Cell. Mol. Life Sci. 64 (2007) 1329 – 1340
1420-682X/07/111329-12
DOI 10.1007/s00018-007-6565-5

Cellular and Molecular Life Sciences

Review

Conotoxins of the O-superfamily affecting voltage-gated sodium channels

S. H. Heinemann* **and E. Leipold**

Center of Molecular Biomedicine, Department of Biophysics, Friedrich Schiller University Jena, Drackendorfer Str. 1, 07747 Jena (Germany), Fax: +49 3641 932 5682, e-mail: Stefan.H.Heinemann@uni-jena.de

Received 27 December 2006; received after revision 30 January 2007; accepted 19 February 2007
Online First 24 March 2007

Abstract. The venoms of predatory cone snails harbor a rich repertoire of peptide toxins that are valuable research tools, but recently have also proven to be useful drugs. Among the conotoxins with several disulfide bridges, the O-superfamily toxins are characterized by a conserved cysteine knot pattern: C-C-CC-C-C. While ω-conotoxins and κ-conotoxins block Ca^{2+} and K^+ channels, respectively, the closely related δ- and μO-conotoxins affect voltage-gated Na^+ channels (Na_V channels). δ-conotoxins mainly remove the fast inactivation of Na_V channels and, thus, functionally resemble long-chain scorpion α-toxins. μO-conotoxins are functionally similar to μ-conotoxins, since they inhibit the ion flow through Na_V channels. Recent results from functional and structural assays have gained insight into the underlying molecular mechanisms. Both types of toxins are voltage-sensor toxins interfering with the voltage-sensor elements of Na_V channels.

Keywords. Na^+ channel, inactivation, cone snails, conotoxin, scorpion toxin, receptor sites, channel block, pain.

Classification of conotoxins

Predatory cone snails hunt fish and mollusks using a cocktail of peptide toxins, referred to as conotoxins. Such toxins are classified according to their number of cysteine residues, resulting in disulfide-rich and disulfide-poor toxins. Those conotoxins harboring many disulfides are grouped into superfamilies according to the pattern of disulfide-forming cysteines [1]. The O-superfamily toxins consist of about 25–35 residues, and 6 cysteines are distributed according to the cysteine knot pattern C-C-CC-C-C (Fig. 1). These cysteines form three disulfide bridges (C_1–C_4, C_2–C_5, C_3–C_6) resulting in compact globular structures (Fig. 2). M-superfamily toxins also harbor three disulfide bridges with the pattern CC-C-C-CC (Fig. 1). Both, O- and M-superfamiliy conotoxins target voltage-gated ion channels. In addition, ψ-conotoxins of the M-superfamily are active on nicotinic acetylcholine receptor channels.

Among the O- and M-superfamily conotoxins, there are three families that target voltage-gated Na^+ channels (Na_V channels). In the M-superfamily these are the so-called μ-conotoxins that block Na_V channels by occluding the channel pore (reviewed in [2, 3]). Such toxins compete with the Na_V channel antagonists tetrodotoxin (TTX) and saxitoxin (STX) and obstruct the ion flow by binding in the outer vestibule of the pore, thereby making contact with residues of all four domains of the channel protein (Fig. 3). Some μ-conotoxins, such as GIIIA from *Conus geographus* (see Fig. 2d), are specific for Na_V channels from skeletal muscle ($Na_V1.4$).

* Corresponding author.

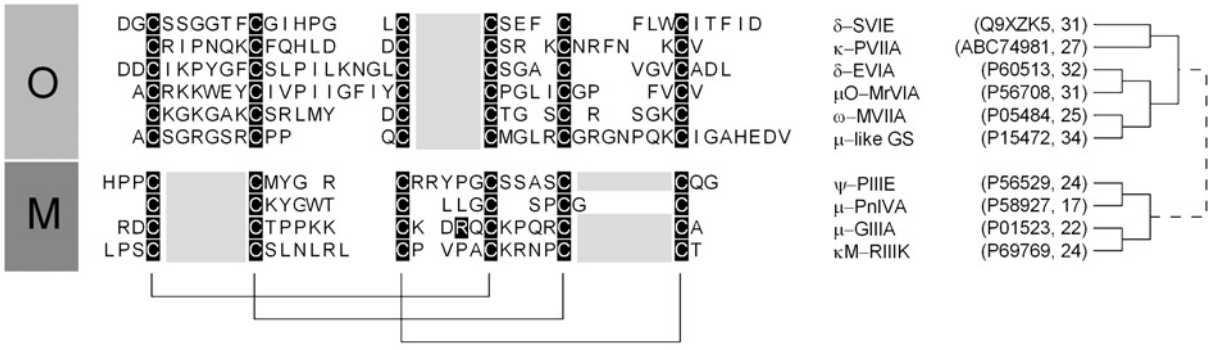

Figure 1. Multiple sequence alignment of a selected set of conotoxins that belong to the O- and the M-superfamily according to their cysteine knot pattern. Cysteines, marked in black, were enforced to align. The gray boxes indicate a lack of a cysteine-cysteine linker. The accession numbers and the number of residues per toxin are provided in parentheses. The cladogram on the right provides an assessment of the relative similarity of these toxins. Prolines and hydroxyprolines are not discriminated in this illustration because the existence of hydroxyprolines has not been proven in all cases.

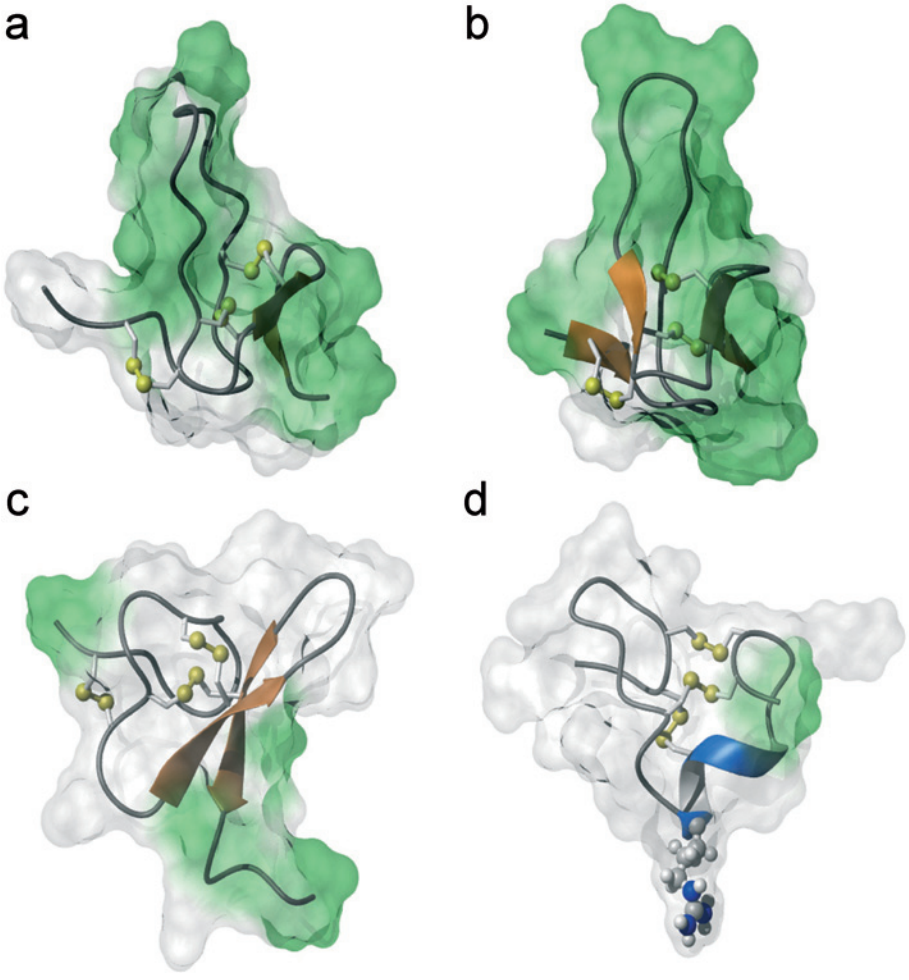

Figure 2. Structures of some selected conotoxins. (*a*) δ-EVIA, pdb 1G1P; (*b*) μO-MrVIB, pdb 1RMK; (*c*) μ-like GS, pdb 1AG7; (*d*) μ-GIIIA, pdb 1TCG. Graphics were generated with MOLMOL [50]. The backbones of the toxins are indicated by the ribbon structure. The underlying shading gives an impression on the space-filling surface. Hydrophobic toxin surfaces are indicated in green. Yellow spheres indicate cysteines forming disulfide bridges. In μ-GIIIA (*d*), arginine-13 is presented explicitly because this residue was shown to be essential for the stabilization of the toxin in the sodium channel pore.

