LETTERS

Enzymatic activation of voltage-gated potassium channels

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Voltage-gated ion channels in excitable nerve, muscle, and endocrine cells generate electric signals in the form of action potentials[1]. However, they are also present in non-excitable eukaryotic cells and prokaryotes, which raises the question of whether voltage-gated channels might be activated by means other than changing the voltage difference between the solutions separated by the plasma membrane. The search for so-called voltage-gated channel activators is motivated in part by the growing importance of such agents in clinical pharmacology. Here we report the apparent activation of voltage-gated K^+ (Kv) channels by a sphingomyelinase.

While screening various sources we found that the venom of the brown spider, *Loxosceles reclusa*, causes Kv channels (for example, Kv2.1 (ref. 2)) to conduct robust outward current at potentials typical for many resting cells, where they otherwise remain largely deactivated (Fig. 1a). The same effect is observed in a scorpion toxin-sensitive mutant Kv2.1 channel (Kv2.1-Δ7 (ref. 3)) that contains seven mutations in the linker between the fifth and sixth transmembrane segments (S5 and S6) (Fig. 1b), and which we used for the studies described below.

To purify the channel-activating activity, we ran the venom through a size-exclusion fast-flow and low-pressure liquid chromatography (FPLC) column and subjected one of the active fractions to reverse-phase high-performance liquid chromatography (HPLC) (Fig. 1c, d). Analysis by HPLC coupled with tandem mass spectrometry (LC–MS/MS) of a trypsin digest of the HPLC-purified material(s) identified fragments (see Supplementary Fig. 1) that matched those of the Lr1 (11 peptides) and Lr2 (8 peptides) isoforms[4] of an enzyme commonly called sphingomyelinase D (SMase D), which is known to be present in the venom and to cause severe dermonecrosis[5,6] (some bacteria also produce SMase D[7–9]). The observed masses of the active materials (31.15 and 31.36 kDa) matched the theoretical masses of Lr1 and Lr2 (31.18 and 31.33 kDa). We infer that the active agents in the venom are in fact SMase D isoforms. Supporting this conclusion, the recombinant Lr2 isoform[10], like the native materials, induces robust outward current in the wild-type and mutant Kv2.1 channels while the membrane is held at −50 mV throughout (Fig. 1e, f; see Methods for the production of recombinant Lr2). Furthermore, we observe no current activation by SMase D that has been pre-exposed to low pH or has had its two critical histidine residues mutated (Fig. 2a, b), two manipulations known to inactivate SMase D[10–12].

Although most phenomena discussed below were originally observed with native materials, the actual data shown were gathered subsequently with recombinant SMase D(−Lr2) (4 ng μl^{-1} unless otherwise specified). SMase D catalyses a reaction in which the positively charged choline group of zwitterionic sphingomyelin is removed, yielding the negatively charged lipid ceramide 1-phosphate[5] (Fig. 2d; SMase D also catalyses the cleavage of choline from lysophosphatidylcholine bound to albumin in plasma, yielding lysophosphatidic acid[7,10,13]). Although phosphatidylinositol-4,5-bisphosphate, which affects the activity of many types of ion channel[14], is naturally present in the inner leaf of the plasma membrane, sphingomyelin is present mainly in the outer leaf. SMase D contains a Mg^{2+} ion in its catalytic centre[15]. Like its lipase activity, the channel-activating action of SMase D is Mg^{2+} dependent (Fig. 2c).

The charge on lipid headgroups contributes to the membrane surface potential and affects the functional properties of some K^+ channels[16–18]. It is conceivable that the positively charged choline group in the lipid substrates of SMase D, which are located near the outer surface of the membrane, interferes energetically with

