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Molecular interaction of δ-conotoxins with voltage-gated sodium channels

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Abstract Various neurotoxic peptides modulate voltage-gated sodium (Na_V) channels and thereby affect cellular excitability. δ-Conotoxins from predatory cone snails slow down inactivation of Na_V channels, but their interaction site and mechanism of channel modulation are unknown. Here, we show that δ-conotoxin SVIE from *Conus striatus* interacts with a conserved hydrophobic triad (YFV) in the domain-4 voltage sensor of Na_V channels. This site overlaps with that of the scorpion α-toxin Lqh-2, but not with the α-like toxin Lqh-3 site. δ-SVIE functionally competes with Lqh-2, but exhibits strong cooperativity with Lqh-3, presumably by synergistically trapping the voltage sensor in its "on" position.

Keywords: Conotoxin; Inactivation; Receptor site; Scorpion toxin; Sodium channel; Neurotoxin

1. Introduction

Na_V channel proteins are responsible for the rapid electrical signaling of neurons and muscle cells. They consist of four homologous domains and respond to changes in the transmembrane electric field by a translocation of their voltage sensor elements formed by the segments S3/S4 in each domain. The investigation of Na_V channels and the discrimination of different isoforms was largely facilitated by various neurotoxins that affect channel function in specific ways. In addition, neurotoxins have attracted attention with respect to their use as drugs, e.g., for pain treatment. Studies of the physical interaction between Na_V channels and neurotoxins have revealed various binding sites for neurotoxins on the channel proteins (termed receptor site-1 through site-9 [1,2]), where only sites 1–5 are molecularly defined. Several classes of neurotoxins of completely different structure affect Na_V channel inactivation. However, it is not known whether they exert their effects according to a common mechanism and if there is a functional coupling between them. Long-chain scorpion α-toxins, for example, consisting of 60–70 amino acids, target receptor site-3 mainly located in the S3/S4 linker of domain-4 and inhibit rapid channel inactivation. Cone-snail δ-conotoxins [3], peptides consisting of about 30 amino acid residues, exert a similar effect on Na_V channels. However, previous competition experiments measuring the binding of radioactively labeled toxins to membrane preparations containing Na_V channels have suggested that their molecular receptor site ("site-6") is distinct from that of scorpion α-toxins [4–6].

Many δ-conotoxins, such as δ-GmVIA and δ-TxVIA [7–10]], are specific for mollusks; δ-EVIA is poorly active on mammals, but exhibits some subtype specificity [11]. In this report, we show that the δ-conotoxin SVIE from *Conus striatus* affects mammalian Na_V channels. In addition, we identify the interaction motif of δ-SVIE with the voltage sensor of domain-4 in Na_V channels and show that this motif is not shared with the α-like toxin Lqh-3 while it is shared with the α-toxin Lqh-2. The interaction pattern with the voltage sensor determines whether neurotoxins functionally compete (δ-SVIE and Lqh-2) or even exhibit a strong functional synergism (δ-SVIE and Lqh-3).

2. Materials and methods

2.1. Channel constructs and mutagenesis

The Na_V channel type used was rat skeletal muscle sodium channel I, $rNa_V1.4$ (M26643) [12]. For single site-directed exchanges of amino acids 1431–1440 against cysteines, an *Nhe*I restriction site was introduced between basepairs 4268 and 4273 of the $rNa_V1.4$-encoding ORF [12], using the QuikChange mutagenesis kit (Stratagene, LaJolla, CA, USA). PCR primers containing this *Nhe*I site and the respective mutation were used in PCRs together with a primer binding on the opposite strand at the end of the ORF, including a second *Nhe*I site following the stop codon. The obtained PCR fragments were ligated into the pGemT vector (Promega, Madison, USA) and then sequenced prior to transferring the *Nhe*I-digested fragments into the $rNa_V1.4$-*Nhe*I-pcDNA3 expression plasmid. Correct insertion was verified by sequencing.

2.2. Cell culture and transfection

HEK 293 cells (CAMR, Porton Down, Salisbury, UK) were maintained in 45% Dulbecco's Modified Eagle's Medium (DMEM) and 45% F12, supplemented with 10% fetal calf serum in a 5% CO_2 incubator at 37 °C. They were transiently transfected with a 5:1 ratio of the Na_V channel expression plasmids and a vector encoding the CD8 antigen using the Superfect transfection kit (Quiagen, Hilden, Germany). Dynabeads (Deutsche Dynal GmbH, Hamburg, Germany) were used for visual identification of individual transfected cells.

