatic mechanism by which removal of the positively charged choline favours 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) decreases Kv2.1 current by about 90% (Fig. 2a–c) and the decrease is independent of voltage for both partial and complete enzymatic treatment (Fig. 2d). SMase C of *Staphylococcus aureus*[15]

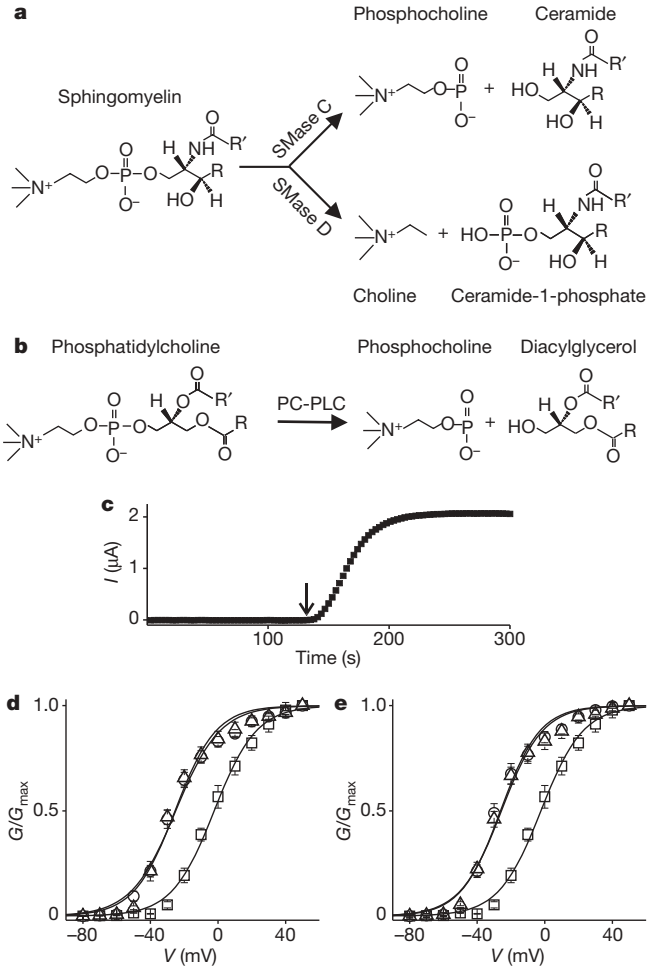

Figure 1 | Reaction schemes of lipid hydrolysis and the effect of SMase D on Kv2.1 channels. a, **b**, Hydrolysis of sphingomyelin by SMases C or D (**a**) and of phosphatidylcholine by PC-PLC (**b**). R and R′ represent acyl chains. **c**, Amplitude of Kv2.1 current, repeatedly elicited by stepping membrane voltage from −80 to −40 mV, where the arrow indicates the addition of recombinant SMase D to the bath. **d**, G–V relations obtained before treatment with SMase D (squares), and 30 min (circles) or 24 h (triangles) after exposure of oocytes to SMase D for 10 min. **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 h after the injection (circles), and again after an additional 15 min of treatment with SMase D (triangles). The control G–V relation was obtained without SMase D treatment (squares). All data in **d** and **e** are presented as means ± s.e.m. ($n = 5$), and the controls were pooled.

[1]Department of Physiology, University of Pennsylvania, 3700 Hamilton Walk, Philadelphia, Pennsylvania 19104, USA.

(^{Sa}SMase C) caused a comparable level of inhibition but with faster kinetics, and only the kinetics—not the extent—of inhibition are dependent on enzyme concentration (Fig. 2b). SMase C therefore inhibits the channels by means of its enzymatic activity rather than by direct binding (more evidence below).

