Localization of the Voltage-Sensor Toxin Receptor on KvAP†

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ABSTRACT: A variety of venomous animals produce small protein toxins that impair the function of voltage-dependent cation channels by affecting the motions of the voltage-sensor domains and altering the energetics of the opening of the channel. In this study, we investigate the location of the receptor for tarantula venom voltage-sensor toxins on the voltage-dependent K^+ channel from *Aeropyrum pernix* (KvAP), an archeabacterial channel that is functionally inhibited by members of this toxin family. We show that it is possible to purify the same set of toxins from venom of the tarantula *Grammostola spatulata* using either the purified KvAP voltage-sensor domain or the full-length KvAP channel. The equivalence of toxin retention profiles for the two channel proteins implies that the tarantula voltage-sensor toxin receptor resides exclusively on the voltage-sensor domain and that the pore is not required for the toxin−channel interaction. We have identified and characterized the functional properties of a subset of the tarantula toxins that bind to the KvAP voltage-sensor domain. Some of these toxins, VSTX1 and GSMTX4, have been previously isolated, while others, VSTX2 and VSTX3, are new members of the tarantula voltage-sensor toxin family. Some but not all toxins that bind to the voltage-sensor domain affect voltage-dependent gating of KvAP channels in lipid membranes.

Voltage-dependent K^+, Na^+, and Ca^{2+} channels form a family of structurally related membrane proteins that open and conduct ions as a function of the membrane voltage (*1*). These channels share a common modular architecture consisting of a central ion conduction pore surrounded by four transmembrane voltage-sensing domains (Figure 1A). Voltage-dependent K^+ (K_v)[1] and some Na^+ (Na_v) channels are formed from the symmetric arrangement of four identical subunits (*2, 3*). Voltage-dependent Ca^{2+} (Ca_v) and Na_v channels from eukaryotes are formed from the arrangement of a single concatenated string of homologous but not identical repeats, each equivalent to a single K_v subunit (*4, 5*). In all cases, each subunit or repeat of a voltage-dependent cation channel contains six hydrophobic segments (S1−S6). The S5−S6 segments line the pore and determine the ion selectivity of the channel, while the S1−S4 segments form each of the four voltage sensors of the channel. The voltage sensors contain highly conserved basic amino acids, known as gating charges, that are concentrated on the S4 segment (Figure 1A,B), as well as conserved acidic amino acids on S2 and S3 (not shown). The gating charges move under the influence of the electric field of the membrane, a conformational change that induces pore opening (*6*−*8*).

The family of voltage-dependent cation channels is essential to the rapid, long-range electrical signaling of the nervous system (*1*). The orchestrated activity of neuronal voltage-dependent cation channels produces a transient change in the membrane potential—an electrical impulse known as an action potential—that propagates at the rate of meters per second over the length of the neuron. Neuronal action potentials ultimately initiate a number of crucial cellular processes including neurotransmitter and hormone release and muscle contraction (*1*). Given the absolutely critical physiological role of this family of channels, it is no surprise that a variety of venomous animals from scorpions to spiders to snakes cripple their prey with selective, high-affinity protein toxins that inhibit voltage-dependent channel function.

Protein toxins known to inhibit voltage-dependent cation channels can be classified into two groups based on the manner in which they impair channel function. Pore-blocker toxins bind to the extracellular entryway of the pore and inhibit the function of the channel by physically occluding the ion conduction pathway (*9*−*16*). Voltage-sensor toxins bind to a separate receptor on the channel, affecting the gating motions of the voltage-sensor domains and altering the energetics of the voltage-dependent gating process (*17*−*27*).

What structural features of a voltage-dependent channel do voltage-sensor toxins recognize? Only one voltage-sensor toxin−channel interface has been thoroughly investigated. Hanatoxin, a well-characterized K_v channel gating modifier toxin isolated from the venom of the Chilean Rose tarantula, *Grammostola spatulata*, inhibits Kv2.1 channel function with

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[1] Abbreviations: K_v channel, voltage-dependent K^+ channel; Na_v channel, voltage-dependent Na^+ channel; Ca_v channel, voltage-dependent Ca^{2+} channel; KvAP, voltage-dependent K^+ channel from *Aeropyrum pernix*; VSTX, voltage-sensor toxin; PATX2, *Phrixotrichus auratus* toxin 2; GSMTX4, *Grammostola* mechano-toxin no. 4; MALDI-TOF mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high-peformance liquid chromatography; TFA, trifluoroacetic acid; DTT, dithiothreitol; MES, 2-(4-morpholino)-ethanesulfonic acid; DM, decylmaltoside; GST, glutathione Sepharose transferase.

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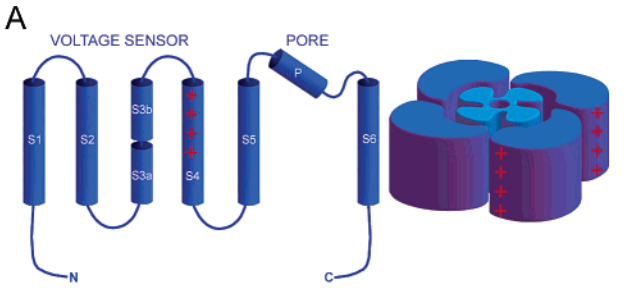

B

	S3B	S4
KvAP	PAGLLALIEGHLAGLG----------	LFRLVRLLRFLRILLIIS
Kv2.1	PYYVTIFLTESNKSVL--QFQNVRRVVQIFRIMRILRILKLAR	
Cav-I	TGILATVGTEFD----------	LRTLRAVRVLRPLKLVSGIP
Cav-II	IIGSIFEVIWAVIKPGTSFGISVLRALRLLRIFKVTKYWASLR	
Cav-III	GALVAFAFTGNSKGK----DINTIKSLRVLRVLRPLKTIKRLP	
Cav-IV	GSITDILVTEFG------	NNFINLSFLRLFRAARLIKLLRQGY
Nav-I	FAYVTEFVNLGN----------	VSALRTFRVLRALKTISVIP
Nav-II	LSLMELGLANVEG----------	LSVLRSFRLLRVFKLAKSWP
Nav-III	VSLVSLTANALGYS-----ELGAIKSLRTLRALRPLRALS---	
Nav-IV	LSIVGMFLAELIEK--YFVSPTLFRVIRLARIGRILRLIKGAK	

