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Research Paper

μO-Conotoxins Inhibit Na_V Channels by Interfering with Their Voltage Sensors in Domain-2

Enrico Leipold[1]
Herbert De Bie[1]
Stefan Zorn[1]
Adolfo Borges[2]
Baldomero M. Olivera[3]
Heinrich Terlau[4]
Stefan H. Heinemann[1,*]

[1]Center for Molecular Biomedicine; Department of Biophysics; Friedrich Schiller University; Jena, Germany

[2]Laboratorio de Toxinas Animales; Centro de Biociencias y Medicina Molecular; Instituto de Estudios Avanzados; and Instituto de Medicina Experimental; Universidad Central de Venezuela; Caracas, Venezuela

[3]Department of Biology; University of Utah; Salt Lake City, Utah USA

[4]Institute of Experimental and Clinical Pharmacology and Toxicology; University of Lübeck; Lübeck, Germany

*Correspondence to: Stefan H. Heinemann; Center for Molecular Biomedicine; Department of Biophysics; Friedrich Schiller University Jena; Drackendorfer St. 1; Jena D 07747 Germany; Tel.: 49.3641.9.32.56.80; Fax: 49.3641.9.32.56.82; Email: stefan.h.heinemann@uni jena.de

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ABSTRACT

The μO-conotoxins MrVIA and MrVIB are 31-residue peptides from *Conus marmoreus*, belonging to the O-superfamily of conotoxins with three disulfide bridges. They have attracted attention because they are inhibitors of tetrodotoxin-insensitive voltage-gated sodium channels (Na_V1.8) and could therefore serve as lead structure for novel analgesics. The aim of this study was to elucidate the molecular mechanism by which μO-conotoxins affect Na_V channels. Rat Na_V1.4 channels and mutants thereof were expressed in mammalian cells and were assayed with the whole-cell patch-clamp method. Unlike for the M-superfamily μ-conotoxin GIIIA from *Conus geographus*, channel block by MrVIA was strongly diminished after activating the Na_V channels by depolarizing voltage steps. Searching for the source of this voltage dependence, the gating charges in all four-voltage sensors were reduced by site-directed mutagenesis showing that alterations of the voltage sensor in domain-2 have the strongest impact on MrVIA action. These results, together with previous findings that the effect of MrVIA depends on the structure of the pore-loop in domain-3, suggest a functional similarity with scorpion β-toxins. In fact, MrVIA functionally competed with the scorpion β-toxin Ts1 from *Tityus serrulatus*, while it did not show competition with μ-GIIIA. Ts1 and μ-GIIIA did not compete either. Thus, similar to scorpion β-toxins, μO-conotoxins are voltage-sensor toxins targeting receptor site-4 on Na_V channels. They "block" Na^+ flow most likely by hindering the voltage sensor in domain-2 from activating and, hence, the channel from opening.

INTRODUCTION

The venoms of fish- and mollusk-hunting cone snails contain a rich repertoire of toxic peptides varying in length and the number of disulfide bridges. Many of these toxins target ion channels and are therefore useful tools for studying ion channel function. Conotoxins of the O-superfamily are characterized by three disulfide bridges with the corresponding cysteine residues arranged like C-C-CC-C-C.[1] O-superfamily toxins target voltage-gated ion channels; families with several known members are ω-conotoxins blocking voltage-gated Ca^{2+} channels and δ-conotoxins removing the inactivation of voltage-gated sodium channels (Na_V channels). Currently, there is only one member described for κ-conotoxins blocking voltage-gated K^+ channels.[2] The fourth family is formed by μO-conotoxins. Their name arises from their potency to inhibit Na_V channels, like μ-conotoxins of the M-superfamily, and their structural classification as O-superfamily conotoxins. To date there are only two μO-conotoxins known: MrVIA and MrVIB, both from *Conus marmoreus*.[3] These toxins have attracted attention because they appear to be valuable molecules to block tetrodotoxin-resistant Na_V channels in dorsal root ganglia and, therefore, may serve as lead structures for the development of novel Na_V channel-specific analgesics.[4,5]

Na_V channels are glycosylated membrane proteins composed of one large pore-forming α-subunit and up to two small β-subunits (reviewed in ref. 6). They open in response to a depolarization of the membrane. The resulting influx of Na^+ into the cells accentuates the depolarization and finally triggers action potentials. Therefore, Na_V channels are of prime importance for the generation and propagation of electrical signals in nerve and muscle cells. Given this physiological relevance it is not surprising that poisonous animals have developed specific toxins targeting Na_V channels. Such Na_V channel-specific toxins usually are classified according to so-called receptor sites (RS) that biochemically characterize the interaction sites at the channel protein (example in ref. 7). Peptide toxins acting on

Na_V channels either block the channel pore (RS1) or they interfere with the voltage-sensor elements consisting of the transmembrane segments S3/S4 of the four homologous domains of the α-subunit. Scorpion α-toxins interact with the voltage sensor in domain-4 (RS3) and cause an elimination of channel inactivation, which is coupled to that voltage sensor.[8,9] Similarly, δ-conotoxins inhibit Na_V channel inactivation making use of an interaction epitope at the domain-4 S3/S4 linker largely overlapping with that of scorpion α-toxins (originally referred to as RS6).[10] Scorpion β-toxins interfere with RS4, constituted in part of the voltage sensor in domain-2 and cause a shift in the voltage dependence of activation and can arrest the voltage sensor in its activated position (voltage-sensor trapping).[11,12]