The Na_V channel-specific toxins of the O-superfamily are δ-conotoxins that mostly inhibit the fast channel inactivation and μO-conotoxins that inhibit ion conductance. The latter received their name because of the functional similarity to μ-conotoxins combined with their structural association to the O-superfamily. In this review we focus on these two types of conotoxins because recent results have started to elucidate their mode of action. In particular, μO-conotoxins have attracted interest because of their potential to serve as a lead structure for novel analgesics [4, 5]. Further members of the O-super-

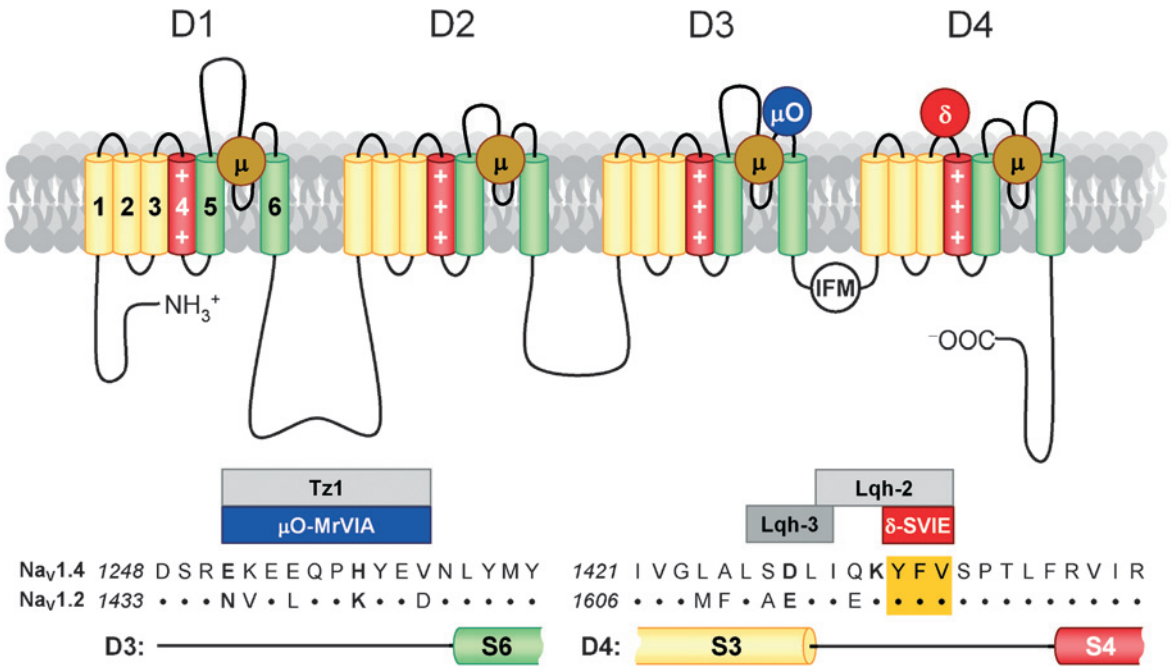

Figure 3. Topology model of a Na_V channel α-subunit. (*Top*) The voltage sensors (S4, red), the pore regions forming the selectivity filter, the inactivation motif "IFM" and the extracellular linkers taking part in forming toxin receptor sites are indicated. Putative interaction sites for μ-, δ- and μO-conotoxins are also marked. (*Bottom*) Comparison of the amino acid sequences forming interaction sites for μO- and δ-conotoxins (numbering of amino acids refer to the rat isoforms of the indicated Na_V channels). μO-MrVIA and scorpion β-toxin Tz1 (from *Tityus zulianus*) interact with an overlapping epitope in linker SS2/S6 of domain-3 (*left*) [30, 38]. δ-SVIE competes with the scorpion α-toxin Lqh-2 for an epitope in the S3/S4 linker of domain-4, while the interaction sites of δ-SVIE and Lqh-3 do not seem to overlap (*right*) [37]. The conserved hydrophobic triad "YFV", important for the function of δ-SVIE, is highlighted. Bold letters mark residues essential for the function of the indicated scorpion toxins.

family toxins are κ-conotoxins acting on voltage-gated K^+ channels and ω-conotoxins inhibiting voltage-gated Ca^{2+} channels (Fig. 1).

Voltage-gated Na^+ channels

Voltage-gated Na^+ channels are large glycosylated membrane protein complexes consisting of a single pore-forming α-subunit and one or two modulatory β-subunits (for review see [6]). The α-subunit is composed of four homologous domains (D1–D4) each consisting of six putative transmembrane segments (S1–S6) (Fig. 3). The ion pore and the intracellular gate are mainly formed by the S5–S6 elements of all four domains, arranged in a pseudo-tetrameric complex. The selectivity of the pore is determined by specific residues in the center of the pore loops, which fold back into the interior of the channel, exposing the residues "DEKA" into the center of the pore [7]. The linker between segments S5 and S6 can be split in two parts, SS1 and SS2, before and after the actual pore region. The remaining segments S1–S4 serve as a voltage sensor, where the gating charge is mainly contributed by S4, harboring a high density of arginine and lysine residues.

By means of these voltage-sensor elements, Na_V channels undergo conformational changes upon membrane depolarization to open an intracellular gate ultimately opening the Na^+-selective permeation pathway. The subsequent influx of Na^+ ions into neurons or muscle cells causes further depolarization with the effect of initiating an action potential. Channel activation is paralleled with a process of rapid inactivation closing the channel pore, and thereby assuring a directed propagation of electrical signals. Like activation, inactivation is regulated by changes in the transmembrane voltage. Although the molecular mechanisms are not known in very much detail, it is well established that the intracellular linker between domains D3 and D4 is essential for rapid inactivation, in particular the triad of residues "IFM" plays a key role and is expected to occlude the channel in a hinged-lid like fashion [8–10]. Experiments employing mutagenesis of residues in the voltage sensors indicate that channel inactivation is tightly coupled to the voltage sensors in domains D3 and D4 [11–13].

Thus, in comparison with voltage-gated K^+ channels, which in case of homomeric complexes are formed by four α-subunits with rotational symmetry, Na_V channels only show a pseudo-symmetry: The voltage

sensors in the four domains are different both with respect to their molecular structure and their functional relevance. In addition, the process of inactivation is coupled to more than one voltage sensor in an asymmetric manner.

Na_V channels are found in almost all higher animals, the number of genes coding for them, however, differs strongly. While, for example the worm, *C. elegans* does not express Na_V channels at all, insects typically have one major form (*e.g.*, the para Na^+ channel in the fruit fly, *D. melanogaster, Dm*Na_V1). In humans there are nine well-characterized channels $Na_V1.1$–$Na_V1.9$ and an additional gene (NaX, $Na_V2.1$) that has not yet been expressed functionally [14, 15]. The Na_V1 channels are typically classified according to their major site of expression (central nervous system: $Na_V1.1$–$Na_V1.3$, $Na_V1.6$; peripheral nerves: $Na_V1.6$–$Na_V1.9$; skeletal muscle: $Na_V1.4$; heart muscle: $Na_V1.5$) or their sensitivity towards the channel blocker TTX ($Na_V1.5$ low sensitivity; $Na_V1.8$ and $Na_V1.9$ insensitive; the rest is sensitive).