FIGURE 1: Part A shows the architecture of a voltage-dependent cation channel. Topology of a single K_v and Na_v channel subunit or Ca_v and Na_v channel homologous repeat consists of six hydrophobic segments (S1−S6)—the S1−S4 segments form the voltage-sensor domain, and the S5−S6 segments contribute to the pore. Four subunits or repeats assemble to form a central ion conduction pore surrounded by four voltage-sensor domains. Part B shows the sequence comparison of the interaction site for voltage-sensor toxins in the voltage-sensor domain. Sequence of the S3B−S4 segments from a KvAP channel subunit (GI 5104624), a Kv2.1 channel subunit (GI 24418849), and the four homologous repeats from αIA-Ca_v (GI 13386498) and brain type II Na_v channels (GI 116448) are aligned. Gating charge residues are highlighted in red. Residues in S3B, highlighted blue, alter the affinity of the channel for voltage-sensor toxins when mutated. Mutations of I273, F274, and E277 of Kv2.1 decrease the affinity of the channel for tarantula toxins (*24*, *27*), mutation of E1658 of the αIA Ca_v channel decreases the affinity of the channel for a funnel-web spider toxin (*30*), and mutations of G845, E1613, and E1616 of the brain type II Na_v channel decrease the affinity of the channel for a *β*-scorpion toxin, sea-anenome and the α-scorpion toxins, and a wasp toxin, respectively (*31*−*33*).

10−100 nM affinity (*22*). Extensive mutagenesis has been carried out on the Kv2.1 channel in search of the full determinants of the hanatoxin receptor. The results of this mutational scan, which included all residues of the voltage-sensor domain and peripheral regions of the pore, show that only three residues on the C-terminal portion of the S3 segment (I273, F274, and E277 on S3B, Figure 1B) appear to dominate the energetics of the toxin−channel interaction—all other residues investigated have negligible contributions (*24*, *28*). This result, although suprising considering that the dimensions of a face of hanatoxin (~20 Å × 25 Å) and relatively high-affinity inhibition imply a larger channel interface (*22*, *29*), suggests that this family of toxins binds to a very localized receptor on the channel. Less is known about other voltage-sensor toxin−channel interfaces. However, the S3 segment appears to form a common target for a number of structurally diverse voltage-sensor toxin families isolated from other venomous animals including sea anemones, scorpions, and wasps. Mutations at nearly equivalent positions on the S3B segment of different voltage-dependent cation channels (residues highlighted in blue in Figure 1B) markedly reduce the affinity of tarantula toxins for a K_v channel (*24*, *27*), a funnel-web spider toxin for a Ca_v channel (*30*), and sea anenome, α-scorpion, *β*-scorpion, and wasp toxins for a Na_v channel (*31*−*33*).

With the first crystal structure of a voltage-dependent cation channel, KvAP, an archeabacterial K_v channel, we have a structural model for the part of the voltage sensor that is sensitive to voltage-sensor toxins. Based on its amino acid sequence, functional characteristics, and pharmacological properties, the KvAP channel is unquestionably similar to eukaryotic K_v channels (*34*). The structure of the KvAP channel shows that the S3B segment and the N-terminal portion of the S4 segment, including the gating charges, form closely packed helix-turn-helix structures termed voltage-sensor paddles that lie at the perimeter of the channel (*35*). Functional experiments aimed at measuring motions of the KvAP voltage-sensor paddles in the membrane indicate that the paddles move a substantial distance through the lipid during the gating process (*36*). The residues of the S3B segment that affect voltage-sensor toxin affinity map to the voltage-sensor paddle of KvAP, a highly mobile region of the channel intimately involved in the gating process.

In the present study, we ask is the receptor for the tarantula voltage-sensor toxin family exclusively localized to the voltage-sensor domain of KvAP? We can answer this question by comparing the toxin retention profile of the isolated voltage-sensor domain with that of the full-length channel. If the pore-forming domain contributes to the tarantula voltage-sensor toxin receptor, we would expect that the retention profiles of the two channel proteins would be different. We find that this is not the case—the isolated voltage-sensor domain interacts with essentially the same complement of tarantula voltage-sensor toxins on its own or in the context of the full-length channel. This result implies that the voltage-sensor domain contains the sole determinants for binding of tarantula voltage-sensor toxins, supporting the idea suggested by mutational studies of eukaryotic K_v channels that this family of toxins recognizes a very localized receptor on the channel.

MATERIALS AND METHODS

Reverse-Phase HPLC. Analytical gradient HPLC was performed on an Agilent 1100 series instrument with UV detection using a Vydac C18 column (5 *μ*m, 4.6 mm × 250 mm) at a flow rate of 1 mL/min using a two-component mobile phase system in which mobile phase A is 0.1% TFA in water and mobile phase B is 90% acteonitrile and 0.1% TFA in water. Absorbance was monitored at 214 and 280 nm.

Mass Spectrometry. MALDI-TOF mass spectrometry was used for characterization of toxin fractions to determine mass and estimate purity. Toxin samples were diluted into a saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 30% acetonitrile, 0.1% TFA in water to a final concentration of approximately 10−100 nM. Spectra were acquired using a MALDI-TOF mass spectrometer, Voyager-DE STR (PE Biosystem), operating in reflectron delayed extraction mode. Spectra from 200 individual laser shots were averaged (using 0.5 ns data channel width) with software provided by the manufacturer. The spectra were calibrated and

analyzed using Data Explorer (PE Biosystem). For tandem mass spectrometry, proteolytic fragments of reduced and alkylated toxins were crystallized with 2,5-dihydroxybenzoic acid matrix in 60% methanol, 2% acetic acid in water and analyzed on a MALDI−Ion Trap MS Thermo Finnigan LCQ-DECAXP mass spectrometer with a homemade MALDI source (*37*).