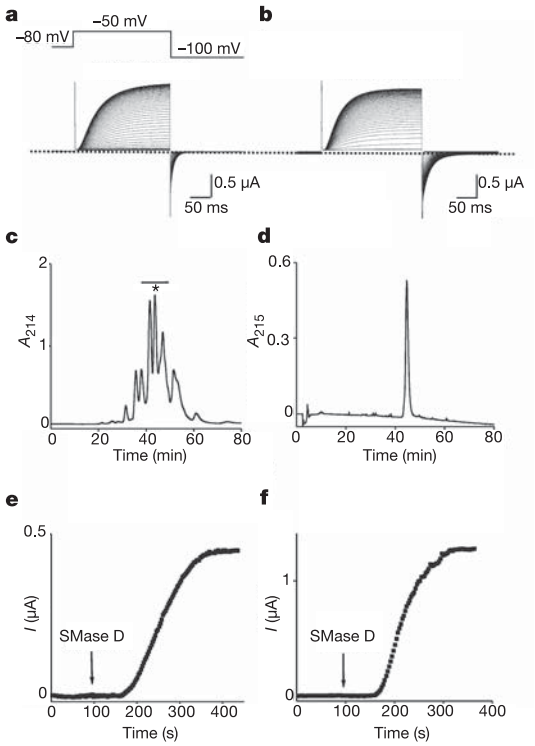

Figure 1 | Identification of Kv channel-stimulating activity. a, **b**, Wild-type (**a**) and Δ7 mutant (**b**) Kv2.1 currents elicited at 3-s intervals with the voltage protocol shown, which gradually appeared after venom addition (1:3,000 dilution). The dotted line indicates the zero current. **c**, Size-exclusion chromatography of 10 μl of venom; the bar identifies active fractions. **d**, The asterisk-marked peak in **c** was further purified on a reverse-phase column, yielding a major peak containing the activity. **e**, **f**, Kv2.1 (**e**) and Kv2.1-Δ7 (**f**) currents induced by recombinant SMase D (4 ng μl^{-1}) as the membrane voltage V_m was held at −50 mV throughout.

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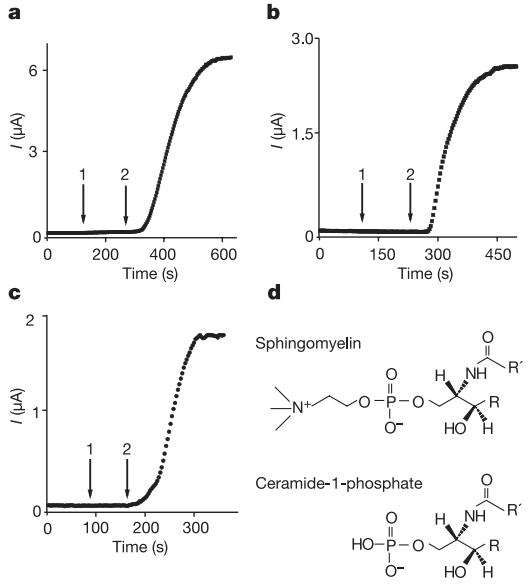

Figure 2 | Effects of pH, histidine mutations and Mg^{2+} on SMase D activity. Amplitudes of Kv2.1-Δ7 currents, repeatedly elicited as in Fig. 1a, are plotted against time. **a**, Arrows indicate successive addition of SMase D-Lr2 that was preincubated (1) or not (2) in 0.1% trifluoroacetic acid (pH < 3) for 14 h. **b**, Successive addition of SMase D-Lr2 with (1) or without (2) the H11A and H47A mutations. **c**, No Mg^{2+} was present in the bath solution during the first ~3 min. Arrows indicate successive additions of SMase D-Lr2 (1) and 1 mM Mg^{2+} (2). **d**, Chemical structures of sphingomyelin and ceramide-1-phosphate; R and R′ indicate acyl chains.