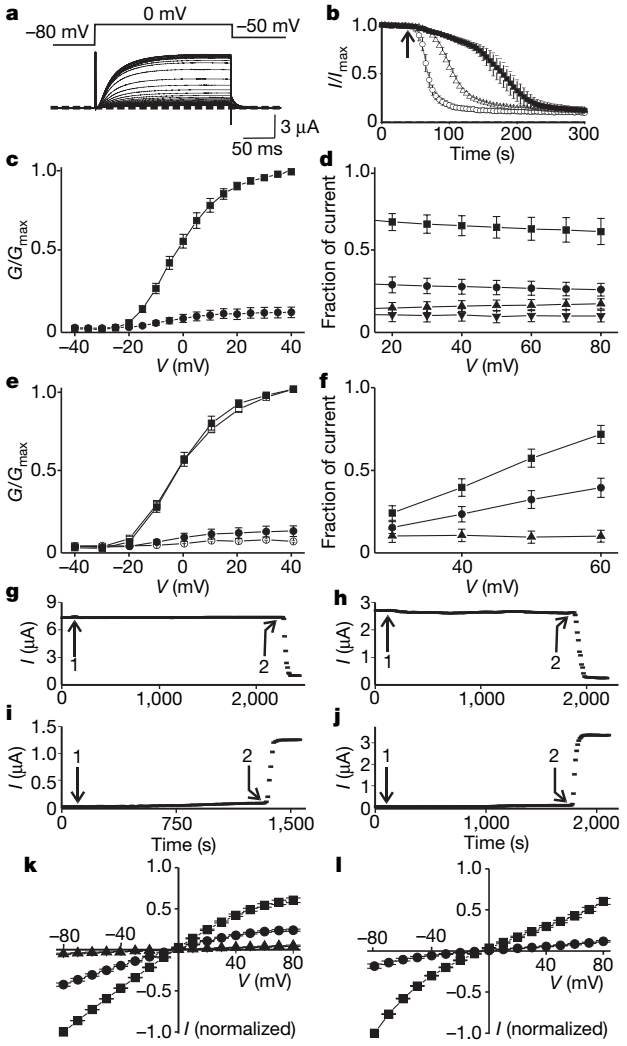

Figure 2 | Effects of SMase C on Kv2.1, Kir1.1 and a KcsA–Kir2.1 chimaera. **a**, Kv2.1 currents elicited at 5-s intervals with the voltage protocol shown, gradually declining after the addition of recombinant ^{Ba}SMase C to the bath. The dotted line indicates zero current level. **b**, Time course of current, where the arrow indicates the addition of 14 ng μl^{-1} (open circles, left) or 1.4 ng μl^{-1} (open triangles, middle) ^{Sa}SMase C, or 40 ng μl^{-1} ^{Ba}SMase C (filled squares, right). **c**, *G–V* curves of Kv2.1 before (squares) and after (circles) treatment with SMase C. **d**, Fraction of current remaining at various times after the addition of ^{Sa}SMase C, plotted against voltage: the 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 the addition of SMase C at the corresponding voltages. **e**, *G–V* curves without (open symbols) or with (filled symbols) incubation for 2 h in a solution containing 5 mM MβCD, and before (squares) or after (circles) treatment with ^{Sa}SMase C. **f**, Fraction of current remaining after the addition of 3 μM hanatoxin (squares, top) and subsequent treatment with ^{Sa}SMase C (circles, middle), or after treatment with ^{Sa}SMase C alone (triangles, bottom). **g–j**, Kv2.1 currents plotted against time, where the arrows labelled 1 indicate the addition of 10 mM phosphocholine (**g**), 0.25 mg ml^{-1} ceramide (C_8) pre-dissolved in ethanol (**h**), 10 mM choline (**i**) or 0.25 mg ml^{-1} ceramide-1-phosphate (C_{12}) pre-dissolved in dodecane/methanol (1:49 by vol.) (**j**). SMase C (**g**, **h**) or SMase D (**i**, **j**) were used as positive controls (arrows labelled 2). Similar results were observed in at least three experiments for each case. **k**, *I–V* curves of Kir1.1 before (squares) and after (circles) treatment with SMase C, and after subsequent application of 0.5 μM pore-blocking tertiapin-Q (triangles). **l**, *I–V* curves of a KcsA–Kir2.1 chimaera before (squares) and after (circles) treatment with SMase C. All data in **c-f**, **k** and **l** are presented as means ± s.e.m. (n = 5–8).

Sphingomyelin and cholesterol may form membrane domains called 'lipid rafts'. We used the cholesterol-extracting agent methyl-β-cyclodextrin (MβCD)[16] to test whether the disruption of sphingomyelin–cholesterol interactions underlies the SMase C effect. Exposure of oocytes to 5 mM MβCD for 2 h (a common regimen) had little effect on the *G–V* curves obtained before and after SMase C treatment (Fig. 2e). The channel-inhibitory effect of SMase C therefore does not seem simply to reflect the 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 (six-transmembrane) bacterial channel KvAP, unlike the non-voltage-gated (two-transmembrane) bacterial channel MthK, is non-functional when reconstituted in a non-phospholipid bilayer[9], 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 (two-transmembrane) K^+ channels: Kir1.1 and a KcsA pore with the Kir2.1 cytoplasmic termini attached[17]. Our finding that SMase C profoundly inhibits K^+ conduction in both channels (Fig. 2k, l) indicates that sphingomyelin head groups are in fact crucial for the proper function of these two-transmembrane channels that lack voltage sensors. More direct approaches are therefore needed to assess how SMase C inhibits voltage-gated Kv2.1 channels.