Receptor sites on Na^+ channels

Given the pivotal role of Na_V channels in the rapid electrical signaling, it does not come as a surprise that many venomous animals have developed toxins that specifically interfere with Na_V channel proteins. Such toxins are usually classified according to their biochemical binding to the channels and the functional consequences. Binding studies have revealed a number of so-called receptor sites (RS) (for review see [16–18]). RS1, for example, is the interaction epitope in the extracellular entry to the channel pore at which TTX and STX bind to block the channel pore. In a similar fashion, although much bigger molecules, μ-conotoxins bind to RS1 and block the channels. The remaining receptor sites are less well defined.

Receptor site-3 (RS3) is defined as the interaction epitope of scorpion α-toxins and sea anemone toxins [19]. Such toxins slow down inactivation of Na_V channels by interacting with the S3/S4 linker of domain-4 (Fig. 3, [20, 21]). At the first glance, it is not obvious how a toxin that binds extracellularly to Na_V channels should affect channel inactivation, which is mainly mediated by the intracellular linker between domains-3 and -4. Although the underlying molecular mechanism is still not understood in detail, it seems clear that a movement of the voltage sensor in domain-4 triggers the inactivation process. Upon depolarization, the voltage sensor in domain-4 moves in two steps: The first step is necessary for channel activation, while inactivation only occurs after the second step has been performed [22]. In the presence of site-3 toxins this second step of the voltage sensor movement is restricted, thus impairing rapid channel inactivation. The interaction of the toxin with the voltage sensor has the consequence that the binding of the toxin to the channels is voltage dependent. Strong depolarizations that drive the domain-4 sensor into the open configuration result in toxin dissociation and loss of toxin effect [23–26]. The physiological response of site-3 toxins is that Na_V channels stay open for longer time leading to hyperexcitability. In contrast to channel blockade, such a gain-of-function effect is very efficient and, hence, toxins modifying Na_V channel inactivation very potently cause pain and uncontrolled neuronal signaling.

δ-conotoxins also impair inactivation of Na_V channels (see Fig. 4a) and therefore show functional similarity to site-3 toxins. However, since δ-TxVIA from *Conus textile* only partially competed with the site-3 sea anemone toxin ATXII in a radioactive binding assay [27], a receptor site-6 was defined as interaction epitope of δ-conotoxins with Na_V channels. Owing to the relatively weak effects that δ-conotoxins exert on mammalian Na_V channels and a poor subtype specificity, little was known about the underlying molecular mechanisms until recently.

Scorpion β-toxins target receptor site-4 on Na_V channels [19]. Their primary effect is to lower the activation threshold of the channels, thus leading to channel opening at normal cellular resting potentials. The physiological effect is, therefore, also hyperexcitability. As with site-3 toxins, long-lasting cell depolarization induced by the toxins can lead to accumulated inactivation of Na_V channels and, hence, initial hyperexcitability may turn into depolarization block of the neurons and paralysis of muscle cells. In addition, some scorpion β-toxins also tend to "block" channels and are therefore classified "depressant" toxins [28]. The molecular mechanism of scorpion β-toxin action is most likely similar to that of scorpion α-toxins. However, scorpion β-toxins interact with the voltage sensor (S3/S4 linker) of domain-2 (Fig. 3). Since this domain is not strongly coupled to inactivation, β-toxins leave the inactivation unaffected. Instead, the toxins apparently trap the domain-2 voltage sensor in an activated state, and thus shift the entire channel into an activated configuration [29]. Unlike site-3 toxins, site-4 toxins do not show appreciable dissociation upon depolarization [30]. Apparently the toxin remains bound to the channel independently on the state of the domain-2 voltage sensor configuration. Such a scenario is supported by Leipold et al. [31], who found that the specificity of the scorpion β-toxin Tz1 to various mammalian Na_V channel types is determined by the structure of the SS2/S6 loop in domain-3 (Fig. 3).

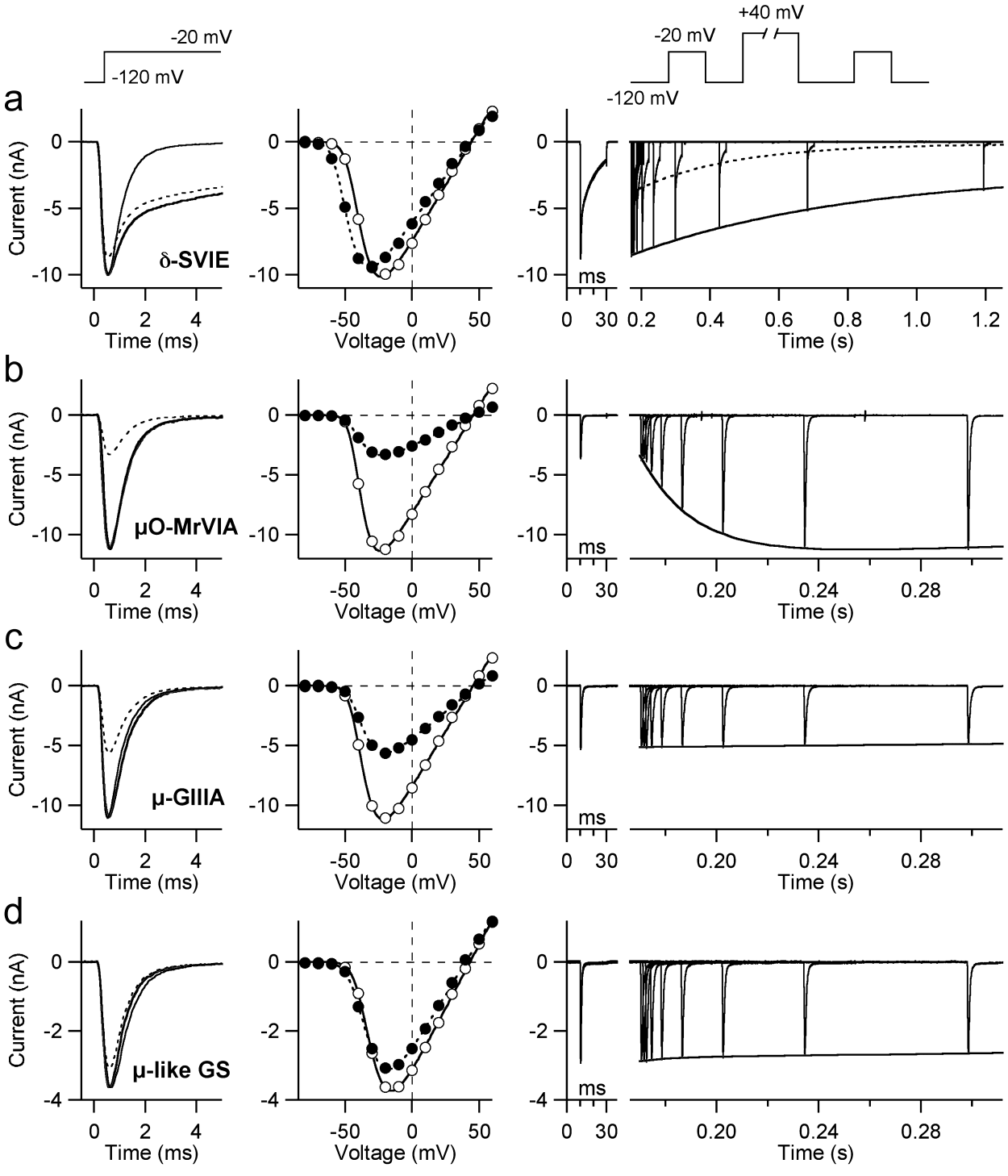

Figure 4. Toxin effects on rat skeletal muscle Na^+ channel ($Na_V1.4$). The functional impact of 2 μM δ-SVIE (*a*), 400 nM μO-MrVIA (*b*), 100 nM μ-GIIIA (*c*), and 300 μM μ-like GS (*d*) is illustrated. $Na_V1.4$ channels were expressed in HEK 293 cells and currents were measured in the whole-cell configuration of the patch clamp method, low-pass filtered at 5 kHz. The left panels show current responses at a depolarization of –20 mV before (solid) and after (dashed) toxin application. The gray traces are the toxin traces, scaled to match the peak of the control currents. The middle panels show current-voltage relationships before (open circles) and after (filled circles) toxin application. The continuous curves are fits according to a Hodgkin & Huxley gating formalism (*e.g.*, [39]). The panels on the right contain superpositions of current traces in response to the pulse protocol indicated at the top. Note that the time axes are split to blank out the conditioning pulse segments. The gray continuous curves are exponential fits to the peak currents measured in the second test pulse. In (*a*) (*right*), the dashed curve indicates the current level 5 ms after start of the depolarization, *i.e.*, indicating those channels that are affected by δ-SVIE to inactivate slowly. δ-SVIE, μO-MrVIA, and μ-GIIIA were kindly provided by, B. M. Olivera (Salt Lake City), μ-like GS was from Alexis Biochem. (San Diego, CA, USA).

