A distinct sodium channel voltage-sensor locus determines insect selectivity of the spider toxin Dc1a

Niraj S. Bende¹, Sławomir Dziemborowicz², Mehdi Mobli³, Volker Herzig¹, John Gilchrist⁴, Jordan Wagner⁴, Graham M. Nicholson², Glenn F. King¹ & Frank Bosmans⁴,⁵

β-Diguetoxin-Dc1a (Dc1a) is a toxin from the desert bush spider *Diguetia canities* that incapacitates insects at concentrations that are non-toxic to mammals. Dc1a promotes opening of German cockroach voltage-gated sodium (Naᵥ) channels (BgNaᵥ1), whereas human Naᵥ channels are insensitive. Here, by transplanting commonly targeted S3b–S4 paddle motifs within BgNaᵥ1 voltage sensors into Kᵥ2.1, we find that Dc1a interacts with the domain II voltage sensor. In contrast, Dc1a has little effect on sodium currents mediated by PaNaᵥ1 channels from the American cockroach even though their domain II paddle motifs are identical. When exploring regions responsible for PaNaᵥ1 resistance to Dc1a, we identified two residues within the BgNaᵥ1 domain II S1–S2 loop that when mutated to their PaNaᵥ1 counterparts drastically reduce toxin susceptibility. Overall, our results reveal a distinct region within insect Naᵥ channels that helps determine Dc1a sensitivity, a concept that will be valuable for the design of insect-selective insecticides.
nsect voltage-gated sodium (Na\textsubscript{v}) channels share a common architecture with their mammalian orthologues\textsuperscript{1}. The pore-forming subunit consists of four connected domains (DI–IV), each with six transmembrane segments (S1–S6). These homologous, but not identical, domains each contain a voltage sensor (S1–S4) and a portion of the pore through which Na\textsuperscript{+} can diffuse (S5–S6)\textsuperscript{2}. While they have yet to be identified in insect Na\textsubscript{v} channels, each voltage-sensing domain within mammalian and bacterial Na\textsubscript{v} channels contains an S3b–S4 helix-turn-helix motif, the voltage-sensor paddle, which drives voltage-sensor activation\textsuperscript{3–6}. Aside from its vital role in channel gating, the paddle motif is also an important pharmacological target, as peptide toxins interact with this region to modify channel opening\textsuperscript{7–9}. Insect Na\textsubscript{v} channels likely possess similar motifs since residue substitutions in homologous regions can abolish channel susceptibility to toxins found in animal venoms\textsuperscript{1}. Even though voltage-sensing domains are extensively targeted by naturally occurring peptides, commercially available insecticides such as pyrethroids and oxadiazines typically interact with the channel pore region or intracellular linker between S4 and S5 to disrupt opening or closing (that is, gating)\textsuperscript{8,9}. However, insects have responded to this threat by mutating residues at strategic locations within the channel that result in a reduced sensitivity to these compounds\textsuperscript{9}. Moreover, the conserved nature of the Na\textsubscript{v} channel pore throughout the animal kingdom often leads to undesired biological activity of insecticides in beneficial insect orders or mammals\textsuperscript{8–10}. Since the amino acid composition of voltage-sensing domains varies considerably between related Na\textsubscript{v} channel isoforms, these regions may replace the pore as a target for designing insecticides with a higher degree of phyletic selectivity, an intriguing notion that has yet to be fully explored.

**Figure 1 | Recombinant production of Dc1a.** (a) Primary structure of rDc1a. The non-native N-terminal Ser residue that is a vestige of the TEV protease cleavage site used for recombinant toxin production is highlighted in grey. Disulphide bridge connectivity is shown above the sequence. (b) Schematic representation of the pLIC-NSB3 vector used for periplasmic expression of Dc1a. The coding region includes a MalE signal sequence (MalEss) for periplasmic export, a His\textsubscript{6} affinity tag, an MBP fusion tag and a codon-optimized gene encoding rDc1a, with a TEV protease recognition site inserted between the MBP and toxin-coding regions. The locations of key elements of the vector are shown, including the ribosome-binding site (RBS). (c) SDS-polyacrylamide gel electrophoresis gels illustrating different steps in the purification of Dc1a. Lanes contain: M, molecular weight markers; lane 1, *E. coli* cell extract before IPTG induction; lane 2, *E. coli* cell extract after IPTG induction; lane 3, soluble periplasmic extract (the His\textsubscript{6}-MBP-rDc1a fusion protein is evident at ~ 50 kDa); lane 4, Ni-NTA beads after loading the cell lysate; lane 5, eluate-1 from washing Ni-NTA resin with 600 mM imidazole; lane 6, eluate-2 from washing Ni-NTA resin with 600 mM imidazole; lane 7, eluate-3 from washing Ni-NTA resin with 600 mM imidazole; lane 8, Ni-NTA beads after elution-3; lane 9, fusion protein sample before TEV protease cleavage; lane 10, fusion protein sample after TEV protease cleavage showing almost complete cleavage of fusion protein to His\textsubscript{6}-MBP. (d) RP-HPLC chromatogram showing the final step in the purification of rDc1a. The arrow head denotes the peak corresponding to correctly folded recombinant rDc1a. Inset is a MALDI-TOF MS spectrum showing the [M + H\textsuperscript{+}] ion for the purified recombinant toxin (obs. = 6,484 Da; calc. = 6,485.39 Da).
**Results**