Looking for evidence that the observed inhibition of Kv2.1 by SMase C results from the removal of sphingomyelin phospho-heads around voltage sensors, we tested whether hanatoxin, which is known to bind to voltage sensors[18,19], prevents SMase C from reaching the lipids and thus mitigates its effect (kinetics of hanatoxin inhibition and recovery[18] are slow compared with the rate of inhibition caused by SMase C). Exposure to 3 μM hanatoxin inhibits Kv2.1 current by about 70% at 30 mV and by about 30% at 60 mV (Fig. 2f). (The extent of current reduction does not reflect the extent of hanatoxin binding; this is because a channel bound with hanatoxin can still be activated by a depolarization that is stronger than usual[19,20].) In the absence of hanatoxin, SMase C decreases current by 90% at all voltages, but in the presence of hanatoxin the observed decrease in current is much smaller (Fig. 2f). Hanatoxin binding therefore protects a large fraction of channels against SMase C, which supports 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 movement[21]. Unfortunately, measuring the gating current of Kv2.1 is technically challenging as a result of 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(-IR[22]) Kv channel for ionic current, and its non-conducting V478W mutant[23] for gating current measurements. SMase C treatment decreases both ionic and gating currents by about half (Fig. 3a–d). Such a proportional decrease could occur if about half of 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 decrease might be a coincidence, peculiar to this particular Kv subtype, such that half of the gating charges in individual channels remain functional and these channels are therefore susceptible to partial activation. However, as shown below, we similarly observed an approximately proportional decrease in ionic and gating currents in Kv1.3 channels. Additionally, SMase C does not decrease 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 of the Shaker channels (more than half for Kv1.3 and Kv2.1), sphingomyelin