Toxin Extraction from Venom Using Immobilized KvAP Voltage-Sensor Domain and Full-Length KvAP Channel. Isolated KvAP voltage-sensor domain and full-length channel were expressed and purified as described (*34*, *35*) but without thrombin cleavage so that the channel proteins contained a C-terminal hexahistidine tag. To generate channel protein affinity columns, 0.1 mL of Talon Co^{2+} resin was saturated with purified protein (15−20 mg/mL resin) and packed into a Bio-Rad Micro Bio-Spin Chromatography Column by gravity flow. Venom from *Grammostola spatulata* spiders (SpiderPharm, Yarnell, AZ) was diluted 10-fold into 20 mM Tris, pH 8.0, 100 mM KCl, and 10 mM DM and applied (0.1 mL) either to a column saturated with isolated voltage-sensor domain, to a column saturated with full-length channel, or to a control column with Co^{2+} resin alone. All three columns were washed under gravity flow (over the course of several minutes) to minimize nonspecifically bound toxins, first in four column volumes of the above buffer, then in four column volumes of the above buffer with 10 mM imidazole. With less stringent washing nonspecific binding of protein ultimately reduced the signal of this assay. Protein remaining on the columns was eluted with 0.1 mL of 400 mM imidazole in the above buffer and reduced with 50 mM DTT at 37 °C for 2 h to improve separation by HPLC. Equal volumes of eluted reduced protein from the three pull-down columns were run on HPLC using a 2 min isocratic flow of 25% mobile phase B, followed by a 25%−45% buffer B linear gradient over 40 min. Channel proteins loaded onto the C18 column do not elute with this buffer system and presumably remain bound to the HPLC column (without detectable affects on column capacity or resolution) until the column is treated with harsher solvents. When the above protocol is used, the toxin retention profiles of the channel proteins and Co^{2+} resin are very reproducible.

Toxin Identification. Individual peaks from the toxin pull-down assay were collected and analyzed by MALDI-TOF mass spectrometry and in some cases Edman sequencing. The peak corresponding to VSTX1 has, within error of measurement, the same mass and retention time as the reduced native toxin run on the HPLC gradient used for the pull-down assay. The peak corresponding to VSTX2 from the pull-down assay was subjected to Edman sequencing (Rockefeller University Protein/DNA Technology Center), which yielded the N-terminal 30 residues. The toxin was reduced with 10 mM DTT for 2 h at 37 °C, alkylated with 100 mM iodoacetamide in the dark for an additional 2 h, and then repurified by HPLC using a 22%−56% buffer B linear gradient over 40 min. Reduced, alkylated VSTX2 was dried under vacuum and resuspended in 100 mM potassium phosphate buffer, pH 7.0 and cleaved with Asp-N endoproteinase for 45 min. The identity and order of the final two residues was then determined by tandem mass spectrometry analysis of the 2981.4 Da fragment of reduced and alkylated VSTX2. The peak corresponding to GSMTX4 from the pull-down assay was also subjected to Edman sequencing, which provided the N-terminal 23 residues. The initial sequence and mass corresponded to that of GSMTX4 (*38*). The peak corresponding to GSMTX4 from the pull-down assay has the same retention time as reduced native GSMTX4, purified from whole venom as described (*38*). The mass of VSTX3 determined from the pull-down assay guided us in isolating impure fractions of native VSTX3 from whole venom. These venom fractions were reduced and purified on the same gradient used for the toxin pull-down assay. A single peak had, within error of measurement, the same retention time and mass as the peak corresponding to VSTX3 from the pull-down assay. Edman sequencing of this peak provided the first 32 residues of the VSTX3 sequence. VSTX3 was reduced, alkylated with iodoacetamide, and repurified as described above, resuspended in 50 mM MES, pH 6.0, and treated with Glu-C endoproteinase for 1 h. The identity and order of the final two residues of VSTX3 were determined by tandem mass spectrometry analysis of the 2260.1 Da fragment of the reduced alkylated toxin.

Toxin Purification from Venom. VSTX1 and GSMTX4 were purified as described (*34*, *38*). To purify VSTX2 and VSTX3, whole venom was fractionated by HPLC using a linear 22%−56% buffer B gradient over 150 min. The fraction containing VSTX2 eluted between 46 and 52 min. VSTX2 was purified to homogeneity on a second gradient consisting of a 2 min isocratic period at 25% buffer B, followed by a linear 25%−45% buffer B gradient over 40 min. The fraction of whole venom containing VSTX3 eluted between 38 and 45 min. Extensive attempts to purify VSTX3 on a second gradient using a variety of different mobile phase systems were unsuccessful. A fraction of whole venom in which the VSTX3 was the dominant mass signal was used for functional experiments.

Production of Recombinant Toxins. The genes for both VSTX1 and VSTX3 were synthesized using two oligonucleotide duplexes ligated into pGEX-4T-2 (Amersham Biosciences) with *Sal*I and *Bam*HI restriction sites and confirmed by sequencing. The sense strand for VSTX1 was as follows: 5′GAATGCGGTAAATTTATGTGGAAATGCAAAAACAGCAACGATTGCTGCAAAGATTTAGTGTGCAGCAGCCGCTGGAAATGGTGCGTGTTAGCCAGCCCGTTT3′. The sense strand for VSTX3 was as follows: 5′GATTGCTTAGGCTGGTTTAAAGGCTGCGATCCGGATAACGATAAATGCTGCGAAGGCTATAAATGCAACCGCCGCGATAAATGGTGCAAATATAAATTATGG3′. Recombinant toxin fusion protein was expressed in BL21-DE3 cells grown in LB medium on induction with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. Expressed fusion protein was maintained in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl and purified on glutathione Sepharose resin (Amersham Biosciences) by batch method. Following elution from the resin with 5 mM glutathione, the toxin was cleaved from the fusion protein with thrombin (1 unit per 2 mg of fusion protein) overnight at room temperature. Recombinant VSTX1 was purified from the cleavage reaction by HPLC using a 2 min isocratic step at 30% buffer B, followed by a 30%−42% buffer B linear gradient over 40 min. Recombinant VSTX3 was initially fractionated using a 2 min isocratic step at 25% buffer B, followed by a 25%−45% buffer B linear gradient over 40 min. The fractions eluting between 12.5 and 30.5 min all corresponded to toxins of the correct recombinant VSTX3