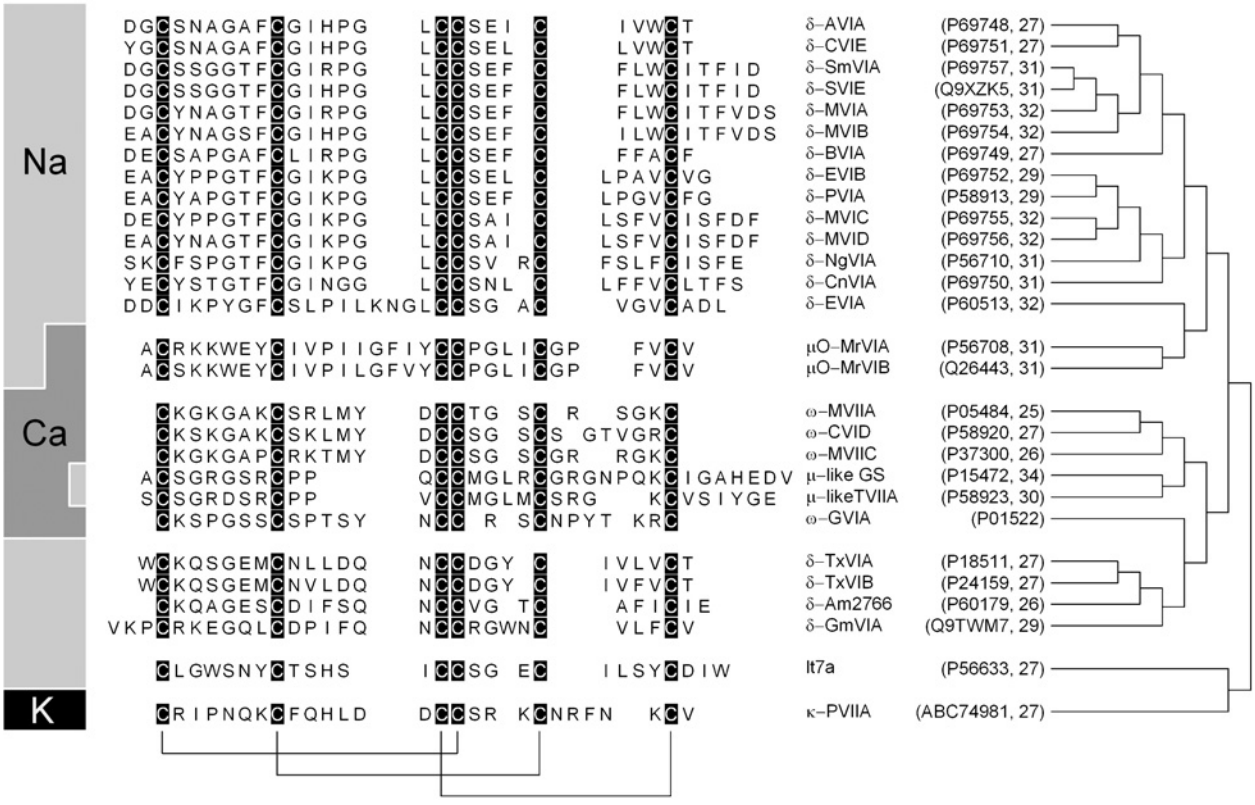

Figure 5. Comparison of O-superfamily conotoxins. Multiple alignment of all known δ-, μO-, and κ-conotoxins together with some selected ω-conotoxins (out of 36 deposited in the NCBI database). The μ-like toxins GS and TVIIA belong to the O-superfamily according to the know cysteine pattern, but thus far their primary target is not unequivocally established. Cysteines, marked in black, were enforced to align. Prolines and hydroxyprolines are not discriminated in this illustration because the existence of hydroxyprolines has not been proven in all cases. Accession numbers and the number of residues per toxin are provided in parentheses. The bars on the left assign the major targets to the toxins shown. In some cases it is established that toxins are promiscuous, *i.e.*, act on Na_V and Ca_V channels.

Little information is available for conotoxins of the μO-family [32, 33]. They inhibit Na_V channels (see Fig. 4b) by an unknown mechanism but apparently do not compete with the site-1 toxin STX [34]. There is no specific receptor site assigned to μO-conotoxins but, as shown later, they exhibit some functional similarity to site-4 toxins.

Molecular mechanism of δ-conotoxin action

Functional studies have been performed for various δ-conotoxins (see Fig. 5) on a diverse set of preparations. However, since these toxins are in the first place specific for fish or mollusks, systematic studies on recombinant Na_V channels that provide insight into the underlying molecular mechanisms, are sparse. An exception is δ-EVIA from *Conus ermineus*. Compared with other δ-conotoxins, δ-EVIA is unusual in the sense that its linker between cysteines 2 and 3 (L2) is longer than those of other δ-conotoxins (Fig. 5). The structure of δ-EVIA was determined (Fig. 2a; [35]) and functional studies were performed on mammalian Na_V channels expressed in *Xenopus* oocytes [36]. δ-EVIA was without effect on Na_V channels from skeletal muscle ($Na_V1.4$) and the heart ($Na_V1.5$). However, albeit at relatively high concentration (10 μM), it impaired the rapid inactivation of the neuronal channels $Na_V1.2$, $Na_V1.3$, and $Na_V1.6$ [36]. In a radioactive binding assay to synaptosomes, it competed with the more typical δ-conotoxin δ-TxVIA, but not with the scorpion α-toxin Lqh-2 [36]. Thus, δ-EVIA is not particularly active on mammalian Na_V channels, but shows a clear preference for neuronal channels. Since δ-conotoxins do not exhibit a clear preference for a specific mammalian Na_V channel, the subtype specificity of δ-EVIA may be utilized to identify interaction sites at the channel protein.

In a study using recombinant $Na_V1.4$ channels expressed in mammalian cells, the functional effect of δ-SVIE from *Conus striatus* was assayed in detail [37]. The toxin slows down inactivation as shown in Figure 4a (*left*), while the voltage dependence of channel activation is only slightly shifted towards hyperpolarized potentials (Fig. 4a, *middle*). Half-

maximal removal of channel inactivation was obtained at 500 nM [37], showing that δ-SVIE potently interacts with mammalian Na_V channels. Since the functional effect of δ-SVIE is reminiscent of the functional impact exerted by scorpion α-toxins, the influence of receptor site-3, *i.e.*, the S3/S4 linker of domain-4, was evaluated. Mutations in this linker that strongly affected the action of scorpion α-toxins Lqh-2 and Lqh-3 [21] were without effect for δ-SVIE [37]. A cysteine scanning mutagenesis, however, revealed that a conserved triad of hydrophobic residues in the S3/S4 linker of domain-4 ("1433YFV" in $rNa_V1.4$) is important for δ-SVIE (Fig. 3). This epitope overlaps with residues that are also essential for Lqh-2. It does not overlap, however, with regions that are essential for the function of Lqh-3. These results suggested that not all scorpion α-toxins share the same interaction epitope at the domain-4 S3/S4 linker; in addition, it clearly shows that δ-SVIE acts similarly to the site-3 toxins. Therefore, there does not seem to be a real molecular reason for defining a completely separate receptor site-6 for δ-conotoxins. We rather argue that both scorpion α-toxins and δ-conotoxins are site-3 toxins in the sense that they exert their effect by interacting with the domain-4 S3/S4 linker. The exact nature of the binding epitope inside the domain-4 S3/S4 linker, however, depends on the toxin under consideration. In addition, further binding epitopes on the channel surface may be different between scorpion α-toxin and δ-conotoxins.