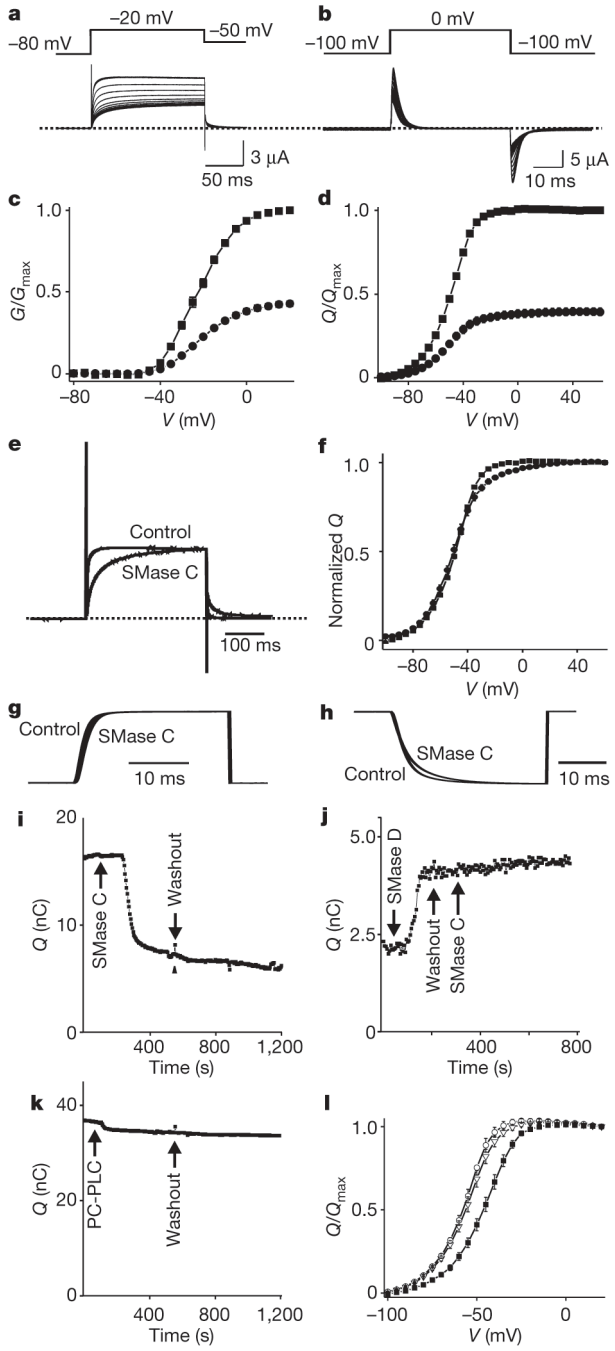

Figure 3 | Effect of SMase C and PC-PLC on ionic and gating currents of Shaker channels. **a**, **b**, Ionic currents of Shaker(-IR) (**a**) and gating currents of the V478W Shaker mutant (**b**) elicited with the protocols shown, gradually decreasing on addition of recombinant ^{Ba}SMase C. **c**, **d**, G–V (**c**) and Q_{on}–V (**d**) relations before (squares) or after (circles) treatment with SMase C. **e**, Trace of ionic current after treatment with SMase C, normalized in amplitude to that before treatment in **a**. **f**, Normalized Q–V curves before (squares) and after (circles) treatment with SMase C in **d**. **g**, **h**, Integrals of on (**g**) and off (**h**) gating charges (before or after treatment with SMase C) against 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 the voltage from −100 mV to 0 mV (**i**, **k**) or −60 mV (**j**). The arrows indicate the addition and washout of SMase C (**i**), the addition and washout of SMase D and subsequent addition of SMase C (**j**), and the addition and washout of purified native PC-PLC (**k**). The current was on average decreased by 11.2 ± 2.8% (means ± s.e.m., $n = 3$) after the addition of PC-PLC. **l**, Q–V curves of control (filled squares) or after treatment with SMase D (open triangles) and subsequent treatment with SMase C (open circles). All data in **c**, **d**, **f** and **l** are presented as means ± s.e.m. ($n = 5$–11); the error bars in **c**, **d** and **f** are generally smaller than the symbols.

phospho-heads are essential in allowing voltage sensors to undergo early transitions involving the bulk of gating-charge movement. Phospholipids other than sphingomyelin presumably fulfil 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 that is 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 is markedly slowed (Fig. 3e), whereas that of the remaining gating currents is barely affected (Fig. 3g, h). Removal of phospho-head groups of sphingomyelin must therefore also increase (but not insuperably) the energy barrier for one or more gating transitions downstream of 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 the occurrence of only 5–10% of total gating charge movement 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 an S4 mutant (L370V) whose late transition is altered[24].

Consistent with our early conclusion that SMase C acts by means of its lipase activity was our observation that gating current did not recover after washout of SMase C (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); that is, it allows the mobilization of gating charges at more negative voltages (Fig. 3j). Indeed, pretreatment 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 one or both of the enzymes continues to interact with the channel protein for the duration of the experiment. The persistence probably reflects strong channel–lipid interactions and/or the confinement of relevant lipid molecules to microdomains. Consistent with this view was our observation that channel current was not significantly affected by the acute addition of products of sphingomyelin hydrolysis catalysed by either enzyme (Fig. 2g–j).

To rule out the possibility that the observed effect of SMase C 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 ± 2.8%) gating current depression (Fig. 3k), in contrast with about 50% for SMase C. SMase C therefore does not cause channel inhibition by hydrolysing PC. The small inhibition caused by PC-PLC is, again, consistent with the interaction of some channels with phospholipids other than sphingomyelin.

SMase C was originally termed β-haemolysin after its haemolytic effect *in vitro*[10], yet its role in pathogenesis remains largely unknown. The fact that inhibition of Kv1.3 in lymphocytes[26,27] is immunosuppressive[28] 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 mutant[29] (Fig. 4), in an approximately proportional manner as with Shaker channels. This finding raises the intriguing possibility that the SMase C action against Kv1.3 helps *S. aureus* to neutralize host defences.

Thus, 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 seems to be preferred over PC (these lipids differ in hydrogen-bonding