mass. These fractions were pooled together, dried under vacuum, and resuspended in 20 mM Tris, pH 8.0, 100 mM KCl, and 10 mM DM for affinity purification on a KvAP voltage-sensor domain column generated as detailed above. Pooled recombinant VSTX3 fractions retained by the voltage-sensor domain were isolated using the same protocol to affinity purify toxins from whole venom described above except that following elution from the resin, bound toxins were not reduced but run on HPLC using a 2 min isocratic flow of 0% buffer B followed by a linear 0%−73% buffer B gradient over 30 min.

Electrophysiology. Electrophysiological studies of KvAP channels in planar lipid bilayers were performed as described (*34*). For investigation of toxin function, the membrane holding potential was −100 mV and the membrane stepped to +100 mV for 200 ms every 2 min to monitor the extent of channel inhibition. Toxin concentrations were determined using the calculated extinction coefficients for 280 nm absorbance. Bovine serum albumin (0.5 mg/mL) was added to the recording chamber prior to toxin addition to prevent nonspecific interaction of the toxin with surfaces of the recording chamber.

RESULTS

Venom from the tarantula *G. spatulata* is a rich mixture of voltage-sensor toxins in the ~3.5−5.0 kDa range. Which of these toxins recognize a receptor on KvAP? Figure 2 shows the strategy that we used to screen for interacting toxins. Briefly, we generated small affinity columns containing Co^{2+} resin saturated with either purified isolated voltage-sensor domain or full-length channel bound to the resin through a hexahistidine C-terminal tag. We ran diluted venom over these two columns or a control column that contained only Co^{2+} resin and no bound protein. After the columns were washed to minimize nonspecific interactions, the channel proteins and the bound toxins were eluted with imidazole and reduced with DTT to improve separation by reverse-phase HPLC. Parts A, B, and C of Figure 3 compare the retention profiles obtained when equal volumes of the reduced eluate are run under the same HPLC conditions. The Co^{2+} resin nonspecifically retains some toxins, but this background signal does not obscure the identification of a number of toxin peaks that bind only to the voltage-sensor domain and the full-length channel.

The retention profile of the voltage-sensor domain is remarkably similar to that of the full-length channel. No additional toxins are retained due to the presence of the pore domain. The toxins most prominently retained by the full-length channel are equally well retained by the isolated voltage-sensor domain. This argues that the voltage-sensor domain alone is sufficient to isolate voltage-sensor toxins and the pore does not contribute to the toxin receptor. The slight differences in toxin retention that are seen between the two retention profiles might reflect subtle differences in the structure or exposure of the toxin receptor depending on whether the voltage-sensor domain is attached to the pore.

We chose to identify and functionally characterize a subset of the toxins specifically retained by both the voltage-sensor domain and full-length channel in our assay, corresponding to the four peaks labeled in Figure 3A. Two of these toxins, VSTX1 and GSMTX4, have been previously isolated and identified (*34*, *38*). Two other prominently retained toxins, VSTX2 and VSTX3, were sequenced through a combination of Edman sequencing and tandem mass spectrometry analysis of proteolytic fragments.

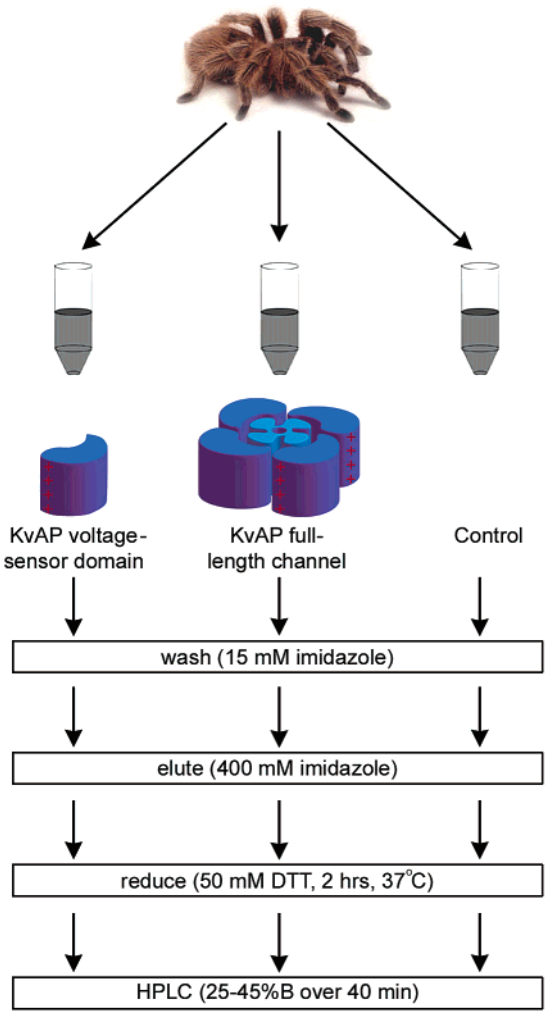

FIGURE 2: Experimental strategy used to isolate tarantula toxins from whole venom that specifically bind to the KvAP voltage-sensor domain and full-length channel. Venom is applied to Co^{2+} resin columns saturated with either KvAP voltage-sensor domain or full-length channel or a control column that contains Co^{2+} resin alone. After the columns are washed, the bound protein is eluted, chemically reduced, and analyzed by analytical reverse-phase HPLC.