A consequence of this scenario is that some site-3 toxins may compete for a common binding site, while others may not. To test for this, Leipold et al. [37] measured how quickly the toxin effect is removed when the channels are held in an activated configuration. As shown in Figure 4a (*right*), such a depolarization (at +40 mV) results in an apparent dissociation of the toxin from the channel because fast inactivation is regained in a subsequent test depolarization. Measurements of toxin dissociation using mixtures of Lqh-2, Lqh-3, and δ-SVIE revealed that Lqh-2 functionally competes with Lqh-3 and δ-SVIE. However, δ-SVIE acts synergistically with Lqh-3, suggesting that both toxins can interact with the domain-4 S3/S4 linker simultaneously as indicated in Figure 3.

We conclude at this point that δ-SVIE is apparently not very subtype specific because its major target in the domain-4 S3/S4 linker is a conserved triad of residues (YFV). Among the mammalian $Na_V1.1$–1.7 channels, only the cardiac form ($Na_V1.5$) shows a conservative alteration of this motif (YFF); the TTX-resistant channel $Na_V1.8$ exhibits a truncation of this motif (YF), giving rise to speculations regarding its sensitivity towards δ-conotoxins. In addition, δ-conotoxins are so-called voltage-sensor toxins; their binding to the channel is destabilized by channel activation, most likely by a conformational change of the voltage sensor in domain-4.

Molecular mechanism of μO-conotoxin action

Because two toxins from *Conus marmoreus*, MrVIA and MrVIB, potently inhibit Na_V channels [33], they inherited the term "μ" of the site-1 μ-conotoxins from the M-superfamily. However, according to the cysteine-knot pattern they belong to the O-superfamily and, therefore, they form a separate family termed "μO". These toxins are remarkable as they are promiscuous regarding the target: They block Na_V channels, but in the μM range they also affect Ca_V channels [32]. In this respect, they seem to be an intermediate between δ- and ω-conotoxins. Functionally, however, these toxins do not resemble δ-conotoxins since they do not affect the time course of Na_V channel inactivation (Fig. 4b). Instead, both μO-MrVIA and μO-MrVIB seem to "block" the channel without marked effects on channel gating kinetics. When tested on recombinant channels expressed in mammalian cells, this blocking potency was about fivefold higher for $Na_V1.4$ than for the neuronal $Na_V1.2$ channels. Zorn et al. [38] used this difference to search for a potential interaction epitope at the channel protein. Domain-swapping experiments revealed that the SS2/S6 loop in domain-3 (see Fig. 3) harbors the determinants for the subtype specificity of these toxins – a motif also used by scorpion β-toxins to discriminate between various Na_V channel isoforms [30]. Thus, it appears as if μO-conotoxins and scorpion β-toxins, similarly to the couple of δ-conotoxins and scorpion α-toxins, share some functional properties. In fact, μO-conotoxins are also voltage-sensor toxins since they affect Na_V channels in a strongly voltage-dependent manner [39]. As shown in Figure 4b (*right*), the blocking effect of 400 nM μO-MrVIA on $Na_V1.4$ channels is progressively removed by application of depolarizing pulses (+40 mV) of increasing lengths.

This voltage-dependent block of $Na_V1.4$ channels by μO-MrVIA is qualitatively very different from a channel block by typical μ-conotoxins. As shown in Figure 4c, 100 nM μ-GIIIA (from *Conus geographus*) also blocks skeletal muscle channels. However, even strong depolarizations do not relieve the block, *i.e.*, under the chosen experimental conditions the block neither noticeably depends on voltage nor on the channel state. The mechanism of μ-conotoxin action is well characterized; the toxins bind to the outer pore vestibule and, hence, obstruct ion flow. In some μ-

conotoxins a positively charged residue plays a central role in guiding the toxin into the channel. In μ-GIIIA this is residue R13, highlighted in the alignment of Figure 1 and in the structure of Figure 2d. The exact mechanism of how μO-conotoxins inhibit Na_V channel activity is not known. Given their voltage dependence (Fig. 4c), the overlap of an interaction epitope with scorpion β-toxins in domain-3 [38, 30], and the lack of competition with the site-1 toxin STX [34], it is expected that they interact with at least one of the voltage sensors.

μO-conotoxins have attracted considerable attention because of their potency to block TTX-resistant Na_V channels in dorsal root ganglia [40]. These channels ($Na_V1.8$) play a pivotal role in transmitting pain sensation and, hence, are potential targets for novel analgesics. In fact, treatment of rats with μO-MrVIB alleviated chronic pain symptoms without compromising motor behavior [4, 5]. The latter aspect may be attributed to the fact that μO-MrVIA blocked $Na_V1.8$ channels about ten times more efficiently than other neuronal channels [5]. Thus, μO-conotoxins are currently considered as potential lead structures for analgesics. The applicability of conotoxins for the treatment of chronic pain has been proven by the successful introduction of the N-type Ca^{2+}-channel specific ω-conotoxin ω-MVIIA as Prialt® (or ziconotide) [41].

Prediction of function from structure?

To date many sequences of conotoxins have been published and there are certainly more to come. However, it is by no means trivial to gain a complete overview of their functional properties. This is largely accounted to a rather circumstantial screening for the functional effects. For many conotoxins only limited tests in very diverse physiological preparations have been performed. Studies on recombinant ion channels in a systematic manner, both with respect to the channels selected and the methods of assay, are only at the very beginning. Therefore, one is often left with speculations about the "real" function of a particular conotoxin, largely motivated by structural features.

In the alignment of Figure 5, we compare various conotoxins of the O-superfamily, concentrating on δ- and μO-conotoxins, *i.e.*, those most specifically acting on Na_V channels. When an alignment of the conserved cysteine residues is enforced, the resulting relational cladogram provides some insight into putative structure-function relationships of these toxins. This becomes even more obvious when the physical properties of the toxins are evaluated in more detail. In Figure 6, we show the same toxins as in Figure 5, although ordered differently, and provide information on the hydrophobicity and the charge of the total toxins, the N- and C-terminal ends, and the loops connecting the cysteines (L1–L4). The cladogram (Fig. 5) and the charge distribution (Fig. 6) provide some structural reasoning for the establishment of the δ-, μO-, κ-, and ω-conotoxin families.

μO-conotoxins of the O-superfamily as well as μ-conotoxins of the M-superfamily suppress the flow of Na^+ through Na_V channels, but are considerably different with respect to their molecular mechanisms. Whereas the members of the M-superfamily, *e.g.*, μ-GIIIA, plug the Na^+ permeation pathway, the O-superfamily toxin μO-MrVIA inhibits Na_V channels by interfering with the channels' voltage sensors. μ- and μO-conotoxins are structurally different (Figs 1, 2); most remarkable is the lack of a linker between cysteines C1/C2 and C5/C6 in μ-conotoxins and between C3/C4 in μO-conotoxins. Therefore, it seems obvious to predict the association of a given Na_V channel inhibiting conotoxin to either the μ- or μO-family from its amino acid sequence. An interesting special case is PnIVA from mollusk-hunting *Conus pennaceus* [42]. This toxin blocks Na_V channels in *Lymnaea* neurons but, compared with μ-conotoxins, exhibits an extra glycine residue between cysteines 5 and 6 (Fig. 1) and was therefore termed here "μ-like PnVIA".