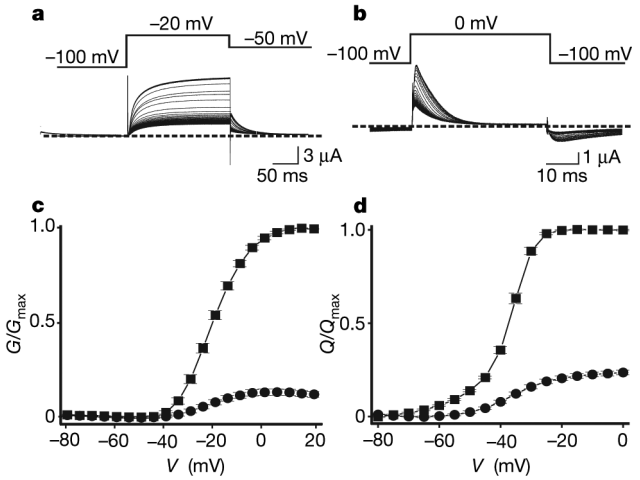

Figure 4 | Inhibition of Kv1.3 by SMase C. a, **b**, Ionic currents of wild-type Kv1.3 (**a**) and gating currents of the W384F mutant (**b**) elicited at 5-s intervals with the voltage protocol shown, gradually declining after the addition of recombinant ^{Sa}SMase C (the off-gating current is largely immobilized). **c**, **d**, G–V (**c**) and Q_{on}–V (**d**) relations before (squares) and after (circles) treatment with ^{Sa}SMase C. The data are presented as means ± s.e.m. (n = 9–11).

characteristics). The apparent preference probably reflects both inherent channel–lipid affinity and/or relative abundance in the channels' lipid microenvironments. Some interactions between voltage sensors and phospholipids are important for the early gating transitions, and others are important 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 counter-charges 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 SUMMARY

Channel currents were recorded under two-electrode voltage clamping from oocytes injected with cRNA encoding relevant channels. The bath solution contained (in mM): 95/5 NaCl/KCl (or 80/20 for tail current measurements), 0.3 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.6. Chemical reagent and lipase stock solutions (2 μl) were added manually to a 100-μl recording chamber. Unless specified otherwise, the final concentrations of recombinant[30] ^{Ba}SMase C, ^{Sa}SMase C, SMase D and native PC-PLC were 40, 14, 4 and 50 ng μl^{-1}, respectively.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 15 October; accepted 17 December 2007.

1. Aggarwal, S. K. & MacKinnon, R. Contribution of the S4 segment to gating charge in the Shaker K^+ channel. *Neuron* **16,** 1169–1177 (1996).
2. Seoh, S. A., Sigg, D., Papazian, D. M. & Bezanilla, F. Voltage-sensing residues in the S2 and S4 segments of the Shaker K^+ channel. *Neuron* **16,** 1159–1167 (1996).
3. Armstrong, C. M. Sodium channels and gating currents. *Physiol. Rev.* **61,** 644–683 (1981).
4. Papazian, D. M. *et al.* Electrostatic interactions of S4 voltage sensor in Shaker K^+ channel. *Neuron* **14,** 1293–1301 (1995).
5. Long, S. B., Campbell, E. B. & MacKinnon, R. Voltage sensor of Kv1.2: structural basis of electromechanical coupling. *Science* **309,** 903–908 (2005).
6. Cuello, L. G., Cortes, D. M. & Perozo, E. Molecular architecture of the KvAP voltage-dependent K^+ channel in a lipid bilayer. *Science* **306,** 491–495 (2004).
7. Freites, J. A., Tobias, D. J., von Heijne, G. & White, S. H. Interface connections of a transmembrane voltage sensor. *Proc. Natl Acad. Sci. USA* **102,** 15059–15064 (2005).
8. Ramu, Y., Xu, Y. & Lu, Z. Enzymatic activation of voltage-gated potassium channels. *Nature* **442,** 696–699 (2006).
9. Schmidt, D., Jiang, Q. X. & MacKinnon, R. Phospholipids and the origin of cationic gating charges in voltage sensors. *Nature* **444,** 775–779 (2006).
10. Glenny, A. T. & Stevens, N. F. Staphylococcal toxins and antitoxins. *J. Pathol. Bacteriol.* **40,** 201–210 (1935).
11. McNamara, P. J., Cuevas, W. A. & Songer, J. G. Toxic phospholipases D of *Corynebacterium pseudotuberculosis*, *C. ulcerans* and *Arcanobacterium haemolyticum*: cloning and sequence homology. *Gene* **156,** 113–118 (1995).
12. Kurpiewski, G., Forrester, L. J., Barrett, J. T. & Campbell, B. J. Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of *Loxosceles reclusa*. *Biochim. Biophys. Acta* **678,** 467–476 (1981).
13. Doery, H. M., Magnusson, B. J., Cheyne, I. M. & Sulasekharam, J. A phospholipase in staphylococcal toxin which hydrolyses sphingomyelin. *Nature* **198,** 1091–1092 (1963).
14. Read, T. D. *et al.* The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423,** 81–86 (2003).
15. Projan, S. J. *et al.* Nucleotide sequence: the β-hemolysin gene of *Staphylococcus aureus*. *Nucleic Acids Res.* **17,** 3305 (1989).
16. Kilsdonk, E. P. *et al.* Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270,** 17250–17256 (1995).
17. Lu, Z., Klem, A. M. & Ramu, Y. Ion conduction pore is conserved among potassium channels. *Nature* **413,** 809–813 (2001).
18. Swartz, K. J. & MacKinnon, R. Mapping the receptor site for hanatoxin, a gating modifier of voltage-dependent K^+ channels. *Neuron* **18,** 675–682 (1997).
19. Phillips, L. R. *et al.* Voltage-sensor activation with a tarantula toxin as cargo. *Nature* **436,** 857–860 (2005).
20. Swartz, K. J. & MacKinnon, R. Hanatoxin modifies the gating of a voltage-dependent K^+ channel through multiple binding sites. *Neuron* **18,** 665–673 (1997).
21. Armstrong, C. M. & Bezanilla, F. Currents related to movement of the gating particles of the sodium channels. *Nature* **242,** 459–461 (1973).
22. Hoshi, T., Zagotta, W. N. & Aldrich, R. W. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science* **250,** 533–538 (1990).
23. Kitaguchi, T., Sukhareva, M. & Swartz, K. J. Stabilizing the closed S6 gate in the Shaker Kv channel through modification of a hydrophobic seal. *J. Gen. Physiol.* **124,** 319–332 (2004).
24. Schoppa, N. E., McCormack, K., Tanouye, M. A. & Sigworth, F. J. The size of gating charge in wild-type and mutant Shaker potassium channels. *Science* **255,** 1712–1715 (1992).
25. Loboda, A. & Armstrong, C. M. Resolving the gating charge movement associated with late transitions in K channel activation. *Biophys. J.* **81,** 905–916 (2001).
26. DeCoursey, T. E., Chandy, K. G., Gupta, S. & Cahalan, M. D. Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis? *Nature* **307,** 465–468 (1984).
27. Matteson, D. R. & Deutsch, C. K^+ channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature* **307,** 468–471 (1984).
28. Chandy, K. G. *et al.* K^+ channels as targets for specific immunomodulation. *Trends Pharmacol. Sci.* **25,** 280–289 (2004).
29. Perozo, E., MacKinnon, R., Bezanilla, F. & Stefani, E. Gating currents from a nonconducting mutant reveal open-closed conformations in Shaker K^+ channels. *Neuron* **11,** 353–358 (1993).
30. Ramu, Y., Xu, Y. & Lu, Z. Inhibition of CFTR Cl-channel function caused by enzymatic hydrolysis of sphingomyelin. *Proc. Natl Acad. Sci. USA* **104,** 6448–6453 (2007).