**Production of recombinant Dc1a.** We first produced recombinant Dc1a peptide (rDc1a) using a novel approach that addresses the challenge of correctly folding disulphide-rich spider toxins in *E. coli*. To this end, we created an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible construct (Fig. 1b) that allows for export of a His6-MBP-Dc1a fusion protein to the *E. coli* periplasm, where the enzymes involved in disulphide-bond formation are located. On recovery from the soluble cell fraction, the fusion protein was purified using nickel affinity chromatography (Fig. 1c). Subsequent cleavage and chromatographic purification yielded a single major disulphide-bond isomer with a purity of >98% as assessed by SDS-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Fig. 1d). Note that, with four disulphide bonds, there are 105 possible disulphide-bond isomers of rDc1a. Overall, the final yield was ~1.1 mg of toxin per litre of culture. Since native Dc1a was unavailable to us, we confirmed the insecticidal activity of rDc1a by injection into the blowfly (*Musca domestica*), which yielded LD50 values of 231 ± 32 pmol g−1 and 493 ± 52 pmol g−1, respectively (n = 3) (Supplementary Fig. 1). We therefore conclude that rDc1a has a similar lethality to agricultural pests when compared with native toxin.

**Solution structure of rDc1a.** The development of an efficient bacterial expression system allowed us to produce uniformly 13C/15N-labelled rDc1a protein for structure determination using heteronuclear NMR. 1H N, 15N, 13C, 13C, and 13C resonance assignments for the toxin were obtained from analysis of amide-proton strips in three-dimensional (3D) HNCA, CBCA(CO)NH, and HNCO spectra. Sidechain 1H and 13C chemical shifts were obtained using a four-dimensional (4D) HCC(CO)NH-TOCSY experiment, which has the advantage of providing sidechain 1H–13C connectivities. Complete chemical shift assignments have been deposited in BioMagResBank (Accession Number [19666](https://www.biomagresbank.org)). CYANA was used for automated NOESY assignment and structure calculation. The disulphide-bond pattern (1–4, 2–5, 3–8, 6–7) was unambiguously determined from preliminary structures calculated without disulphide-bond restraints and is identical to the framework determined from random starting conformations, 20 conformers with high stereochemical quality (as judged by MolProbity) were selected to represent the solution structure of rDc1a (Fig. 2a). Atomic coordinates for the final ensemble of 20 structures are available from the Protein Data Bank (PDB; Accession Number 2MI5).

Statistics highlighting the high precision and stereochemical quality of the ensemble of rDc1a structures are shown in...
Supplementary Table 1. The average MolProbity score of 1.24 places the ensemble in the 99th percentile relative to all other structures ranked by MolProbity. The high stereochemical quality of the ensemble stems from a complete absence of bad close contacts and an excellent Ramachandran plot quality (> 99% of residues in the most favoured region). The structural ensemble is also highly precise with backbone and heavy-atom root-mean-square deviation (r.m.s.d.) values over all the structural ordered regions (residues 3–42, 52–56) of 0.27 ± 0.07 Å and 0.70 ± 0.11 Å, respectively. The ensemble of rDc1a structures ranks as ‘very high resolution’ based on these measures of precision and stereochemical quality28.

Three of the four disulphide bonds in rDc1a form a classical inhibitor cysteine knot (ICK) motif in which the Cys13–Cys26 and Cys20–Cys40 disulphide bonds and the intervening sections of the polypeptide backbone form a 23-residue ring that is pierced by the Cys25–Cys54 disulphide bond (Fig. 2b). This ICK motif is commonly found in spider toxins, and this particular scaffold provides these peptides (so-called ‘knottins’) with an unusually high degree of chemical, thermal and biological stability15. However, the structure of rDc1a differs markedly from other ICK toxins, and a DALI search29 of the PDB returned no structural homologues. First, rDc1a contains an additional disulphide bond (Cys42–Cys52) that appears to serve as a molecular staple which limits the flexibility of a disordered serine-rich hairpin loop (residues 43–51) (Fig. 2b). Stapled hairpins of this kind have been observed in only a small number of spider toxins30. Second, the extended N terminus of rDc1a along with an unusually large loop between Cys26 and Cys40 enables the formation of an amino-terminal (N-terminal) three-stranded antiparallel β-sheet that is not found in any other knottin (Fig. 2b,c). The uniqueness of the rDc1a structure can readily be seen by comparison with the structure of As1a, a typical spider venom–derived knottin that modulates the activity of insect NaV channels30. The two toxins can be aligned over their core ICK regions with an rmsd of 1.9 Å (Fig. 2c) consistent with them both being members of the knottin family. However, when aligned over this ICK region, the N-terminal β-sheet of rDc1a is entirely separate to the region of structural overlap (Fig. 2c). Despite its structural uniqueness, the molecular surface of rDc1a contains a relatively uniform distribution of charged residues (Fig. 2d); moreover, there are no distinct clusters of hydrophobic residues that might mediate an interaction with lipid bilayers7,31 (Supplementary Movie 1).