The toxins that interact with KvAP are aligned with a number of known tarantula voltage-sensor toxins in Figure 4. Conserved within these new toxins are many of the amino acids known to underlie structural features characteristic of this toxin family. Completely conserved cysteines highlighted in gray form three disulfide bonds and dictate the toxin fold (*29*, *39*−*41*). Conserved aromatic residues contribute to a hydrophobic surface that is functionally important (*42*, *43*). Charged residues surrounding the hydrophobic surface are also thought to be important in the toxin−channel interaction. These newly identified toxins are clearly members of the tarantula voltage-sensor toxin family. VSTX2 is in fact identical through the first 27 residues to a previously identified toxin, PATX2 isolated from a related tarantula species, *Phrixotrichus auratus*, as an inhibitor of eukaryotic K_v channels (*44*).

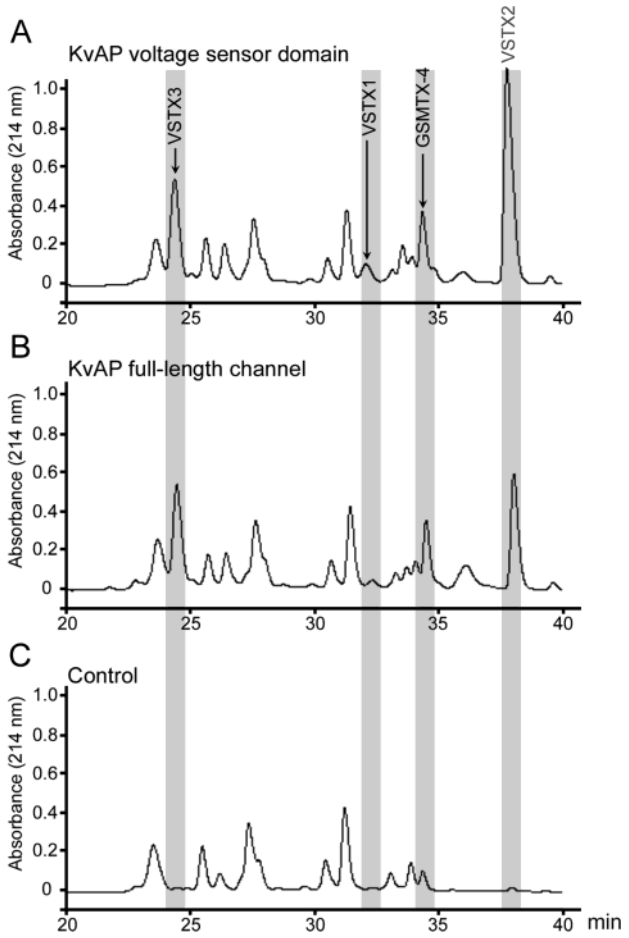

FIGURE 3: Comparison of the tarantula toxins purified from whole venom by the KvAP voltage-sensor domain and the KvAP full-length channel. Analytical reverse-phase HPLC chromatograms of toxins retained by a KvAP voltage-sensor domain affinity column (A), a full-length KvAP channel affinity column (B), and a resin only control column (C) show that the channel proteins specifically retain a number of tarantula voltage-sensor toxins. Toxins retained by the three columns were eluted and chemically reduced to enhance separation on a linear 25%−45% buffer B acetonitrile/H_2O gradient over 40 min. See Materials and Methods for details on mobile and stationary phase composition. The gray bars highlight toxins that bind only to the channel proteins and were identified and characterized further in this study.

Do the toxins that bind to the purified KvAP voltage-sensor domain in our biochemical assay affect channel function? One of the toxins retained by the voltage-sensor domain is a previously identified inhibitor of KvAP channel function (*34*). VSTX1 was isolated in a functionally based assay in which individual venom fractions were screened for activity when applied to KvAP channels reconstituted into planar lipid membranes. VSTX1 exhibits a characteristic feature of voltage-sensor toxins—a slow apparent rate of inhibition that makes it technically difficult to reach equilibrium with subsaturating concentrations of toxin. However, Figure 5B shows that 25 nM VSTX1 is an approximate half-inhibitory concentration of toxin, reducing KvAP channel currents by 50% 60 min after application to the extracellular side of the membrane. Thus VSTX1, as previously shown, appears to inhibit KvAP channel function in membranes with high affinity (*34*).

For functional assays, VSTX1 was purified from whole venom to a single peak on HPLC (Figure 5A) and a single mass by mass spectrometry. However, this is not a rigorous