Acknowledgements 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 the manuscript; and P. De Weer for review and discussion of the manuscript. This study was supported by a grant from the National Institute of General Medical Sciences to Z.L.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to Z.L. (zhelu@mail.med.upenn.edu).

METHODS

The cDNAs of Kv2.1, Shaker-IR, Kir1.1 and the KcsA–Kir2.1 chimaera were cloned in the pGEMHess vector, and Kv1.3 in the pSP64 vector. The mutant channel cDNAs were produced through PCR-based mutagenesis and confirmed 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 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES; the pH was adjusted to 7.6 with NaOH. Chemical reagent and lipase stock solutions (2 µl) were added manually to the 100-µl recording chamber. Ceramide-C_8 (Cayman Chemicals) was pre-dissolved in ethanol, and ceramide-1-phosphate-C_{12} (Avanti Polar lipids) in dodecane/methanol (1:49 by vol.). 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^{-1}, respectively. PC-PLC from *B. cereus* was purchased from Sigma-Aldrich. To produce recombinant[30] SMases C and D, *Escherichia coli* BL21 (DE3) cells were transformed with the respective cDNAs cloned into pET30 vector, grown in Luria–Bertani medium to an attenuance of about 0.6 at 600 nm, and induced with 1 mM isopropyl β-D-thiogalactoside for 2 h. The bacteria were harvested, resuspended and sonicated. The resulting samples were loaded on a cobalt-affinity column and eluted by stepping the imidazole concentration from 50 mM to 500 mM (all SMase proteins contain amino-terminal and carboxy-terminal histidine tags). The imidazole was later removed by dialysis. Hanatoxin was purified as described previously[31]. Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich/Fluka.

31. Swartz, K. J. & MacKinnon, R. An inhibitor of the Kv2.1 potassium channel isolated from the venom of a Chilean tarantula. *Neuron* **15**, 941–949 (1995).