**rDc1a opens the German cockroach Na\textsubscript{v} channel BgNa\textsubscript{v}1.** *Digieta* spiders are generalist predators and their diet consists of various insects ranging from small ants to sizeable prey such as grasshoppers and cockroaches16,32. As such, we decided to test whether rDc1a influences the gating properties of BgNa\textsubscript{v}1, a well-studied Na\textsubscript{v} channel isoform cloned from the German cockroach8,21. We functionally expressed BgNa\textsubscript{v}1 in *Xenopus* oocytes and applied various concentrations of rDc1a. At 1 μM, rDc1a produced robust opening of BgNa\textsubscript{v}1 at voltages where the channel is normally closed. This was achieved by a drastic shift in channel activation to more negative potentials (V\textsubscript{1/2} was shifted from −27.3 ± 0.2 mV (mean ± s.e.m.) (slope 4.0 ± 0.2) to −49.4 ± 0.4 mV (slope 3.5 ± 0.3) in the presence of 1 μM rDc1a; Fig. 3a) whereas steady-state inactivation (or channel availability) was only slightly affected (V\textsubscript{1/2} was shifted from −52.2 ± 0.1 mV to −55.1 ± 0.1 mV in the presence of 1 μM rDc1a; Fig. 3a). The rate of recovery from fast inactivation was also not significantly altered (τ = 2.1 ± 0.1 ms versus 2.2 ± 0.1 ms; Fig. 3b). On addition of 1 μM rDc1a to channels depolarized to −60 mV every 5 s, sodium currents became rapidly visible as a result of the −22 mV shift in voltage-dependent opening of BgNa\textsubscript{v}1 (Fig. 3c). Moreover, rDc1a similarly affects the channel when voltage-steps to −60 mV were applied every 50 s, suggesting that the toxin can access its binding site when BgNa\textsubscript{v}1 is in the resting state1,2. Channels completely recovered following washout in toxin-free solution (Fig. 3c). The persistent nature of the emerging current does not stem from the inhibition of fast inactivation by rDc1a since wild-type (WT) BgNa\textsubscript{v}1 inherently possesses these characteristics at mildly depolarizing voltages33. Fitting the Hill equation to the concentration dependence for toxin-induced current potentiation determined from shifts in the midpoint of channel activation (V\textsubscript{1/2}), Line represents a fit of the data with the Hill equation resulting in a slope factor of 1.19 ± 0.02; n = 5 and error bars represent s.e.m. (e) On addition of 1 μM rDc1a to channels depolarized to −60 mV every 5 s (red open circles) or 50 s (green open circles; holding voltage was −100 mV), BgNa\textsubscript{v}1-mediated sodium currents become rapidly visible. Channels completely recover after toxin washout. Inset shows current trace before (black), and after (red), addition of 1 μM rDc1a at −60 mV (5 s pulses). Noticeable is the persistent current that appears at this voltage, even in control experiments. Abscissa scale is 10 ms, ordinate scale is 0.3 μA. (d) Concentration dependence of rDc1a-induced current potentiation determined from shifts in the midpoint of channel activation (V\textsubscript{1/2}).
The domain III BgNav1/Kv2.1 construct is bimodal, suggesting and interactions with S3b–S4 paddle motifs within BgNav1 voltage sensors in a manner analogous to previously described β-scorpion toxins. In order to test this hypothesis, we first needed to establish whether BgNav1 contains paddle motifs with similar functions as in K_+ channels and in mammalian Na_+ channel isoforms where they have been identified (rNa_+1.2a, rNa_+1.4, rNa_+1.8, rNa_+1.9, and hNa_+1.9). To this end, we employed a previously reported approach in which specific S3b–S4 regions from each voltage-sensing domain of a Na_+ channel were transplanted into homotetrameric K_+ channels (Fig. 4a). After several attempts (Fig. 4a), we were able to generate functional chimeras between BgNav1 and Kv2.1 using known paddle motif boundaries. All of the constructs contain the crucial basic residues that contribute to gating charge movement in K_+ channels, suggesting that each of the four voltage-sensing domains in BgNav1 contain paddle motifs that are capable of sensing membrane voltage changes.

Examination of the conductance–voltage (G–V) relationships for the BgNav1/Kv2.1 chimeras reveals that each of the four voltage-sensor paddles has a distinct effect on the gating properties of Kv2.1. This was evidenced by marked differences in the midpoints of activation for the G–V relations for the domain I, II and IV constructs which are ≥50 mV, 13 ± 2 mV, and −9 ± 2 mV, respectively (Fig. 4c). The G–V relationship for the domain III BgNav1/Kv2.1 construct is bimodal, suggesting that the functional coupling between the voltage-sensing domains and the pore has been considerably altered. Although the underlying mechanism is unclear, it is possible that pore opening in this particular chimera may occur when: (1) not all four voltage sensors are fully activated; or (2) each voltage sensor has transferred only a portion of its charge across the hydrophobic septum. The latter scenario seems more likely since in the related Shaker K_+ channel, all four voltage sensor need to be in the active position before the pore opens. Moreover, intermediate states have also been observed when measuring gating currents of the Shaker K_+ channel in which a mutation immobilized voltage-sensor movement between the resting and activated states, thereby shifting the voltage activation of the ionic currents. Finally, it is worth mentioning that the domain III BgNav1/Kv2.1 construct may display multiple open states according to a model that has been described for N-type Ca_+ channels.

Remarkably, domain IV paddle motifs from rNa_+1.2a and rNa_+1.4 slow activation kinetics when transplanted into K_+ channels. These observations support the notion that the domain IV paddle motif contributes substantially to the overall rate of voltage-sensor activation. To explore whether the domain IV paddle motif serves a similar role in BgNav1, we measured the kinetics of activation and deactivation of the four BgNav1 paddle constructs in response to membrane depolarization and repolarization, respectively. Similar to previously studied chimeric channels, the activation and deactivation kinetics observed for the domain IV construct of BgNav1/Kv2.1 are slower over a wide voltage range when compared with the other domains (Fig. 4d), suggesting that this particular domain may indeed contribute to fast inactivation in insect Na_+ channels. We obtained additional evidence for the functional role of the domain IV paddle motif in BgNav1 gating from experiments with BomIV, a classic insect-selective α-scorpion toxin from the venom of Buthus occitanus mardochei that inhibits fast inactivation in insect Na_+ channels.
American cockroach neurons\textsuperscript{52}. To determine whether BomIV influences BgNa\textsubscript{1} in a similar fashion, we applied 10 nM to channel-expressing Xenopus oocytes and observed that the toxin indeed slows down fast inactivation (Supplementary Fig. 2b). Subsequent testing on the BgNa\textsubscript{1}/K\textsubscript{2.1} chimeras revealed that BomIV does not affect WT K\textsubscript{2.1} or the domain I, II, and III constructs. However, a dramatic toxin-induced inhibition of the domain IV chimera corroborates the importance of this particular voltage-sensing domain in toxin binding and BgNa\textsubscript{1} fast inactivation (Supplementary Fig. 2b).