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VSTX1   : -ECGKFMWKCKNSND-CCKDLV-CSSRWKW--CVLASPF- : 34
VSTX2   : -YCQKWMWTCDEERK-CCEGLV-CRLW-----CKKKIEEG : 32
VSTX3   : -DCLGWFKGCDPPNDKCCEGYK-CNRRDKW--CKYKLW-- : 34
GSMTX4  : -GCLEFWWKCNPNDDKCCRPKLKCSKLFKL--CNFSF--- : 34
HATX1   : -ECRYLFGGCKTTSD-CCKHLG-CKFRDKY--CAWDFTFS : 35
HATX2   : -ECRYLFGGCKTTAD-CCKHLG-CKFRDKY--CAWDFTFS : 35
SGTX1   : -TCRYLFGGCKTTAD-CCKHLA-CRSDGKY--CAWDGTF- : 34
GRTX    : -DCVRFWGKCSQTSD-CCPHLA-CKSKWPRNICVWDGSV- : 36
PROTX1  : -ECRYWLGGCSAGQT-CCKHLV-CSRRHGW--CVWDGTFS : 35
PROTX2  : -YCQKWMWTCDSERK-CCEGMV-CRLW-----CKKKLW-- : 30
PATX1   : -YCQKWMWTCDSARK-CCEGLV-CRLW-----CKKII--- : 29
PATX2   : -YCQKWMWTCDEERK-CCEGLV-CRLW-----CKRIINM- : 31
HPTX1   : -DCGTIWHYCGTDQSECCEGWK-CSRQL----CKYVIDW- : 33
HPTX2   : DDCGKLFSGCDTNAD-CCEGYV-CRLW-----CKLDW--- : 30
HPTX3   : -ECGTLFSGCSTHAD-CCEGFI-CKLW-----CRYERTW- : 31
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FIGURE 4: Sequence comparison of toxins that bind to the KvAP voltage-sensor domain with previously identified tarantula voltage-sensor toxins. Cysteines that determine the tarantula toxin fold are highlighted in gray. The following toxin sources were used: tarantula toxins VSTX1, VSTX2, and VSTX3, voltage-sensor toxins from *Grammostola spatulata*; GSMTX4, *Grammostola* mechanotoxin no. 4 from *Grammostola spatulata* (*38*); HATX1 and HATX2, hanatoxins from *Grammostola spatulata* (*22*); SGTX1 from *Scodra griseipes* (*46*); GRTX, omega-grammotoxin-SIA from *Grammostola spatulata* (*26*); PROTX1 and PROTX2 from *Thrixopelma pruriens* (*45*); PATX1 and PATX2, phrixotoxins from *Phrixotrichus auratus* (*44*); HPTX1, HPTX2, and HPTX3, heteropodatoxins from *Heteropoda venatoria* (*47*).

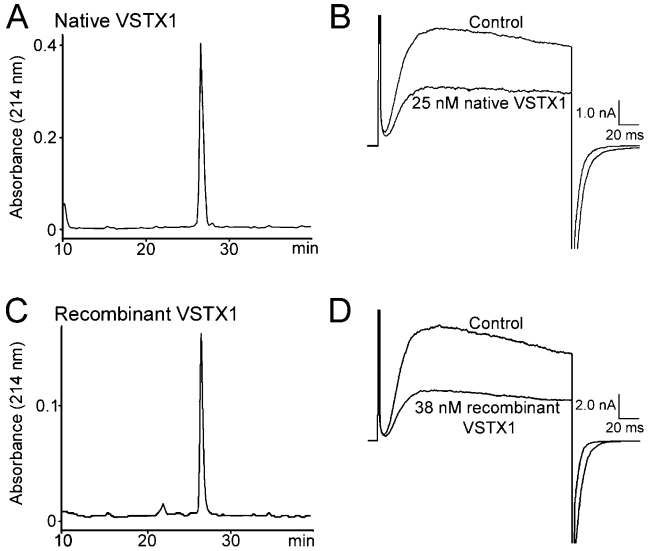

FIGURE 5: Native and recombinant forms of VSTX1 are high-affinity inhibitors of KvAP channel function. As shown in part A, VSTX1 can be purified from whole venom with two successive reverse-phase HPLC gradients. The chromatogram of the second HPLC purification step (a linear 30%−42% buffer B acetonitrile/H_2O gradient over 40 min) is shown. Part B shows the effect of native VSTX1 on KvAP channels reconstituted into planar lipid membranes. Currents elicited by depolarization to +100 mV in the absence (control) or presence of 25 nM native VSTX1 purified from venom are shown. Part C shows the reverse-phase HPLC chromatogram of recombinantly expressed VSTX1 following cleavage from purified GST-fusion protein run under the same HPLC conditions as the native toxin shown in part A. The majority of recombinant VSTX1 elutes with the same retention time as the native toxin. As shown in part D, recombinant VSTX1 inhibits KvAP channels. KvAP channel currents elicited by depolarization to +100 mV in the absence or presence of 38 nM recombinant VSTX1 are shown. For all current records shown, depolarization steps were from and back to a membrane holding voltage of −100 mV. All traces shown are an average of three measured traces before and after addition of voltage-sensor toxins.

demonstration that we have correctly isolated the inhibitory component from whole venom that affects KvAP channel function. It is possible that a copurifying inhibitory contaminant might be present but below the detection limit. To