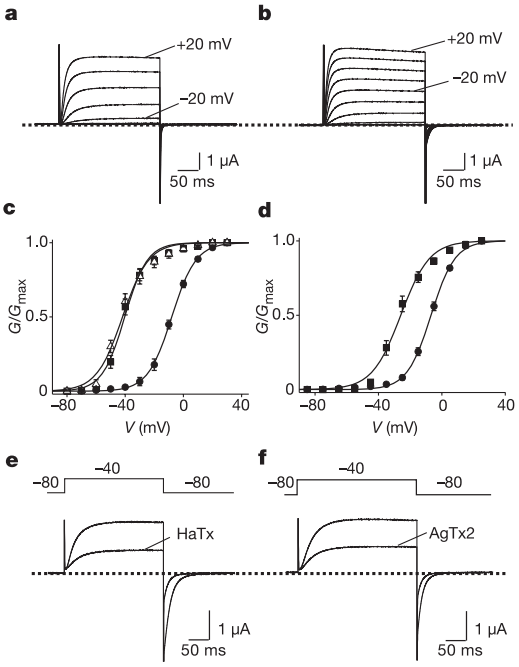

Figure 3 | Effect of SMase D on the *G–V* curve of Kv2.1-Δ7 channels. **a**, **b**, Currents before (**a**) and after (**b**) SMase D treatment, in which V_m was stepped from the −80-mV holding voltage ($V_{holding}$) to various test voltages (V_{test}) up to +20 mV in 10-mV increments ($V_{increment}$) ($[K^+]_{ext}$ = 20 mM). **c**, **d**, Plots of G/G_{max} (determined from the tail currents) against V_{test} are fitted with Boltzmann functions, yielding for the case of 1 mM extracellular Mg^{2+}, $V_{1/2} = -8.0 \pm 0.3$ mV (mean ± s.e.m.; $n = 6$) and $Z = 3.1 \pm 0.1$ before treatment with SMase D (filled circles), $V_{1/2} = -40.0 \pm 0.3$ mV, $Z = 3.3 \pm 0.3$ after treatment with SMase D (filled squares), and $V_{1/2} = -41.8 \pm 0.3$ mV and $Z = 2.9 \pm 0.3$ ($n = 4$) 1 h after SMase D wash-out (open triangles) (**c**), and for the case of 10 mM Mg^{2+}, $V_{1/2} = -7.0 \pm 0.2$ mV ($n = 6$) and $Z = 3.5 \pm 0.2$ before the treatment (filled circles) and $V_{1/2} = -26.0 \pm 0.8$ mV and $Z = 2.9 \pm 0.2$ after the treatment (filled squares) (**d**). **e**, **f**, Currents (after treatment with SMase D, $[Mg^{2+}]_{ext}$ = 1 mM, $[K^+]_{ext}$ = 10 mM) evoked by stepping V_m from $V_{holding}$ of −80 mV to −40 mV and then back to −80 mV without or with 25 nM HaTxs (**e**) and 0.2 nM AgTx2 (**f**).

depolarization-induced conformational changes and/or with the movement of the voltage sensors, which contain many positively charged residues (reviewed, for example, in ref. 19; for crystal structure see ref. 20). If that is so, SMase D-catalysed removal of the choline group will eliminate this interference, perhaps even favouring movement of the voltage sensors. SMase D does indeed cause a −30-mV shift in the conductance–voltage (*G–V*) curve, which allows activation of the channels at a more negative—typically resting—potential (Fig. 3a–c), and which, as expected, does not recover even 1 h after enzyme wash-out (open symbols; Fig. 3c). If the channels themselves are not primarily affected by the SMase D treatment, they should remain inhibitable by toxins that interfere with their gating (for example hanatoxins (HaTxs) (refs 21, 22)) or block their pore (for example agitoxin 2 (AgTx2) (ref. 23)). This is indeed what we observe (Fig. 3e, f).