We next investigated whether rDc1a modulates the activity of any of the four BgNa\textsubscript{1}/K\textsubscript{2.1} paddle chimeras and found that 1 \mu M rDc1a exclusively interacts with the domain II construct whereas domains I, III, IV, and WT K\textsubscript{2.1} are unaffected (Fig. 4c, Supplementary Fig. 2c). This result suggests that rDc1a specifically targets the domain II voltage sensor within BgNa\textsubscript{1} to influence channel opening.

**rDc1a distinguishes between cockroach Na\textsubscript{1} channel.** Since the domain II paddle motif in BgNa\textsubscript{1} (ref. 21) is identical to that in PaNa\textsubscript{1} (ref. 22), we expected a similar effect of rDc1a in whole-cell patch-clamp recordings from American cockroach dorsal unpaired median (DUM) neurons. Surprisingly, 1 \mu M rDc1a had little effect on sodium current amplitude or kinetics in DUM neurons as reflected in the threshold, or V\textsubscript{1/2}, of Nav channel activation or the V\textsubscript{1/2} of steady-state inactivation measurements (Supplementary Fig. 3). Furthermore, there were no significant use-dependent effects on sodium current amplitude or kinetics.

Due to the unexpectedly weak effects of 1 \mu M rDc1a on voltage-dependent activation of P. americana DUM neurons compared with BgNa\textsubscript{1} channels from B. germanica, acute toxicity assays were expanded to include both cockroach species (Fig. 5a,b). Remarkably, these bioassays revealed lethal toxicity of rDc1a in B. germanica compared with only mild, reversible effects in P. americana. At doses up to 5 nmol g\textsuperscript{-1}, rDc1a generated only minor spastic contractions of the abdomen, some shaking and evidence of reduced motor activity in P. americana. Even though these effects were completely reversible, a very small percentage of cockroaches did develop paralysis after 24 h (7 \pm 7% knockdown, n = 3). One explanation for these observations may be toxin binding to a subset of Na\textsubscript{v} channel splice variants\textsuperscript{53} or the presence of endogenous pharmacologically active auxiliary subunits\textsuperscript{54}. Conversely, B. germanica underwent dose-dependent flaccid or contractile paralysis, and lethality was observed after 8 h at all doses tested (Fig. 5a). The KD\textsubscript{50} and LD\textsubscript{50} values at 24 h post injection were 3.0 \pm 1.2 nmol g\textsuperscript{-1} and 4.2 \pm 3.4 nmol g\textsuperscript{-1} (n = 3), respectively. These observations suggest that residues in regions outside of the domain II paddle motif may underlie the remarkably different sensitivity of BgNa\textsubscript{1} and PaNa\textsubscript{1} to rDc1a.

**The S1–S2 loop modulates Na\textsubscript{v} channel sensitivity to rDc1a.** Sequence alignment of BgNa\textsubscript{1} with PaNa\textsubscript{1} reveals a highly conserved S1–S4 voltage sensor in domain II with only three amino acid substitutions (Fig. 6a; Supplementary Fig. 4). Of those, two residues in the S1–S2 loop are potentially accessible for a peptide toxin applied from the extracellular environment (His\textsuperscript{805} and Asp\textsuperscript{812} in BgNa\textsubscript{1} which correspond to a Tyr and Glu in PaNa\textsubscript{1}, respectively). On mutating these residues in BgNa\textsubscript{1} to their PaNa\textsubscript{1} counterparts, the resulting BgNa\textsubscript{1}\textsuperscript{YE} construct functionally expressed in Xenopus oocytes with a voltage-dependent activation V\textsubscript{1/2} of −41.5 \pm 0.3 mV and a steady-state inactivation V\textsubscript{1/2} of −56.4 \pm 0.2 mV (Fig. 6b). Unfortunately, a functional PaNa\textsubscript{1} clone was unavailable to undertake gain-of-function experiments. Strikingly, 100 nM rDc1a was no longer sufficient to open BgNa\textsubscript{1}\textsuperscript{YE} whereas this concentration generated large inward sodium currents when applied to WT channels (Fig. 6c, inset). When surveying higher concentrations of rDc1a, it becomes clear that concentrations of more than 1 \mu M are required to achieve a potentiation with BgNa\textsubscript{1}\textsuperscript{YE} that is comparable to the WT channel (EC\textsubscript{50} \sim 65 nM; Fig. 6c). Although we cannot exclude the possibility that rDc1a interacts with other PaNa\textsubscript{1} domains at such concentrations, administering high doses of the toxin is indeed mildly toxic to P. americana (Fig. 5a,b). Thus, our mutagenesis experiments have uncovered a novel region within insect Na\textsubscript{v} channels that helps determine their sensitivity to spider toxins. This locus contributes to the drastically reduced sensitivity of the American cockroach to rDc1a from the venom of the desert bush spider whereas its German counterpart is highly susceptible.