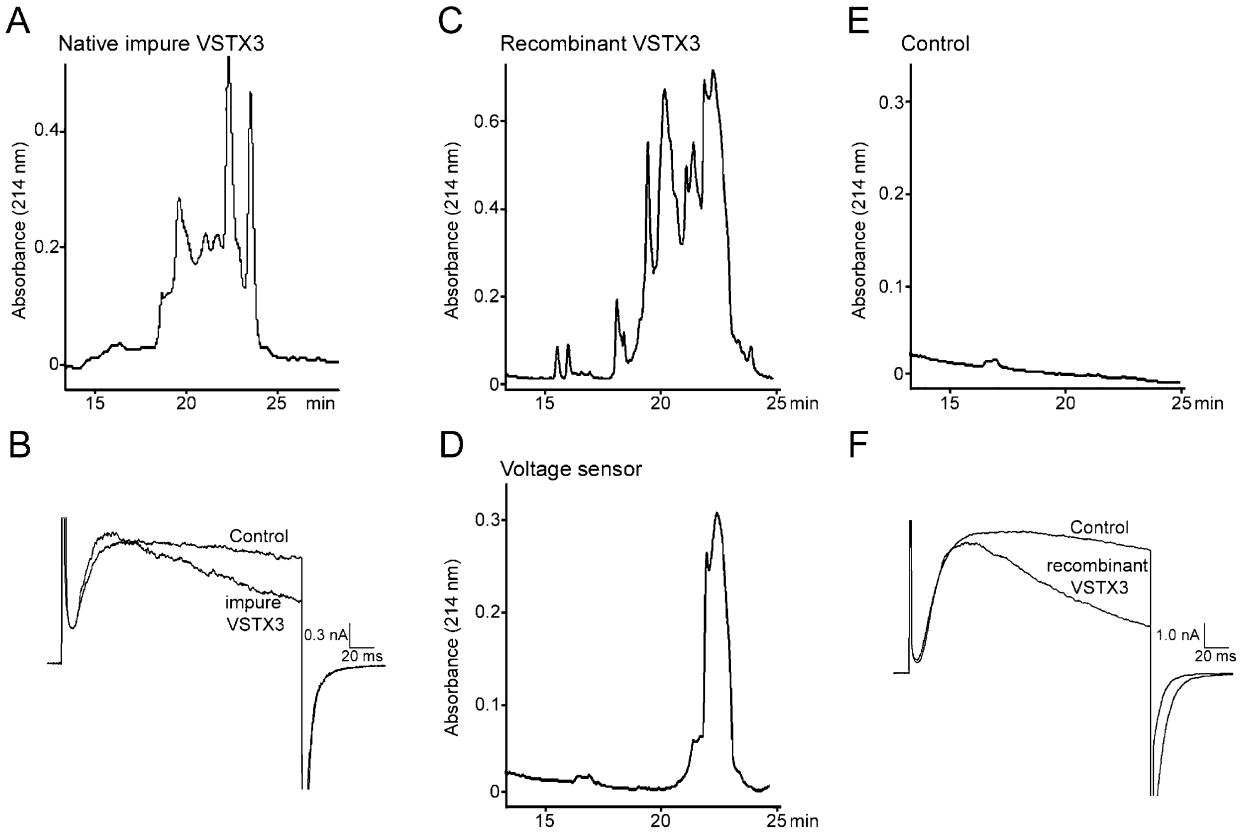

FIGURE 6: Native and recombinant forms of VSTX3 alter KvAP channel kinetics. Part A presents the reverse-phase HPLC chromatogram of an impure fraction of VSTX3 from venom used for functional experiments. Fractions of venom containing VSTX3 were pooled together and run on a linear 25%−45% buffer B acteonitrile/H_2O gradient over 40 min. As shown in part B, venom fractions containing VSTX3 alter the kinetics of KvAP channel gating. Currents from reconstituted KvAP channels elicited by depolarization to +100 mV in the absence and presence of impure VSTX3 are shown. Part C presents the reverse-phase HPLC chromatogram of recombinantly expressed VSTX3 following cleavage from purified GST-fusion protein run on a linear 0%−73% buffer B acteonitrile/H_2O gradient over 30 min. Toxin peaks eluting between 18 and 24 min have the correct mass for recombinant VSTX3 and presumably correspond to different toxin folds. As shown in part D, the KvAP voltage-sensor domain purifies the correct fold of recombinant VSTX3. Recombinant VSTX3 fractions were pooled together and passed over a column of Co^{2+} resin saturated with purified KvAP voltage-sensor domain (D) or a control column of only Co^{2+} resin (E). Toxins retained by the two columns were analyzed by analytical reverse-phase HPLC run under the same conditions as the HPLC chromatogram shown in part C. As shown in part F, Recombinant VSTX3 fractions purified by the KvAP voltage-sensor domain alter the function of reconstituted KvAP channels. KvAP currents elicited by depolarization to +100 mV prior to and after application of the recombinant VSTX3 fraction retained by the voltage-sensor domain in part D are shown.

verify that VSTX1, which we know physically interacts with the purified voltage-sensor domain, is responsible for channel inhibition, we produced a recombinant version of the toxin. VSTX1 was expressed as a GST-fusion protein in *Escherichia coli* with an N-terminal thrombin protease recognition site. The recombinant toxin was cleaved from the purified fusion protein, and the cleavage reaction was then run on HPLC. Figure 5C shows that the majority of recombinant VTSX1 elutes as a single peak with the same retention time as that of the native toxin despite the addition of two N-terminal residues, glycine and serine, resulting from the thrombin cleavage recognition site. This peak corresponds to the correctly folded, functionally active toxin: 38 nM recombinant VSTX1 inhibits slightly more than half the functional KvAP channels in membranes (Figure 5D). We conclude that VSTX1 binds to a receptor on the KvAP voltage-sensor domain and affects channel gating.

VSTX3 is a second functionally active toxin that is selectively retained by the purified voltage-sensor domain and full-length channel in our biochemical assay. In its reduced form (Figure 3A,B), VSTX3 elutes as a single symmetric peak. However in its native folded form, VSTX3 has an unusually broad elution profile (Figure 6A) that hindered our ability to isolate it away from copurifying toxin contaminants. A fraction containing VSTX3 and three other toxins when applied to the extracellular side of the membrane has an interesting effect on KvAP channel function, different from what is seen with VSTX1. Impure VSTX3 alters the gating kinetics of KvAP, speeding the rate of activation and inactivation (Figure 6B).

To further investigate the function of VSTX3 and conclusively determine whether it is responsible for the observed change in channel gating kinetics, we expressed the toxin recombinantly using the same strategy used to produce functional VSTX1. The cleavage reaction of recombinant VSTX3 from the purified fusion protein yielded multiple peaks on HPLC, most with the correct mass for the recombinant toxin. These peaks presumably represent different possible toxin folds with different disulfide connectivity (Figure 6C). We reasoned that only the correctly folded toxins would interact with their receptor on the voltage-sensor domain and that we might be able to purify the correctly folded toxin away from misfolded versions using a voltage-sensor domain affinity column. We pooled together HPLC