But which structural parameters distinguish the δ- and μO-families from the ω- and κ-families? A first hint came from Shon et al. [43]. They observed a remarkably longer elution time from a reversed phase HPLC column for natively folded δ-GmVIA (from *Conus gloriamaris*) compared with its partially reduced form, giving rise to the prediction that natively folded toxins expose hydrophobic residues to the solvent and that such hydrophobic residues may be important for the toxin-channel interaction. This assumption is supported by the three-dimensional structures of δ-TxVIA (*Conus textile*, [44]) and δ-EVIA (*Conus ermineus*, [35], Fig. 2a). Both toxins show an unusual hydrophobic patch on their surfaces (indicated as green surface in Fig. 2a) that is formed by hydrophobic residues of all four cysteine-cysteine linkers.

In addition to structural data, functional experiments with mutated δ-PVIA from *Conus purpurascens* verified the importance of hydrophobic residues for the activity of δ-conotoxins [45]. In a δ-PVIA background, hydrophobic residues that are conserved among the δ-conotoxins of fish-hunting species were substituted with alanine. F9 and I11 were identified as essential for the function of δ-PVIA but not for the binding of the toxin to its target. When injected intracranially into mice, the mutant δ-PVIA_F9A was ineffective, but protected the mice

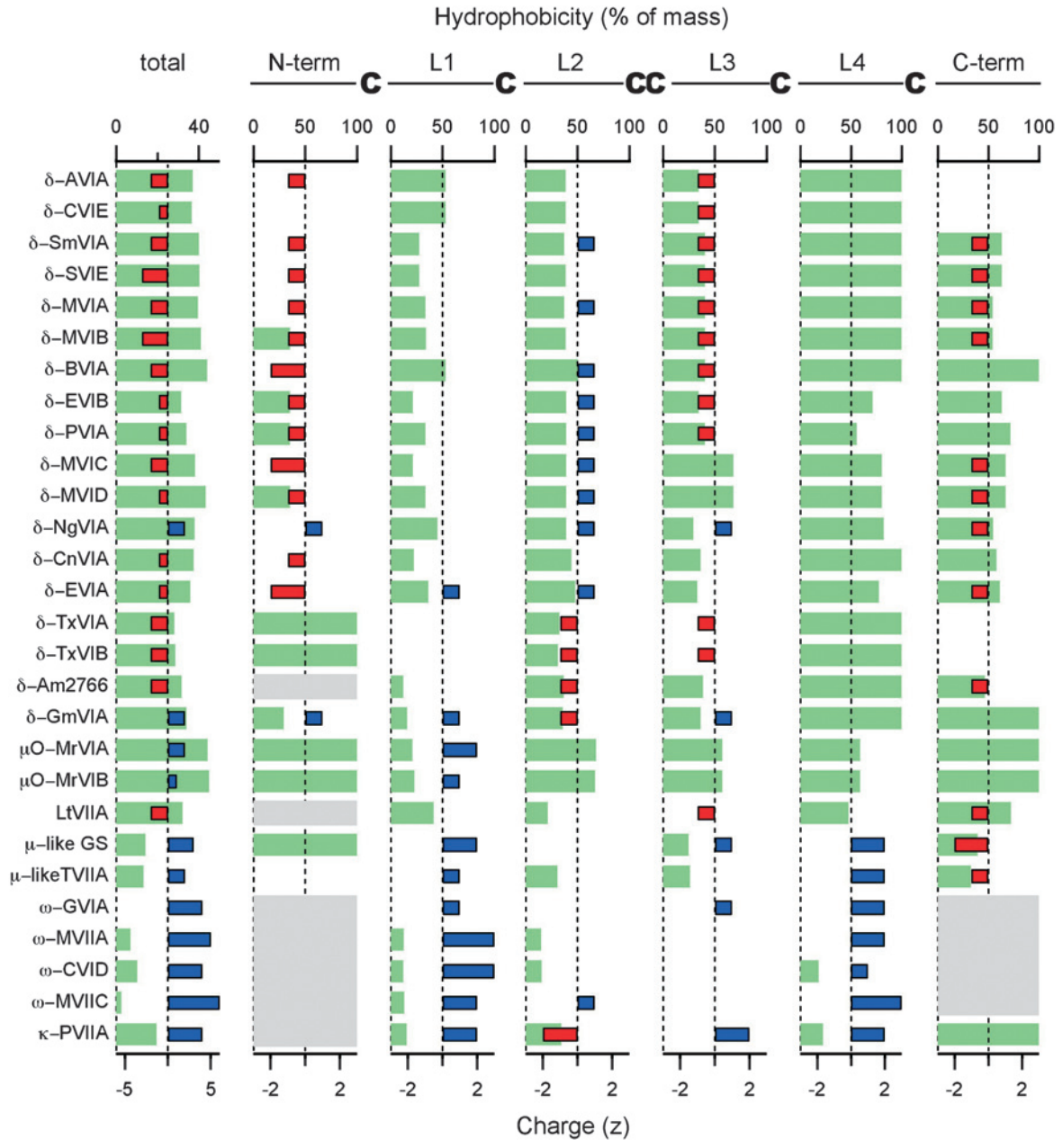

Figure 6. Physical properties of O-superfamily conotoxins. The cysteine knot pattern of O-superfamily conotoxins results in toxin structures with N-terminal and C-terminal ends and four cysteine-cysteine linkers (L1–L4). For the total toxins (*left*) and the individual ends and linkers, the hydrophobicity (A, V, L, I, F, and W were considered hydrophobic, their mass relative to the total mass is given in %; green bars) and the net negative and positive charges (red and blue bars, respectively) are presented. Gray boxes indicate the lack of N- or C-terminal extensions. This presentation provides some insight into the structural criteria used to assign a family to a specific conotoxin, *i.e.*, δ-conotoxins are characterized by a negative charge of the N terminus and a lack of charge in linkers L1 and L4. Such linkers, for example, are positively charged in κ-, ω-, and μ-like conotoxins.

from the excitatory effects that were observed when only the wild-type toxin was injected. However, this protection was not observed on frog sciatic nerve preparations. In addition, wild-type δ-PVIA and the mutants δ-PVIA_F9A and δ-PVIA_I11A were able to displace radiolabeled δ-TxVIA from rat brain preparations. These results fit very well to the identification of hydrophobic interaction sites for δ-SVIE in the S3/S4 linker of the Na_V channel's domain-4 (Fig. 3) [37].

Given the importance of hydrophobic residues for the function of δ-conotoxins, one may speculate whether the hydrophobicity of a certain toxin area is an exclusive feature of δ-conotoxins. When comparing the total hydrophobicity among the O-superfamily toxins (Fig. 6), it becomes evident that the toxins

separate into two groups. While the Na_V channel-specific δ- and μO-conotoxins form a highly hydrophobic cluster, the Ca_V channel-selective ω-conotoxins and the K_V channel-specific κ-conotoxin κ-PVIIA (*Conus purpurascens*) constitute a less hydrophobic group. Low hydrophobicity is also found for μ-like GS and μ-like TVIIA (see below). On the level of individual cysteine-cysteine linkers, this feature can be observed at higher resolution, being most obvious for linker L4, but also apparent for L2 and L3. It seems plausible that the remarkable hydrophobicity of δ- and μO-conotoxins and its distribution on the toxin surface are essential for recognizing Na_V channels. It is also conceivable that the conserved distribution of hydrophobic residues is indicative of a common molecular mechanism. Since both δ- and μO-conotoxins are voltage-sensor toxins, their strong hydrophobicity may be required for an interaction with the voltage sensors. Similarly to voltage-sensor toxins of K^+ channels [46], the Na_V-specific voltage-sensor toxins may at least partially involve the lipid phase of the membrane to access the molecular target at the channel protein.