**Figure 5 | Acute toxicity of rDc1a in two cockroach families.** (a) Dose-response curve for lethal effects of rDc1a determined 24 h after injection into German cockroaches (B. germanica; family Blattellidae, closed circles). Data were fitted according to Equation 5 in Methods and represent mean \pm s.e.m. of three independent experiments. (b) Comparison of the KD\textsubscript{50} (open columns) and LD\textsubscript{50} (grey columns) doses at 24 h after injection of rDc1a in L. cuprina, M. domestica and B. germanica. Note: doses up to 5 nmol g\textsuperscript{-1} failed to produce any signs of knockdown or lethality up to 72 h post injection in American cockroaches (Periplaneta americana; family Blattidae). Data represent mean \pm s.e.m. of three independent experiments.
Concentration dependence for toxin-induced current potentiation (as determined by shifts in $V_{1/2}$) is shown. It is clear that alterations in substitutions (grey background). The T797M substitution within S1 is unlikely to be toxin-accessible. (Shown are the normalized deduced conductance ($G_\text{max}$). Descriptive values can be found in the Results section. Currents were elicited by 5 mV step depolarizations from a holding voltage of $-100 \text{ mV}$. Current traces were evoked by a 50-ms depolarization to 90 mV/C0).

**Discussion**

The initial goal of this study was to explore the mechanism underlying the efficacy of Dc1a, a potent insecticidal peptide toxin produced by desert bush spiders. To this end, we first produced recombinant rDc1a and determined its solution structure using heteronuclear NMR (Figs 1 and 2). Next, we tested rDc1a on the heterologously expressed German cockroach channel BgNav1 and discovered that the toxin dramatically promotes channel opening (Fig. 3). By taking advantage of the portable nature of S3b–S4 paddle motifs within voltage-sensing domains, we demonstrated that such motifs also exist in each of the four voltage-sensing domains of BgNav1, and that rDc1a selectively interacts with the paddle motif in domain II, a feature that it shares with an extensive list of animal toxins that target this particular paddle motif, albeit with a high degree of insect selectivity (Fig. 4). Remarkably, our insect assays revealed that German cockroaches are highly susceptible to rDc1a whereas the closely related American cockroach is virtually insensitive despite the fact that the domain II paddle motif in BgNav1 is identical to that in PaNav1 (Fig. 5; Supplementary Fig. 4). To elucidate the machinery responsible for this discrepancy, we mutated two residues in the S1–S2 loop that differ between BgNav1 and PaNav1 (His805 and Asp812) and found that the susceptibility of the resulting BgNav1,1E construct to rDc1a is dramatically reduced (Fig. 6). Interestingly, mammalian Na+ channel isoforms possess a Tyr and Ser at the corresponding positions, which presumably contributes to their insensitivity to rDc1a; however, their domain II paddle motif differs as well (Supplementary Fig. 4).

It is interesting to consider potential mechanisms that may underlie the insect-family specificity of rDc1a. For example, the B3–B4 hairpin (Fig. 2b) often houses the pharmacophore in spider ICK toxins that target voltage-gated ion channels, but the unusual architecture of rDc1a (Fig. 2c) and its unique ability to dramatically promote opening of insect Na+ channels (Fig. 3) suggests that it might interact with the voltage sensors of these channels in a unique manner. As such, the domain II S1–S2 loop may play a role in position the toxin into a water-filled cavity adjacent to the S3–S4 paddle motif, thereby placing particular toxin residues in strategic positions to stabilize the domain II voltage sensor in an activated position. This hypothesis is strengthened by the observation that the substitution of two residues in the domain II S1–S2 loop within mammalian Na+,2 channels decreases sensitivity to the functionally related β-scorpion toxin CsαIV by a factor of four. Another possible mechanism is that rDc1a binding to the domain II S1–S2 loop in BgNav1 allosterically influences channel opening. Such a hypothesis was postulated when a domain III S1 splice variant of BgNav1 revealed an increased susceptibility to the insect-selective β-scorpion toxin Lqh-dprIT3 (ref. 60). However, one caveat is that a depolarizing prepulse is needed to potentiate the maximal effect of CsαIV and Lqh-dprIT3 (refs 49,50). Similar to the structurally unrelated spider toxin Magi5 which activates rat Na+,1.2 channels by binding to the domain II voltage sensor61, rDc1a does not require such a prepulse.

In summary, the current study provides a molecular explanation for the remarkable insect selectivity of Dc1a, the most potent insecticidal toxin identified in the venom of the desert bush spider. Moreover, we have uncovered a novel toxin receptor site within insect Na+ channels that provides a new framework for the design of molecules capable of targeting specific insect families. This knowledge may be used in the future to develop insecticides that target specific insect pests without affecting beneficial insects or endangering human health.

**Methods**

**Chemicals.** All chemicals were purchased from Sigma-Aldrich Australia (Castle Hill, NSW, Australia), Sigma-Aldrich USA (St Louis, MO, USA), or Merck Chemicals (Kilsyth, Victoria, Australia) with the exception of IPTG and streptomycin (Life Technologies, Victoria, Australia), tetrodotoxin (Alomone Labs, Israel), and high-performance liquid chromatography (HPLC)-grade acetonitrile (RCI Labscan, Bangkok, Thailand). 13C6-glucose and 15NH4Cl were from Sigma-Aldrich and high-performance liquid chromatography (HPLC)-grade acetonitrile (RCI Labscan, Bangkok, Thailand). 13C6-glucose and 15NH4Cl were from Sigma-Aldrich.

**Production of recombinant rDc1a.** A synthetic gene encoding Dc1a, with codons optimized for expression in *Escherichia coli*, was produced and cloned into a variant of the pLIC-MBP expression vector by GeneArt (Invitrogen, Regensburg, Germany). This vector (pLIC-NSB3) encodes a MalE signal sequence for periplasmic export, a His6 tag for affinity purification, a maltose-binding protein.