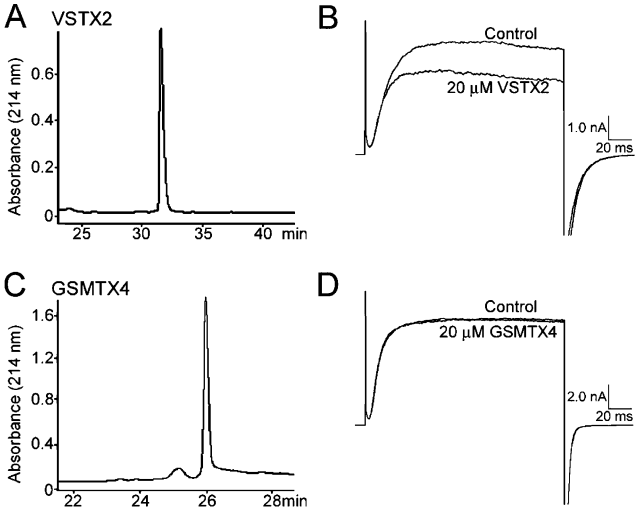

FIGURE 7: Some voltage-sensor toxins that bind the KvAP voltage-sensor domain do not strongly affect the function of KvAP channels. As shown in part A, VSTX2 can be purified from whole venom with two successive reverse-phase HPLC gradients. The chromatogram of the second HPLC purification step (a linear 25%−45% buffer B acetonitrile/H_2O gradient over 40 min) is shown. Part B shows the effect of VSTX2 on reconstituted KvAP channels. KvAP channel currents elicited by depolarization to +100 mV in the absence (control) and in the presence of 20 μM VSTX2 are shown. Part C shows purified GSMTX4 from venom run on a linear 0%−73% buffer B acetonitrile/H_2O gradient over 30 min. As shown in part D, 20 μM GSMTX4 has no affect on KvAP channel function. Traces of KvAP currents elicited by depolarization to +100 mV in the absence and presence of GSMTX4 are superimposed.

fractions of recombinant VSTX3 with the correct mass and applied them to a column of Co^{2+} resin saturated with purified voltage-sensor domain or, as a control, Co^{2+} resin without any bound protein. After washing, we eluted the bound protein from the two columns with imidazole and ran equal volumes of the eluate, without chemical reduction, on the same HPLC gradient. Passage of recombinant VSTX3 over the voltage-sensor domain column has partially purified the recombinant toxin (Figure 6D), which does not adhere to the Co^{2+} resin control (Figure 6E). We cannot explain why affinity purified recombinant VSTX3 shows a multiple-peaked broad chromatographic profile—although it is reminiscent of the elution of the native toxin. Some degree of conformational heterogeneity might be characteristic of certain tarantula voltage-sensor toxins. Native hanatoxin, for example, displays a less severe form of heterogeneity that gives rise to two chromatographically separated peaks in equilibrium. Nevertheless, the fraction of recombinant VSTX3 selectively retained by the voltage-sensor domain was then applied to KvAP channels in membranes to test for functional activity. Figure 6F shows that this fraction changes the kinetics of channel activation and inactivation in a manner very similar to what is seen with impure native VSTX3.

The two remaining toxins identified through their physical interaction with the purified KvAP voltage-sensor domain show negligible effects on channel function. VSTX2 is the most abundant toxin of this family in the *G. spatulata* venom (data not shown). Purified native VSTX2 inhibits KvAP with at best only weak affinity—20 μM VSTX2 appears to inhibit currents by about 30% (Figure 7A,B). However, at such high concentrations of a toxin purified from whole venom, there is a distinct possibility that the low level inhibition that we see is due to a small amount of a higher affinity inhibitory contaminant. GSMTX4 was previously isolated as a low affinity inhibitor of stretch activated channels in astrocytes. Purified GSMTX4 appears to have no effect on the KvAP channel even at micromolar concentrations (Figure 7C,D).

If these toxins bind to the voltage-sensor domains, why do we not see a functional consequence of this interaction on KvAP channels in the membrane? One possibility is that the toxins bind to the KvAP voltage-sensor domains in the membrane but do not affect channel function. Another possibility is that they are unable to access their receptor when the channel is immersed in the lipid bilayer. Alternatively, some toxins, like VSTX2, might bind to the purified voltage-sensor domain with extremely low affinity and are retained in our biochemical assay by virtue of their sheer abundance in the venom. This is a more likely explanation in light of recent evidence that VSTX1 partitions into the membrane and has, in fact, low intrinsic affinity for the purified KvAP channel in the absence of the lipid bilayer (*43*).

DISCUSSION

In this study, we have shown that the determinants for tarantula voltage-sensor toxin binding are contained exclusively within the voltage-sensor domain. The pore and its exterior walls do not appear to contribute to toxin specificity. This conclusion is consistent with functional studies of eukaryotic K_v channels that suggest a four-to-one stoichiometry (four toxins per channel or one toxin per voltage sensor) (*23*) and with mutational studies that point to a very localized region of the channel for toxin binding, limited to the S3B segment of the voltage sensor.

Only two of the four toxins that are retained by both the purified voltage-sensor domain and the full-length channel in our assay affect the function of KvAP channels in membranes. Clearly the tarantula toxins interact with the purified voltage-sensor domain with some specificity, otherwise we would have seen nonspecific selection of toxins by the pore domain, which provides an additional potential interaction surface for the toxins. Instead we see that the same toxin retention profile is obtained for both the KvAP voltage-sensor domain alone and for the full-length channel. Moreover, we are able to purify the correctly folded, functionally active form of recombinant VSTX3 through its affinity for the voltage-sensor domain, implying that the voltage-sensor domain specifically selects for toxins with the correct fold and the correct three-dimensional shape.

The fact that the voltage-sensor domain can purify a mixture of functional and nonfunctional toxins likely reflects that the toxin−channel interaction increasingly appears to be quite complex and is presently not well understood. Recent work has shown that VSTX1 has intrinsically low affinity for purified KvAP channels and much of the binding energy of the toxin−channel interaction comes from interaction of the toxin with lipid membranes (*43*). Our selection for interacting toxins using purified channel protein thus necessarily lacks an important component of the system that these toxins have evolved to target—the lipid bilayer. Our assay might therefore capture only relatively weak interactions and may select for toxins with greater abundance in the spider venom. Despite these limitations in our experimental system and our incomplete understanding of how

voltage-sensor toxins bind to and inhibit voltage-dependent cation channels, we have shown that the determinants for tarantula voltage-sensor toxin binding reside exclusively in the voltage-sensor domain.

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