Close inspection shows that the cladogram does not present δ-conotoxins as a common family. The first set of δ-conotoxins in Figure 5 comes from fish-hunting species, while those of the lower part are from snails that preferentially feed on mollusks. In the first place these are δ-TxVIA and δ-TxVIB from *Conus textile.* These toxins are without marked functional effects on mammalian Na_V channels, but seem to compete with other δ-conotoxins [31], indicating that there must be multiple interaction epitopes for δ-conotoxins at the channel protein. Compared with the other δ-conotoxins, they have a very hydrophobic N terminus and a negative charge in L2. Also deviating from the majority of δ-conotoxins is the mollusk-specific δ-GmVIA (*Conus gloriamaris*) [43]. δ-Am2766 from mollusk-hunting *Conus amadis* was shown to impair inactivation of rat brain sodium channels ($Na_V1.2$) [47]. An "outlier" is δ-EVIA that does not align well with either of the two groups. The charge distribution does not look very different from the other fish-specific δ-conotoxins, except for a positive charge of L1. A more marked difference, however, is the size of L2 (Fig. 5), which is six residues for typical δ-conotoxins and nine residues for δ-EVIA. Interestingly, μO-conotoxins also have nine residues in this linker.

The μO-conotoxins are characterized by a strong hydrophobicity (also see Fig. 2b), which clearly puts them into a group with the δ-conotoxins. The differences from most δ-conotoxins mainly reside in the hydrophobicity of the N terminus, a positive charge in L1, and a lack of negative charge in L3. μO-MrVIA and μO-MrVIB only differ by three amino acid residues and, thus far, only minor functional differences have been reported for these toxins. Since μO-conotoxins have been reported to also block Ca_V channels [32], one may expect some structural similarity with ω-conotoxins. According to Figure 6 this could reside in the common positive charge of L1, which is not present in typical δ-conotoxins.

At a concentration of 250 nM, LtVIIA, a recombinantly produced peptide based on a cDNA library of the worm-hunting *Conus litteratus* was shown to block TTX-sensitive and TTX-insensitive voltage-gated Na_V channels in rat dorsal root ganglia [48]. The alignment and the physical properties do not clearly put this toxin into the μO-family. Instead, there are marked similarities to κ-PVIIA acting on K_V channels. Thus, it will be interesting to know how this toxin affects Ca_V and K_V channels.

Further interesting examples are two O-superfamily toxins that may interact with Na_V channels, here termed μ-like toxins. μ-like GS (*Conus geographus*) apparently competes with site-1 toxins in binding to rat skeletal muscle homogenates with an IC_{50} of about 1 μM [49]. However, the cysteine knot pattern clearly puts this toxin into the O-superfamily and the structure of μ-like GS (Fig. 2c) is clearly different from that of μ-GIIIA (Fig. 2d). In addition, we tested the effect of 300 μM μ-like GS on rat $Na_V1.4$ channels expressed in mammalian cells and only found a very minor blocking effect, which was not relieved by strong depolarizations (Fig. 4d). Thus, it is by no means clear if this toxin is in fact Na_V specific. The physical properties rather put μ-like GS and μ-like TVIIA to a group with ω-conotoxins, acting on Ca_V channels (see, for example, the positive charges in L1 and L4).

Conclusion

Conotoxins of the O-superfamily form a diverse group of peptides that primarily target voltage-gated ion channels. The major targets of δ-conotoxins are Na_V channels, those of ω-conotoxins are Ca_V channels. While δ-conotoxins remove inactivation of Na_V channels by interacting with their voltage sensors in domain-4, the molecular mechanism of the other O-superfamily toxins is less obvious. μO-conotoxins, which have attracted attention because of their ability to block TTX-resistant Na_V channels, are also voltage-sensor toxins and may form a family between δ- and ω-conotoxins. The functional properties assigned to such voltage-sensor toxins will strongly depend on the exact nature of the experimental protocols, necessitating systematic ap-

proaches with standardized voltage-clamp protocols.

An unequivocal association of a conotoxin to a specific family still remains problematic because in most cases the functional assessment is only fragmentary. Therefore, future studies will not only bring to light more conopeptides enriching the O-superfamily, but will also elucidate more precisely the molecular basis for channel specificity or promiscuity. Given a detailed understanding of the functional modules of O-superfamily conotoxins will enable us to rationally design conopeptides with tailored properties as desired for the development of potent and specific drugs.

Acknowledgments. We thank H. Terlau for comments on the manuscript, B. M. Olivera for providing us with conotoxins, and the Deutsche Forschungsgemeinschaft for support (HE 2993/5).

1 Terlau, H. and Olivera, B. M. (2004) Conus venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev. 84, 41 – 68.
2 French, R. J. and Terlau, H. (2004) Sodium channel toxins-receptor targeting and therapeutic potential. Curr. Med. Chem. 11, 3053 – 3064.
3 Li, R. A. and Tomaselli, G. F. (2004) Using the deadly μ-conotoxins as probes of voltage-gated sodium channels. Toxicon 44, 117 – 122.
4 Bulaj, G., Zhang, M.-M., Green, B. R., Fiedler, B., Layer, R. T., Wei, S., Nielsen, J. S., Low, S. J., Klein, B. D., Wagstaff, J. D., Chicoine, L., Harty, T. P., Terlau, H., Yoshikami, D. and Olivera, B. M. (2006) Synthetic μO-conotoxin MrVIB blocks TTX-resistant sodium channel $Na_v1.8$ and has a long-lasting analgesic activity. Biochemistry 45, 7404 – 7414.
5 Ekberg, J., Jayamanne, A., Vaughan, C. W., Aslan, S., Thomas, L., Mould, J., Drinkwater, R., Baker, M. D., Abrahamsen, B., Wood, J. N., Adams, D. J, Christie, M. J. and Lewis, R. J. (2006) μO-conotoxin MrVIB selectively blocks $Na_v1.8$ sensory neuron specific sodium channels and chronic pain behavior without motor deficits. Proc. Natl. Acad. Sci. USA 103, 17030 – 17035.
6 Catterall, W. A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron 26, 13 – 25.
7 Heinemann, S. H., Terlau, H., Stühmer, W., Imoto, K. and Numa, S. (1992) Calcium channel characteristics conferred on the sodium channel by single mutations. Nature 356, 441 – 443.
8 Vassilev, P. M., Scheuer, T. and Catterall, W. A. (1988) Identification of an intracellular peptide segment involved in sodium channel inactivation. Science 241, 1658 – 1661.
9 Stühmer, W., Conti, F., Suzuki, H., Wang, X. D., Noda, M., Yahagi, N., Kubo, H. and Numa, S. (1989) Structural parts involved in activation and inactivation of the sodium channel. Nature 339, 597 – 603.
10 West, J. W., Patton, D. E., Scheuer, T., Wang, Y., Goldin, A. L. and Catterall, W. A. (1992) A cluster of hydrophobic amino acid residues required for fast Na^+-channel inactivation. Proc. Natl. Acad. Sci. USA 89, 10910 – 10914.
11 Chahine, M., George, A. L. Jr., Zhou, M., Ji, S., Sun, W., Barchi, R. L. and Horn, R. (1994) Sodium channel mutations in paramyotonia congenita uncouple inactivation from activation. Neuron 12, 281 – 294.
12 Chen, L. Q., Santarelli, V., Horn, R. and Kallen, R. G. (1996) A unique role for the S4 segment of domain 4 in the inactivation of sodium channels. J. Gen. Physiol. 108, 549 – 556.
13 Cha, A., Ruben, P. C., George, A. L. Jr., Fujimoto, E. and Bezanilla, F. (1999) Voltage sensors in domains III and IV, but not I and II, are immobilized by Na^+ channel fast inactivation. Neuron 22, 73 – 87.
14 Goldin, A. L. (1999) Diversity of mammalian voltage-gated sodium channels. Ann. N. Y. Acad. Sci. 868, 38 – 50.
15 Goldin, A. L. (2002) Evolution of voltage-gated Na^+ channels. J. Exp. Biol. 205, 575 – 584.
16 Janiszewski, L. (1990) The action of toxins on the voltage-gated sodium channel. Pol. J. Pharmacol. Pharm. 42, 581 – 588.
17 Zlotkin, E. (1999) The insect voltage-gated sodium channel as target of insecticides. Annu. Rev. Entomol. 44, 429 – 455.
18 Cestèle, S. and Catterall, W. A. (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883 – 892.
19 Catterall, W. A. (1986) Molecular properties of voltage-sensitive sodium channels. Annu. Rev. Biochem. 55, 953 – 985.
20 Rogers, J. C., Qu, Y., Tanada, T. N., Scheuer, T. and Catterall, W. A. (1996) Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the Na^+ channel α subunit. J. Biol. Chem. 271, 15950 – 15962.
21 Leipold, E., Lu, S., Gordon, D., Hansel, A. and Heinemann, S. H. (2004) Combinatorial interaction of scorpion toxins Lqh-2, Lqh-3, and LqhαIT with sodium channel receptor sites-3. Mol. Pharmacol. 65, 685 – 691.
22 Horn, R., Ding, S. and Gruber, H. J. (2000) Immobilizing the moving parts of voltage-gated ion channels. J. Gen. Physiol. 116, 461 – 476.
23 Strichartz, G. R. and Wang, G. K. (1986) Rapid voltage-dependent dissociation of scorpion α-toxins coupled to Na channel inactivation in amphibian myelinated nerves. J. Gen. Physiol. 88, 413 – 435.
24 Strichartz, G., Rando, T. and Wang, G. K. (1987) An integrated view of the molecular toxinology of sodium channel gating in excitable cells. Annu. Rev. Neurosci. 10, 237 – 267.
25 Catterall, W. A. (1979) Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. Correlation of voltage-dependent binding with activation. J. Gen. Physiol. 74, 375 – 391.
26 Chen, H., Gordon, D. and Heinemann, S. H. (2000) Modulation of cloned skeletal muscle sodium channels by the scorpion toxins Lqh II, Lqh III, and LqhαIT. Pflügers Arch. 439, 423 – 432.
27 Fainzilber, M., Kofman, O., Zlotkin, E. and Gordon, D. (1994) A new neurotoxin receptor site on sodium channels is identified by a conotoxin that affects sodium channel inactivation in molluscs and acts as an antagonist in rat brain. J. Biol. Chem. 269, 2574 – 2580.
28 Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. (1998) Functional anatomy of scorpion toxins affecting sodium channels. J. Toxicol. Toxin Rev. 17, 131 – 159.
29 Cestèle, S., Qu, Y., Rogers, J. C., Rochat, H., Scheuer, T. and Catterall, W. A. (1998) 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.
30 Jover, E., Couraud, F. and Rochat, H. (1980) Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. Biochem. Biophys. Res. Commun. 95, 1607 – 1614.
31 Leipold, E., Hansel, A., Borges, A. and Heinemann, S. H. (2006) Subtype specificity of scorpion β-toxin Tz1 interaction with voltage-gated sodium channels is determined by the pore loop of domain 3. Mol. Pharmacol. 70, 340 – 347.
32 Fainzilber, M., van der Schors, R., Lodder, J. C., Li, K. W., Geraerts, W. P. M. and Kits, K. S. (1995) New sodium channel-blocking conotoxins also affect calcium currents in *Lymnaea* neurons. Biochemistry 34, 5364 – 5371.
33 McIntosh, J. M., Hasson, A., Spira, M. E., Gray, W. R., Li, W., Marsh, M., Hillyard, D. R. and Olivera, B. M. (1995) A new family of conotoxins that blocks voltage-gated sodium channels. J. Biol. Chem. 270, 16796 – 16802.
34 Terlau, H., Stocker, M., Shon, K. J., McIntosh, J. M. and Olivera, B. M. (1996) μO-conotoxin MrVIA inhibits mamma-