![Figure 6](image-url)
(MBP) fusion tag to aid solubility, and a tobacco etch virus (TEV) protease recognition site directly preceding the codon-optimized DclA gene\(^{37}\). The plasmid encoding DclA was transformed into E. coli strain BL21 (DE3) for recombinant toxin production. Protein expression and purification were performed as described previously\(^{38}\) with minor modifications. In summary, cultures were grown in Luria-Bertani broth at 37 °C with shaking. Toxin gene expression was induced with 0.003% IPTG at an OD\(_{600}\) of 1.0–1.1, then cells were grown at 20 °C for a further 12 h before collecting by centrifugation for 15 min at 10,500 g. For production of uniquely \(^{13}\)C\(^{15}\)N-labelled rDclA, cultures were grown in minimal medium supplemented with \(^{13}\)C-glucose and \(^{15}\)NH\(_4\)Cl as the sole carbon and nitrogen sources, respectively. The His\(_{16}\)-MBP-toxin fusion protein was extracted from the bacterial periplasm by cell disruption at 27 kPa (TS Series Cell Disrupter, Constant Systems Ltd, Northants, UK), and then captured by passing the extract (buffered in 40 mM Tris, 450 mM NaCl, pH 8.0) over Ni-NTA Superflow resin (Qiagen, Chadstone, Australia). Proteins bound non-specifically were removed by washing with 10 mM imidazole then the fusion protein was eluted with 600 mM imidazole. The eluted fusion protein was concentrated to 10 ml and the buffer was exchanged to remove imidazole. Reduced and oxidized glutathiones were then added to a final concentration of 0.6 mM and 0.4 mM, respectively, to maintain TEV protease activity and promote folding of the protein. Approximately, 100 µg of His\(_{16}\)-tagged TEV protease was added per mg of rDclA, then the cleavage reaction was allowed to proceed at room temperature for 12 h. The cleared His\(_{16}\)-MBP, and His\(_{16}\)-TEV protease were precipitated by addition of 1% trifluoroacetic acid (TFA), then the sample was centrifuged at 16,000 g. The supernatant was filtered using a 0.45-μm syringe filter (Millipore, MA, USA) and subjected to further purification using RP-HPLC. RP-HPLC was performed on a Vydac C18 column (250 × 4.6 mm, particle size 5 μm) using a flow rate of 1 ml min\(^{-1}\) and a gradient of 20–45% Solvent B (0.043% TFA in (0.05% TFA in water) over 30 min. rDclA contains a non-native N-terminal serine residue (a vestige of the TEV protease cleavage site), making it one residue longer than native DclA.

**Mass spectrometry.** Toxin masses were confirmed by MALDI-TOF mass spectrometry using a Model 4700 Proteomics Bioanalyzer (Applied Biosystems, CA, USA). RP-HPLC fractions were mixed (1:1 v-v) with hydroxycinnamic acid matrix (5 mg ml\(^{-1}\)) and spotted onto a MALDI-TOF 5020 mass spectrometer. Toxin masses were confirmed by MALDI-TOF mass spectrometry using a Model 4700 Proteomics Bioanalyzer (Applied Biosystems, CA, USA). RP-HPLC fractions were mixed (1:1 v-v) with hydroxycinnamic acid matrix (5 mg ml\(^{-1}\)) and spotted onto a MALDI-TOF 5020 mass spectrometer. Liquid junction potentials were calculated using JPCALC, and all data were expressed as mean ± s.e.m. of n independent experiments.

**Electrophysiological measurements on DUM neurons.** DUM neurons were isolated from unsexed adult American cockroaches (P. americana) as described previously\(^{39}\). Briefly, terminal abdominal ganglia were dissected in normal insect saline (NIS) (containing in (mM): NaCl 140, KCl 3, H\(_2\)PO\(_4\) 10, NaHCO\(_3\) 20, and EGTA 1) and transferred to a petri dish containing HEPES (10 mM) and D-glucose 20. Toxin masses were confirmed by MALDI-TOF mass spectrometry using a Model 4700 Proteomics Bioanalyzer (Applied Biosystems, CA, USA). RP-HPLC fractions were mixed (1:1 v-v) with hydroxycinnamic acid matrix (5 mg ml\(^{-1}\)) and spotted onto a MALDI-TOF 5020 mass spectrometer. Liquid junction potentials were calculated using JPCALC, and all data were expressed as mean ± s.e.m. of n independent experiments.

**Insecticidal assays.** rDclA was dissolved in insect saline and injected into the ventro-lateral thoracic region of adult sheep blowflies (Lucilia cuprina), adult houseflies (Musca domestica) and adult German cockroaches (Blattella germanica)\(^{40}\). Injected were made using a 1.0 ml Terumo Insulin syringe (B-D Ultra-Fine, Terumo Medical Corporation, Maryland, USA) with a fixed 29 G needle fitted to an Arnold hand micro-applicator (Burkard Manufacturing Co., Ltd., England). A maximum volume of 2 μl was injected per insect. Toxin injection using the chelator control (insect saline; n=10 insects) was used. The assay was repeated three times. Median knockdown (KD\(_{50}\)) and median lethal (LD\(_{50}\)) doses were calculated as described previously\(^{40}\) and averaged to produce KD\(_{50}\) and LD\(_{50}\) values.