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2.3. Electrophysiological measurements and analysis

Whole-cell voltage clamp experiments and toxin application were performed as described previously [13]. Data were acquired with an EPC-10 patch clamp amplifier operated by PatchMaster software (HEKA Elektronik, Lambrecht, Germany). Data analysis was performed using FitMaster (HEKA) and IgorPro (WaveMetrics, Lake Oswego, OR, USA). The patch pipettes contained (mM): 35 NaCl, 105 CsF, 10 EGTA, 10 HEPES (pH 7.4 with CsOH). The bath solution contained (mM): 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH). The degree of fast inactivation was assayed at 0 mV by measuring the peak current as well as the mean current level between 4.5 and 5 ms after the start of the depolarization. The ratio $I_{5\,ms}/I_{peak}$ gives an estimate of the probability for the channels not to be inactivated after 5 ms [13]. The dose dependence for toxin-induced removal of fast inactivation was measured by plotting $I_{5\,ms}/I_{peak}$ as a function of toxin concentrations. The concentration dependence was described with the Hill equation, $I_{5\,ms}/I_{peak} = a_0 + a_1/(1 + (EC_{50}/[\text{toxin}])^h)$, where h is the Hill coefficient, [toxin] the toxin concentration, a_0 the offset. The amplitude, a_1 plus a_0, provides the maximal value of $I_{5\,ms}/I_{peak}$ indicating the expected maximal effect of the toxin on fast inactivation. EC_{50} provides a measure for the concentration of half-maximal inhibition of fast inactivation. All data are expressed as arithmetic means ± S.E.M. (n = number of independent experiments).

2.4. Toxin purification and synthesis

δ-SVIE (Q9XZK5) was synthesized as previously described [14]. The scorpion α-toxins Lqh-2 (P59355) and Lqh-3 (P56678) [15] from *Leiurus quinquestriatus hebraeus* were generous gifts of D. Gordon (University Tel Aviv, Israel).

3. Results and discussion

3.1. Effect of δ-SVIE on channel inactivation

The δ-conotoxin SVIE investigated here, proved to be highly potent in modifying the gating of rat skeletal muscle $Na_V1.4$ channels expressed in mammalian cells (Fig. 1A). The major effect of δ-SVIE is to impair rapid channel inactivation. The concentration dependence for slowing channel inactivation at 0 mV yielded an apparent K_D value of the toxin effect of 500 ± 130 nM (n = 5; Fig. 1B) and a Hill coefficient close to unity (0.93 ± 0.18) suggesting a simple one-to-one reaction between toxin and channel molecule.

The effect of δ-SVIE on $Na_V1.4$ channel inactivation is similar to that of scorpion α-toxins, which bind to receptor site-3 [13,16–19]. This prompted us to perform an analysis of the functional impact of δ-SVIE on $Na_V1.4$ channel mutants that were previously shown to exhibit quite different properties regarding their interaction with the scorpion α-toxins Lqh-2 and Lqh-3 [15]. Rat $Na_V1.2$, a representative of Na_V channels from the central nervous system is insensitive to Lqh-3, and human $Na_V1.7$ from peripheral nerves, is less sensitive to Lqh-2 than $Na_V1.4$, when expressed in HEK 293 cells [13]. This difference in toxin-channel interaction could be attributed to single charged residues in the S3/S4 linker of domain-4 (Fig. 1C). Mutation D1428E of $Na_V1.4$ reduces the activity of Lqh-3 by about a factor of 1000; Q1431E and K1432T had strong impacts for the action of Lqh-2 ([19], Fig. 1E). However, as illustrated in Fig. 1D and E, these mutations did not result in a reduction of the ability of 2 μM δ-SVIE to remove inactivation of the channels.

