Online Methods

Electrophysiological recording. We carried out expression of Kv channel and Ci-VSP constructs in *Xenopus laevis* oocytes and studied using two-electrode voltage-clamp recording techniques (OC-725C, Warner Instruments) as previously described [33]. Data were filtered at 1 kHz and digitized at 10 kHz. Microelectrode resistances were 0.1–1MΩ when filled with 3M KCl. For ionic current measurements, the external recording solution contained (in mM) 50 KCl, 50 NaCl, 5 HEPES, 1 $MgCl_2$ and 0.3 $CaCl_2$, pH 7.6 with NaOH. For gating current measurements on Ci-VSP, the external recording solution contained (in mM) 100 NaCl, 10 HEPES, 1 $MgCl_2$ and 0.3 $CaCl_2$, pH 7.6 with NaOH. Recombinant SMaseD (final concentration 8 ng μl^{-1}) was added to the recording chamber for 10-15 minutes as previously described[6], using weak depolarizations elicited at 3s intervals to monitor the effects of the enzyme. After reaching equilibrium, the oocyte was extensively washed with the recording solution. All experiments were performed at room temperature (~22 °C). GxTx-1E and VSTx1 were synthesized using solid phase chemical methods as previously described[25], and ProTx-I was purchased from Peptides International (USA). Conductance-voltage relationships were obtained from tail currents measured after a series of membrane depolarizations. For a few mutants that displayed fast deactivation kinetics, conductance was calculated from steady-state current measurements.

Estimating toxin occupancy of channels

Occupancy of closed or resting channels by toxins was examined using negative holding voltages where open probability is low, and the fraction of unbound channels (F_u) was estimated using depolarizations that are too weak to open toxin-bound channels (Supplementary Fig 5), as previously described [26,27,29,33,44,45]. For all channels, we recorded voltage–activation relationships in the absence and presence of different concentrations of toxin. The ratio of currents (I/I_0) recorded in the presence (I) and absence (I_0) of toxin was calculated for various strength depolarizations, typically –70 mV to +10 mV, and the value of I/I_0 measured in the plateau phase at voltages where toxin-bound channels do not open was taken as F_u (see Supplementary Fig 5). For all the experiments, voltage protocols were

adjusted appropriately so that the plateau phase in the I/I_0–voltage relationship was well defined. The apparent equilibrium dissociation constant (K_d) was calculated assuming four independent toxin-binding sites per channel, with single occupancy being sufficient to inhibit opening in response to weak depolarizations: $K_d = ((1/(1-F_u^{1/4})) -1)$ [Toxin].

Toxin partitioning into oocytes membranes.

Twenty defolliculated *X. laevis* oocytes were incubated at room temperature (~ 22°C) in a solution (350 μl) containing 50 mM KCl, 50 mM NaCl mM, 5 mM HEPES, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, pH 7.6 (with NaOH) and 200 nM ^{125}I-GxTx-1E (20 Ci mmol^{-1}; generously provided by W. Schmalhofer and M. Garcia, Merck Research Labs, Rahway, NJ). After 15 min, the oocytes were separated by decantation, washed twice with 1ml ice cold solution without GxTx-1E and the amount of radiolabeled toxin measured in a liquid scintillation counter. To examine the effect of lipid modification on toxin partitioning into lipid membranes, oocytes were preincubated for 30 min with SMaseD (20 ng μl^{-1}). Fraction partitioned (f_p) was calculated as f_p=[$toxin_{bound}$]/[$toxin_{total}$].

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45. Lee, H.C., Wang, J.M. & Swartz, K.J. Interaction between extracellular Hanatoxin and the resting conformation of the voltage-sensor paddle in Kv channels. *Neuron* **40**, 527-36 (2003).