**Electrophysiological measurements on oocytes.** Chimeras and channel mutants were generated using sequential PCR-cuts with K.217AT (ref. 68) and B.na1-3 (ref. 21) as templates. The K217AT construct contains seven point mutations in the outer conductance domain and five of these were altered to alanine as a control for the DNA sequence of all constructs and mutants was confirmed by automated DNA sequencing and cDNA was synthesized using T7 polymerase (mMessage mMachine kit, Life Technologies, NATURE COMMUNICATIONS | DOI: 10.1038/ncomms5350 | www.nature.com/naturecommunications
USA) after linearizing the DNA with appropriate restriction enzymes. Channel constructs including hNa1.1–hNa1.7 and hERG were expressed in Xenopus oocytes by themselves or, in the case of BgNav1, together with the TipE subunit (1:5 molar ratio), and studied following 1–2 days incubation after cRNA injection (incubated at 17 °C in 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM MgCl₂, and 1.8 mM CaCl₂, 50 µg ml⁻¹ gentamycin, pH 7.6 with NaOH) using two-electrode voltage-clamp recording techniques (OC-725C, Warner Instruments, USA) with a 150-μl recording chamber. Data were filtered at 4 kHz and digitized at 20 kHz using pClamp software (Molecular Devices, USA). Microelectrode resistances were 0.5–1 MΩ when filled with 3 M KCl. For most Kv channel experiments, the external recording solution contained (in mM): 50 KCl, 50 NaCl, 5 HEPES, 1 MgCl₂, 0.3 CaCl₂, 7.6 with NaOH. For Na₆ channel experiments, the external recording solution contained (in mM): 96 NaCl, 2 KCl, 5 HEPES, 1 MgCl₂, and 1.8 CaCl₂, pH 7.6 with NaOH. All experiments were performed at room temperature (±2 °C). Leak and background conductances, identified by blocking the channel with intoxicating-2 (Alomone labs) or tetrodotoxin, were subtracted for most of the Kᵥ or BgNav1 currents shown.

Voltage–activation relationships were obtained by measuring tail currents for Kᵥ channels or steady-state currents and calculating conductance for Naᵥ channels. Protocols for other measurements are described in the figure legends. After addition of the toxin to the recording chamber, the equilibration between the toxin and the channel was monitored using weak depolarizations elicited at 5-r intervals. For all measurements, we recorded voltage–activation relationships in the absence and presence of toxin. Off-line data analysis was performed using Clampfit 10 (Molecular Devices, USA), Origin 8.0 (Originlab) and Microsoft Solver (Microsoft Excel).

References

1. King, G. F., Escoubas, P. & Nicholson, G. M. Peptide toxins that selectively target insect Naᵥ and Caᵥ channels. Channels 2, 100–116 (2008).
2. Hille, B. Ion Channels of Excitable Membranes 3rd edn (Sinauer Associates, Inc., 2001).
3. Bosmans, F., Martin-Eauclaire, M. F. & Swartz, K. J. Deconstructing voltage sensor function and pharmacology in sodium channels. Nature 456, 202–208 (2008).
4. Bosmans, F., Pupeolo, M., Martin-Eauclaire, M. F., Bean, B. P. & Swartz, K. J. Functional properties and toxin pharmacology of a dorsal root ganglion sodium channel viewed through its voltage sensors. J. Gen. Physiol. 138, 59–72 (2011).
5. Papavassiliou, J., Scheuer, T., Zheng, N. & Catterall, W. A. The crystal structure of a voltage-gated sodium channel. Nature 475, 353–358 (2011).
6. Zhang, X. et al. Crystal structure of an orthologue of the NaChBac voltage-gated sodium channel. Nature 486, 130–134 (2012).
7. Milesu, M. et al. Interactions between lipids and voltage sensor paddles detected with tarantula toxins. Nat. Struct. Mol. Biol. 16, 1080–1085 (2009).
8. Dong, C. K. Insect sodium channels and insecticide resistance. Invert. Neurosci. 7, 17–30 (2007).
9. Soderlund, D. M. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. Arch. Toxicol. 86, 165–181 (2012).
10. Tan, J. & Soderlund, D. M. Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat NaV1.6 sodium channels. PLoS ONE 8, e63865 (2013).
11. Milesu, M. et al. Tarantula toxins interact with voltage sensors within lipid membranes. J. Gen. Physiol. 130, 497–511 (2007).
12. Eberhard, W. Attack behavior of diguetid spiders and the origin of prey wrapping in spiders. Psyche 74, 173–181 (1967).
13. Liu, Z., Song, W. & Dong, K. Persistent tetrodotoxin-sensitive sodium current resulting from U-to-C RNA editing of an insect sodium channel. Proc. Natl Acad. Sci. USA 101, 11862–11867 (2004).
14. Trudel, M. C., Warmke, J. W., Ganetzky, B. & Robertson, G. A. HERG, a human inward rectifier in the voltage-gated potassium channel family. Science 269, 92–95 (1995).
15. Cestele, S. et al. Structure and function of the voltage sensor of sodium channels probed by a beta-scorpion toxin. J. Biol. Chem. 281, 21332–21344 (2006).
16. Cestele, S. et al. Voltage sensor-trapping: enhanced activation of sodium channels by β-scorpion toxin bound to the S3–S4 loop in domain II. Neuron 21, 919–931 (1998).
17. Swartz, K. J. Tarantula toxins interacting with voltage sensors in potassium channels. Toxicon 49, 213–230 (2007).
18. Alabi, A. A., Bahamonde, M. I., Jung, H. J., Kim, J. I. & Swartz, K. J. Portability of paddle motif function and pharmacology in voltage sensors. Nature 450, 370–375 (2007).
19. Long, S. B., Tao, X., Campbell, E. B. & MacKinnon, R. Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. Nature 450, 376–382 (2007).
20. Aggarwal, S. K. & MacKinnon, R. Contribution of the S4 segment to gating charge transfer center in voltage sensors. Proc. Natl Acad. Sci. USA 102, 1681–1691 (2005).
21. 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).
22. Zagotta, W. N., Hoshi, T. & Aldrich, R. W. Shaker potassium channel gating. III: evaluation of kinetic models for activation. J. Gen. Physiol. 103, 321–362 (1994).
23. Delmotte, L., Tarek, M., Klein, M. L., Amaral, C. & Treptow, W. Intermediate states of the Kv1.2 voltage sensor from atomistic molecular dynamics simulations. Proc. Natl Acad. Sci. USA 108, 6109–6114 (2011).
24. Jensen, M. B. et al. Mechanism of voltage gating in potassium channels. Science 336, 223–233 (2012).
25. Tao, X., Lee, A., Limapichat, W., Dougherty, D. A. & MacKinnon, R. A gating charge transfer center in voltage sensors. Science 328, 67–73 (2010).
26. Gagnon, D. G. & Bezanilla, F. A single charged voltage sensor is capable of gating the Shaker K+ channel. J. Gen. Physiol. 133, 467–483 (2009).
27. Lacroix, J. J. et al. Intermediate state trapping of a voltage sensor. J. Gen. Physiol. 140, 635–652 (2012).
28. Buraei, Z., Anghelescu, M. & Elmslie, K. S. Slow N-type calcium channel (Cav.2.2) deactivation by the cyclin-dependent kinase inhibitor roscovitine. Biophys. J. 89, 1681–1691 (2005).
29. Czap, D. L., Goldschneider-Otm, M. P., Arcisio-Miranda, M., Bezanilla, F. & Chanda, B. Domain IV voltage-sensor movement is both sufficient and rate limiting for fast inactivation in sodium channels. J. Gen. Physiol. 142, 101–112 (2013).
50. Chanda, B. & Bezanilla, F. Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. J. Gen. Physiol. 120, 629–645 (2002).