3.2. Identification of the δ-SVIE interaction site at the Na_V voltage sensor element

Alterations of non-conserved residues in the putative interaction site for scorpion α-toxins only had moderate impacts

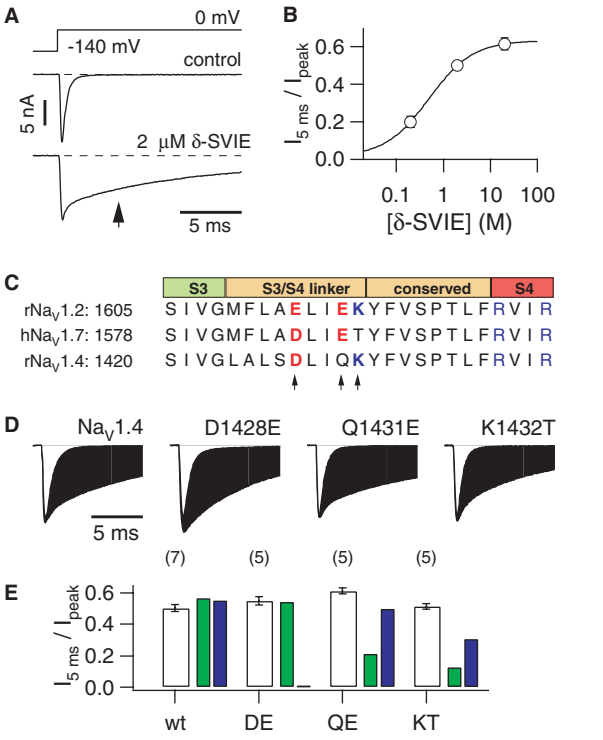

Fig. 1. Effect of δ-SVIE on wild-type $Na_V1.4$ channels and receptor site-3 mutants. (A) Current responses to depolarizations to 0 mV for $Na_V1.4$ channels under control conditions (center) and after application of 2 μM δ-SVIE (bottom). The arrow marks 5 ms, the time where inactivation of the channels was assayed. (B) Dose–response curve for the effect of δ-SVIE to remove inactivation at 0 mV (n = 5). (C) Alignment of the S3/S4 linker of domain-4 of the indicated mammalian Na_V channel types. Residues that were shown to take part in forming receptor site-3 are highlighted (arrows). The residues following to the right, i.e., towards the voltage sensor S4 are rather conserved. (D) Normalized current records at −20 mV of the wild-type channel $Na_V1.4$ as well as the indicated single-point mutants in the background of $Na_V1.4$. The control currents are superimposed to the records taken after application of 2 μM δ-SVIE. The difference between the two traces is shown as filled surface to indicate the increase in current integral upon toxin application. (E) Analysis of the mean inactivation at 0 mV, here expressed as $I_{5\,ms}/I_{peak}$, after application of 2 μM δ-SVIE. The filled bars indicate the corresponding values for the effect of 5 nM Lqh-2 (green) and 5 nM Lqh-3 (blue) (from [18,19]).

on the effect of the δ-conotoxin. Therefore, a cysteine-scanning mutagenesis of the conserved part of the S3/S4 linker of domain-4 was carried out, starting from Q1431C and ending at F1440C, the residue before the first arginine of the voltage sensor (Fig. 1C). All mutants were functionally expressed in HEK 293 cells and the effects of 2 μM δ-SVIE, 5 nM Lqh-2, and 5 nM Lqh-3, concentrations producing about the same effect on the inactivation of wild-type $Na_V1.4$ channels, were tested (Fig. 2A). The potency of δ-SVIE was slightly reduced by K1432C while the toxin was basically inactive on mutants Y1433C and F1434C. Surprisingly, it exhibited stronger effects on mutant V1435C. Thus, the ability of δ-SVIE to functionally modify $Na_V1.4$ channels is mainly determined by the hydrophobic triad Y1433–F1434–V1435 supporting the hypothesis of a hydrophobic interaction of δ-conotoxins with Na_V channels [10,20].

Interestingly, cysteine substitutions in the hydrophobic triad also modulate the effects of the α-toxin Lqh-2, while they leave the effects of the α-like toxin Lqh-3 unchanged (Fig. 2B).