As divalent cations are known to shift the *G–V* curves of voltage-gated channels[24]—an effect commonly understood to reflect lowering of the membrane's negative surface potential[1]—raising their concentration might be expected to mitigate the SMase D effect. We found that a tenfold increase in extracellular Mg^{2+} concentration markedly decreases the SMase D-induced shift in the *G–V* curve (Fig. 3c, d; this difference does not reflect a difference in SMase D activity because in both cases the enzymatic treatment was performed with 1 mM Mg^{2+}). Nevertheless, it is doubtful that the observed effects of SMase D on gating reflect a change in the distributed surface potential of the membrane bilayer. Rather, for the reasons given below, we conclude that they reflect a change in headgroup charge of lipid molecules that interact intimately and (relatively) specifically with the channels. First, under the present experimental conditions, SMase D has little effect on the voltage-gated mEAG K^+ channel[25], or on the mSlo K^+ channel[26] that is gated by both voltage and intracellular Ca^{2+} (Fig. 4a–f). Second, SMase D causes the *G–V* curves of different classic Kv channels to shift by different amounts. For example, it shifts the *G–V* curve of Shaker channels (with inactivation removed[27]) by only about −10 mV (Fig. 4g, i). Incidentally but significantly, the non-conducting W434F Shaker channel[28] allows one to observe the SMase D-caused increase in gating current at a hyperpolarized potential, as well as a roughly −10 mV shift in the gating charge–voltage (*Q–V*) curve (Fig. 4h, j; 8 ng μl^{-1} SMase D was used to reduce the recording time required). Third, the sphingomyelin/phosphatidylcholine abundance ratio in oocyte membranes is only 1:13 (ref. 29) (lysophosphatidylcholine is even scarcer, if present at all). If this ratio holds under our conditions and if phosphatidylcholine is indeed not a substrate for SMase D[7,10,12,13], the fact that removing the choline group of sphingomyelin had a significant impact must mean that the association between these lipids and the channel is (relatively) specific. Lipids associated with a K^+ channel protein have actually been resolved in the crystal structure of KcsA (ref. 18), whose transmembrane portion can substitute for the pore module of a Kv channel[30]. All these findings are consistent with the presence of specific lipid molecules between the voltage-sensing and the pore modules, where oxygen atoms of those lipids may interact with the positively charged residues in the S4. The protein–lipid–protein interaction might explain in part why lipids seem to help crystallized Kv1.2 to maintain a more native conformation[20] and indicates that the use of specific lipids might improve the chances of crystallizing voltage-gated channels in desirable gating states.

Thus, SMase D, by cleaving the positively charged choline group from specific types of phospholipid, shifts the *G–V* (and *Q–V*) curve

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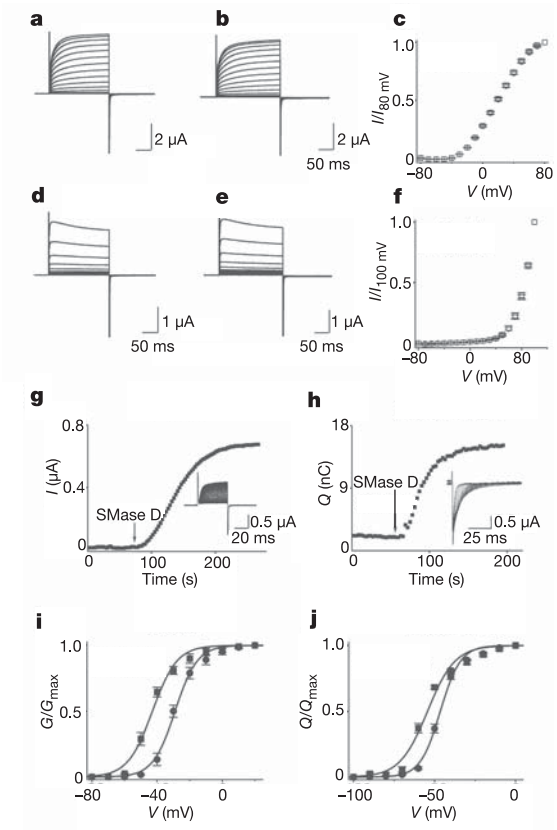