51. Sheets, M. F., Kyle, J. W., Kallen, R. G. & Hanck, D. A. The Na channel voltage sensor associated with inactivation is localized to the external charged residues of domain IV, S4. Biophys. J. 77, 747–757 (1999).

52. Cestele, S. et al. Scorpion α-toxins, toxic to both mammals and insects, differentially interact with receptor site 3 on voltage-gated sodium channels in mammals and insects. Eur. J. Neurosci. 11, 975–985 (1999).

53. Tan, J., Liu, Z., Nomura, Y., Goldin, A. L. & Dong, K. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. J. Neurosci. 22, 5300–5309 (2002).

54. Bourdin, C. M. et al. Intron retention in mRNA encoding ancillary subunit of insect voltage-gated sodium channel modulates channel expression, gating regulation and drug sensitivity. PLoS ONE 8, e67290 (2013).

55. Catterall, W. A. Voltage-gated ion channels and gating modifier toxins. Toxicon 49, 124–141 (2007).

56. Xiao, Y., Jackson, 2nd J. O., Liang, S. & Cummins, T. R. Common molecular determinants of tarantula huwentoxin-IV inhibition of Na+ channel voltage sensors in domains II and IV. J. Biol. Chem. 286, 27301–27310 (2011).

57. Saez, N. J. et al. Spider-venom peptides as therapeutics. Toxins 2, 2851–2871 (2010).

58. Krepkij, D. et al. Structure and hydration of membranes embedded with voltage-sensing domains. Nature 462, 473–479 (2009).

59. Zhang, J. Z. et al. Structure-function map of the receptor site for β-scorpion toxins in domain II of voltage-gated sodium channels. J. Biol. Chem. 286, 33641–33651 (2011).

60. Song, W. et al. Substitutions in the domain III voltage-sensing module enhance the sensitivity of an insect sodium channel to a scorpion β-toxin. J. Biol. Chem. 286, 15781–15788 (2011).

61. Corzo, G. et al. Distinct primary structures of the major peptide toxins from the venom of the spider Macrothele gigas that bind to sites 3 and 4 in the sodium channel. FEBS Lett. 547, 43–50 (2003).

62. Fang, L. et al. An improved strategy for high-level production of TEV protease in Escherichia coli and its purification and characterization. Proteins Expr. Purif. 51, 102–109 (2007).

63. Mobli, M., Maciejewski, M. W., Gryk, M. R. & Hoch, J. C. An automated tool for maximum entropy reconstruction of biomolecular NMR spectra. Nat. Methods 4, 467–468 (2007).

64. Shen, Y., Delaglio, F., Cornilescu, G. & Bax, A. TALOS +: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. J. Biomol. NMR 44, 213–223 (2009).

65. Eitan, M. et al. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. Biochemistry 29, 5941–5947 (1990).

66. Herzig, V. & Hodgson, W. C. Neurotoxic and insecticidal properties of venom from the Australian theraphosid spider Selenotholus foelschei. Neurotoxicology 29, 471–475 (2008).

67. Zhou, W. & Goldin, A. L. Use-dependent potentiation of the Nav1.6 sodium channel. Biophys. J. 87, 3862–3872 (2004).

68. Swartz, K. J. & MacKinnon, R. Hanatoxin modifies the gating of a voltage-dependent K+ channel through multiple binding sites. Neuron 18, 665–673 (1997).

69. Feng, G., Deak, P., Chopra, M. & Hall, L. M. Cloning and functional analysis of TipE, a novel membrane protein that enhances Drosophila para sodium channel function. Cell 82, 1001–1011 (1995).

70. Nguyen, M. N., Tan, K. P. & Madhusudhan, M. S. CLICK—topology-independent comparison of biomolecular 3D structures. Nucleic Acids Res. 39, W24–W28 (2011).