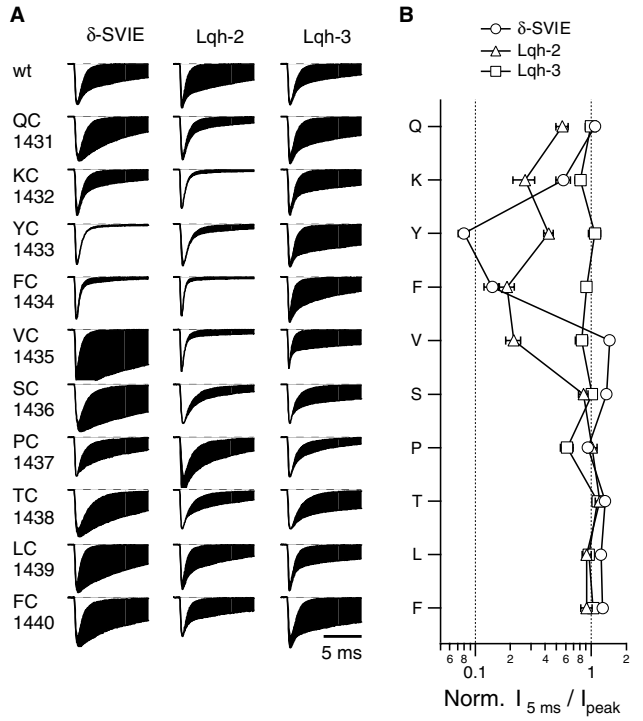

Fig. 2. Cysteine scan of the S3/S4 linker. (A) Cysteine-substituted $Na_V1.4$ channels were expressed in HEK 293 cells and current responses to −20 mV before and after application of 2 μM δ-SVIE, 5 nM Lqh-2, or 5 nM Lqh-3 were recorded. The shaded areas illustrate the increase in current integral upon toxin application. (B) The effect of the toxins on the inactivation time course at 0 mV was normalized to the values obtained for the wild-type ($Na_V1.4$) and is plotted as a function of the position of the mutated residue on a logarithmic scale. While mutagenesis of the channel sequence had virtually no impact on the effect of Lqh-3 (squares), both δ-SVIE (circles) and Lqh-2 (triangles) are strongly affected when cysteines are introduced between residues K1432 and V1435.

Together, these results are compatible with Lqh-2 and δ-SVIE sharing common interaction epitopes on Na_V channels. Considering that the S3/S4 linker in domain-4 of Na_V channels is longer than the S3/S4 linkers in the other domains, it is conceivable that it harbors both, parts of receptor site-3 and site-6, where site-3 is located more towards S3 and site-6 towards S4.

It can be concluded that a broad variety of neurotoxins affecting inactivation of Na_V channels (e.g. [16,21–24]) all function according to a common mechanism. One part of the toxin presumably binds to the surface of the channel protein. This channel section forms the major part of a "receptor site", i.e., receptor site-3 for α-toxins and receptor site-6 for δ-conotoxins. With a second moiety the toxins attack the voltage sensor of domain-4, which is tightly coupled to the process of inactivation.

3.3. Competition and synergism between δ-conotoxin SVIE and scorpion α-toxins

Scorpion α-toxins interact with the channel in a state-dependent manner. When the membranes are subjected to depolarizations strong enough to drive all channels into an open state, the toxin effect is diminished, i.e., the toxin appears to dissociate from its binding site. Such an experiment with the δ-conotoxin δ-SVIE is shown in Fig. 3A, where a 170-ms depolarization to +80 mV removes part of the toxin effect in the following test depolarization. Varying the time of depolarization yields a single-exponential dissociation (Fig. 3B, open circles). Hence, also δ-SVIE appears to bind to Na_V channels depending on the channel's conformational state.

The apparent overlap of residues in the channel protein important for the interaction with δ-SVIE and Lqh-2 should result in functional competition of these toxins for the overlapping site; this may not be the case for δ-SVIE and Lqh-3. To address whether there are functionally competitive interactions between toxins, we measured toxin dissociation when membranes were depolarized to +80 mV for 2 μM δ-SVIE and for 200 nM of the α-toxins, separately and in combination. Dissociation proceeds with a single-exponential time course (Fig. 3B–D) when only one type of toxin is present; the individual toxins had the following dissociation time constants: δ-SVIE, 247 ± 12 ms; Lqh-2, 29 ± 2 ms; Lqh-3, 488 ± 19 ms ($n = 4$ each). Coapplication of δ-SVIE and Lqh-2 resulted in a biphasic disappearance of the toxin effect with roughly the same time constants, consistent with the toxins competing for the same binding domain (Fig. 3B). The same holds true for Lqh-2 and Lqh-3 (Fig. 3C).