Figure 4 | Effects of SMase D on other K^+ channels. a–f, mEAG (**a**, **b**) and mSlo K^+ (**d**, **e**) currents elicited by stepping V_m from $V_{holding}$ of −80 mV to various V_{test} values up to +80 or +100 mV, where $V_{increment} = 10$ mV before (**a**, **d**) and after (**b**, **e**) treatment with SMase D, and the (normalized) currents (mean ± s.e.m., $n = 5$) at the various V_{test} values are shown in **c** and **f**, respectively (circles, control; squares, SMase D). **g**, **h**, Time course of current of Shaker(-IR) (elicited by stepping V_m from −80 mV to −50 mV; inset) (**g**) and the (off-)gating charges of the W434F mutant (elicited by stepping V_m from −60 mV to −100 mV; inset) (**h**). For gating current studies, the current records were corrected for background ionic and capacitive currents with the P/6 protocol. **i**, **j**, *G–V* and *Q–V* curves of Shaker(-IR) (**i**) and W434F mutant (**j**) before (circles) and after (squares) treatment with SMase D ($[K^+]_{ext} = 100$ mM; $V_{holding} = -80$ mV, $V_{test} = -80$ to 20 mV with $V_{increment} = 10$ mV, $V_{tail} = -70$ mV for **i**, and $[K^+]_{ext} = 20$ mM; $V_{holding} = -100$ mV, $V_{test} = -100$ to 0 mV with $V_{increment} = 10$ mV, $V_{tail} = -100$ mV for **j**) are fitted with Boltzmann functions, yielding $V_{1/2} = -30.0 \pm 0.6$ mV (mean ± s.e.m.; $n = 8$) and $Z = 3.8 \pm 0.3$ before treatment and $V_{1/2} = -41.2 \pm 0.9$ mV and $Z = 3.5 \pm 0.3$ after treatment (**i**), and $V_{1/2} = -46.5 \pm 0.5$ mV (mean ± s.e.m.; $n = 7$) and $Z = 4.2 \pm 0.3$ before treatment and $V_{1/2} = -54.5 \pm 1.0$ mV and $Z = 3.6 \pm 0.3$ after treatment (**j**).

of Kv channels in the negative direction, effectively activating the channels at negative potentials at which they would otherwise remain practically deactivated and conduct little current. This previously unknown means of activation of voltage-gated ion channels provides significant insight into how cell excitability may be regulated (or affected pathophysiologically) by altering the properties of lipids in (the outer leaf of) the plasma membrane, and how voltage-gated channels may be activated in non-excitable cells or lower organisms. Additionally, SMase D offers a simple, effective pharmacological way to activate certain voltage-gated channels at membrane potentials near resting.

METHODS

Molecular biology and electrophysiological recordings. The complementary DNAs of Kv2.1 and mSlo were cloned in pBluescript and pcDNA3 plasmids, respectively, whereas Shaker H4 with N-type inactivation removed (Shaker-IR) and mEAG were cloned in the pGEM-HESS plasmid. All mutant cDNAs were obtained through PCR-based mutagenesis and confirmed by 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 the corresponding cRNA) with 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 Na^+ (Cl^- + OH^-), 5 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES; pH was adjusted to 7.6 with NaOH. When K^+ concentration was increased, Na^+ concentration was decreased to keep their sum at 100 mM. SMase D (1–2 μl) was added manually to the recording chamber (100 μl).

Identification, purification and production of recombinant SMase D. For purification, *Loxosceles reclusa* venom samples were loaded on a size-exclusion FPLC column (Superdex G200; Pharmacia) in which the running buffer contained 50 mM NaCl and 5 mM Tris-HCl (pH 6.4). One of the active fractions was subsequently loaded on a reverse-phase C_{18} HPLC column (Beckman) and eluted with a water and acetonitrile gradient (increasing by 1% acetonitrile per minute). The aqueous and organic mobile phases contained 0.1% and 0.07% trifluoroacetic acid, respectively. The sample corresponding to the main peak on the HPLC chromatogram contained the activity and was subsequently analysed by matrix-assisted laser desorption ionization–time-of-flight MS for mass identification. The trypsin digestion products of the HPLC-purified material were then analysed with LC–MS/MS, yielding 11 and 8 partial peptide sequences corresponding to the Lr1 and Lr2 isoforms of SMase D (Supplementary Fig. 1). The SMase D activity was drastically decreased after HPLC purification because of exposure to low pH[11,12]. To produce the recombinant mature form of wild-type and the H11A + H47A mutant SMase D, *Escherichia coli* BL21 (DE3) cells were transformed with the respective cDNA of the Lr2 isoform cloned in pET30 Ek/LIC vector, grown in Luria–Bertani medium to a D_{600} of about 0.6, and induced for 2 h with 1 mM isopropyl β-D-thiogalactoside. The bacteria were harvested, resuspended and sonicated. The resulting sample was loaded onto a cobalt-affinity column and eluted by stepping the imidazole concentration from 50 mM to 500 mM. The imidazole was later removed by dialysis.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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