lian sodium channels, but not through site, I. J. Neurophysiol. 76, 1423 – 1429.
35 Volpon, L., Lamthanh, H., Barbier, J., Gilles, N., Molgo, J., Menez, A. and Lancelin, J.-M. (2004) NMR solution structures of δ-conotoxin EVIA from *Conus ermineus* that selectively acts on vertebrate neuronal Na^+ channels. J. Biol. Chem. 279, 21356 – 21366.
36 Barbier, J., Lamthanh, H., Le Gall, F., Favreau Ph., Benoit, E., Chen, H., Gilles, N., Ilan, N., Heinemann, S. H., Gurevitz, M., Gordon, D., Ménez, A. and Molgó, J. (2004) A δ-conotoxin from *Conus ermineus* venom that inhibits inactivation in vertebrate neuronal Na^+ channels, but not in skeletal and cardiac muscles. J. Biol. Chem. 279, 4680 – 4685.
37 Leipold, E., Hansel, A., Olivera, B. M., Terlau, H. and Heinemann, S. H. (2005) Molecular interaction of δ-conotoxins with voltage-gated sodium channels. FEBS Lett. 579, 3881 – 3884.
38 Zorn, S., Leipold, E., Hansel, A., Bulaj, G., Olivera, B. M., Terlau, H. and Heinemann, S. H. (2006) The μ-O-conotoxin MRVIA inhibits voltage-gated sodium channels by associating with domain-3. FEBS Lett. 580, 1360 – 1364.
39 Leipold, E., Zorn, S., de Bie, H., Borges, A., Olivera, B. M., Terlau, H., Heinemann, S. H. (2007) μO-Conotoxins inhibit Na_V channels by interfering with their voltage sensors in domain-2. Biophysical Society Meetings Abstracts. Biophys. J., 178a, Abstract, 831-Plat.
40 Daly, N. L., Ekberg, J. A., Thomas, L., Adams, D. J., Lewis, R. J. and Craik, D. J. (2004) Structures of μO-conotoxins from *Conus marmoreus*. J. Biol. Chem. 279, 25774 – 25782.
41 Bowersox, S. S. and Luther, R. (1998) Pharmacotherapeutic potential of omega-conotoxin MVIIA (SNX-111), an N-type neuronal calcium channel blockers found in the venom of *Conus magus*. Toxicon 36, 1651 – 1658.
42 Fainzilber, M., Nakamura, T., Gaathon, A., Lodder, J. C., Kits, K. S., Burlingame, A. L. and Zlotkin, E. (1995) A new cysteine framework in sodium channel blocking conotoxins. Biochemistry 34, 8649 – 8656.
43 Shon, K. J., Hasson, A., Spira, M. E., Cruz, L. J., Gray, W. R. and Olivera, B. M. (1994) δ-conotoxin GmVIA, a novel peptide from the venom of *Conus gloriamaris*. Biochemistry 33, 11420 – 11425.
44 Kohno, T., Sasaki, T., Kobayashi, K., Fainzilber, M. and Sato, K. (2002) Three-dimensional solution structure of the sodium channel agonist/antagonist δ-conotoxin TxVIA. J. Biol. Chem. 277, 36387 – 36391.
45 Bulaj, G., DeLaCruz, R., Azimi-Zonooz, A., West, P., Watkins, M., Yoshikami, D. and Olivera, B. M. (2001) δ-Conotoxin structure/function through a cladistic analysis. Biochemistry 40, 13201 – 13208.
46 Lee, S. Y. and MacKinnon, R. (2004) A membrane-access mechanism of ion channel inhibition by voltage sensor toxins from spider venom. Nature 430, 232 – 235.
47 Sudarslal, S., Majumdar, S., Ramasamy, P., Dhawan, R., Pal, P. P., Ramaswami, M., Lala, A. K., Sikdar, S.K., Sarma, S. P., Krishnan, K. S. and Balaram, P. (2003) Sodium channel modulating activity in a δ-conotoxin from an Indian marine snail. FEBS Lett. 553, 209 – 212.
48 Pi, C., Liu, J., Wang, L., Jiang, X., Liu, Y., Peng, C., Chen, S. and Xu, A. (2007) Soluble expression, purification and functional identification of a disulfide-rich conotoxin derived from *Conus litteratus*. J. Biotechnol. 128, 184 – 193.
49 Yanagawa, Y., Abe, T., Satake, M., Odani, S., Suzuki, J. and Ishikawa, K. (1988) A novel sodium channel inhibitor from *Conus geographus:* purification, structure, and pharmacological properties. Biochemistry 27, 6256 – 6262.
50 Koradi, R., Billeter, M. and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. J. Mol. Graphics 14, 51 – 55, 29 – 32.