In the presence of δ-SVIE and Lqh-3, however, the dissociation rate under depolarization was very much slowed down. Although the more slowly dissociating α-toxin Lqh-3 exhibits

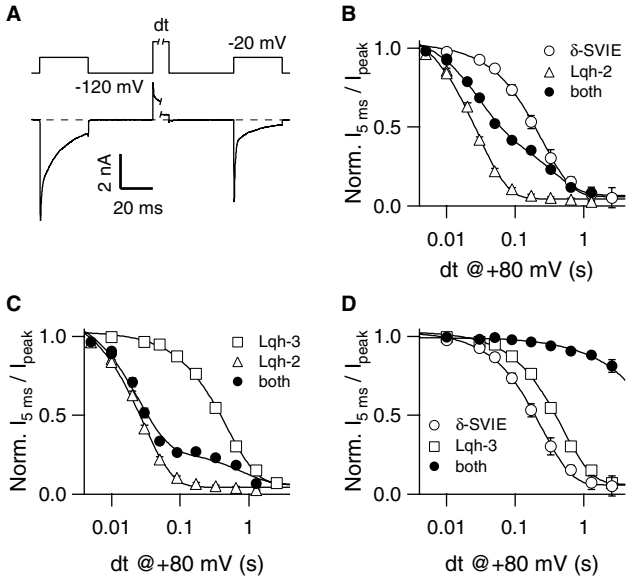

Fig. 3. Competition and synergy between δ-conotoxin SVIE and scorpion α-toxins. (A) Current trace in the presence of 2 μM δ-SVIE for the indicated pulse protocol. The center pulse to +80 mV was given for variable durations, here shown for 170 ms. (B) Time course of depolarization-induced loss of toxin effect for 200 nM Lqh-2, 2 μM δ-SVIE, and both toxins together. The apparent dissociation of the individual toxins was described by single-exponentials yielding time constants of 247 ms (δ-SVIE) and 29 ms (Lqh-2). The combination of both was described by a double-exponential with $\tau_1 = 29$ ms (56%) and $\tau_2 = 340$ ms (44%). (C) Similar experiments as in (B) but with Lqh-3 and Lqh-2, yielding 490 ms for Lqh-3. The combination of both toxins obeyed a double-exponential time course: $\tau_1 = 25$ ms (78%) and $\tau_2 = 810$ ms (22%). (D) Dissociation experiments with Lqh-3 and δ-SVIE. In combination with δ-SVIE the toxin effect could not even be eliminated by very long depolarizations. Only about 3% of the channels showed a dissociation constant of 490 ms. For the remaining 97% a time constant of at least 15 s was estimated. Such long depolarizations cannot be tested experimentally because of slow channel inactivation taking place ($n = 5$).

a dissociation time constant of about 500 ms, a pulse of 2.5 s to +80 mV only removed about 10% of the toxin effect when 2 μM δ-SVIE was coapplied. Therefore, the two toxins clearly do not directly compete for the same binding site at the voltage sensor (Fig. 3D). Instead, they apparently stabilize each other at their target sites such that these toxins affect the gating of Na_V channels in a highly synergistic manner.

When applied together, Lqh-3 and δ-SVIE dissociated at a strikingly slower rate from the channel protein during depolarization than would have been predicted from the off-times of each of the individual toxins. Translocation of the domain-4 S3/S4 voltage sensor element is presumably required before the faster dissociation kinetics can occur. If the voltage sensor with both toxins bound were unable to undergo this conformational change upon depolarization, in effect, Lqh-3 and δ-SVIE immobilized the domain-4 S3/S4 element in a conformation different from that of the fully translocated voltage sensor.

With respect to the interaction motif between the S3/S4 linker and neurotoxins various possibilities are utilized in nature yielding competition or synergism between neurotoxins. δ-SVIE and Lqh-3, which are unrelated in structure and origin, co-operate in immobilizing the channel's voltage sensor. The results, thus, identify the molecular mechanism of δ-conotoxin action on Na_V channels and present an unprecedented synergism of neurotoxins with therapeutic potential.

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