The μO-conotoxins MrVIA and MrVIB consist of 31 natural amino acids and the structure of MrVIB has already been solved by NMR analysis (Fig. 6A).[13] The primary action of μO-conotoxins is to inhibit Na^+ flow through Na_V channels, but thus far no receptor site could be assigned to these toxins. Functionally they are similar to μ-conotoxins that block Na_V channels by binding inside the outer pore vestibule (RS1) (reviewed in ref. 14). However, binding assays involving radioactively labeled saxitoxin (STX) revealed that MrVIA apparently does not compete with STX for RS1.[15]

The molecular mechanism by which μO-conotoxins inhibit Na_V channels is unknown. A typical feature of δ-conotoxins and apparently also of μO-conotoxins is that they do not exhibit a marked subtype specificity when comparing their effect on various types of mammalian Na_V channels. For δ-conotoxins this was mainly attributed to the major interaction site in the domain-4 S3/S4 linker, which is conserved among mammalian Na_V channels.[10] For μO-conotoxins we have previously shown that they have a mild preference for the skeletal muscle ($Na_V1.4$) over the brain-type ($Na_V1.2$) channel. Based on that difference, a systematic approach involving the construction and assay of channel chimeras revealed that the C-terminal pore loop in domain-3 determines the channel phenotype with respect to the block effect of MrVIA.[16] This is exactly the channel epitope determining the subtype specificity of the scorpion β-toxin Tz1 (from *Tityus zulianus*).[17]

The aim of the following study was to identify the mode of action of the μO-conotoxin MrVIA and to test for a functional similarity with μ-conotoxins and scorpion β-toxins.

MATERIALS AND METHODS

Channel constructs. The wild-type channel used in this study was the rat skeletal muscle sodium channel $Na_V1.4$ (SCN4A, M26643.1).[18] In addition, eight mutant channels were constructed in which the two outermost charged residues in the S4 segments of domain-1 to domain-4 of $Na_V1.4$ were replaced individually with cysteines (R219C, R222C, R663C, R666C, K1119C, R1122C, R1441C, R1444C) using a PCR-based strategy. The construction of mutant G658N in the same background was previously described in ref. 17. All mutations were verified by sequencing. Plasmid DNA was isolated from *E. coli* using the Midi- or Maxi-plasmid purification kit (Qiagen, Hilden, Germany).

Cell culture and transfection. HEK 293 cells (CAMR, Porton Down, Salisbury, UK) were maintained in 45% Dulbecco's Modified Eagles Medium (DMEM) and 45% Ham's F12 Medium, supplemented with 10% fetal calf serum in a 5% CO_2 incubator at 37°C. HEK 293 cells were trypsinized, diluted with culture medium, and grown in 35-mm dishes. When grown to 30–50% confluence, cells were transfected with a 5:1 ratio of the Na_V channel expression plasmids and a vector encoding the CD8 antigen[19] using the Superfect transfection kit (Qiagen). Dynabeads (Deutsche Dynal GmbH, Hamburg, Germany) were used for visual identification of individual transfected cells. Electrophysiological recordings were performed 2–3 days after transfection.

Solutions and toxins. The patch pipettes contained (in mM): 35 NaCl, 105 CsF, 10 EGTA, 10 HEPES (pH 7.4 with CsOH). The bath solution contained (in mM): 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH). The toxins were diluted in bath solution containing 1 mg/ml BSA and stored at -20°C until use. The application of toxins was performed with an application pipette as previously described in ref. 20. GIIIA was chemically synthesized according to standard methods,[21] MrVIA was synthesized using a modification of a method previously described in ref. 3. Ts1 was obtained from *T. serrulatus* crude venom (Sigma Chemical Co., St. Louis, USA), which was first fractionated by gel exclusion chromatography using Sephadex G-50 according to Miranda et al.[22] Ts1 was then purified from Sephadex G-50 toxic fraction TstFG50[23] by a combination of ion exchange and reversed phase chromatographic steps according to Chávez-Olórtegui et al.[24]

Electrophysiology. Whole-cell voltage-clamp experiments were performed as (previously described in ref. 20). Briefly, patch pipettes with resistances of 0.9–2.0 MΩ were used. The series resistance was compensated for by more than 80% in order to minimize voltage errors. An EPC-10 patch-clamp amplifier was operated by PatchMaster software (both HEKA Elektronik, Lambrecht, Germany). Leak and capacitive currents were corrected with a p/*n* method. Currents were low-pass filtered at 5 kHz and sampled at a rate of 25 kHz. All experiments were performed at constant temperature (19–21°C). Data analysis was performed using IgorPro (WaveMetrics, Lake Oswego, OR, USA) and FitMaster (HEKA Elektronik). All data were presented as mean ± standard error of the mean (n = number of independent experiments). Data sets were tested for statistical significance using Student's t-test with unequal variances when appropriate.

Current-voltage relationships. From a holding potential of -120 mV test depolarizations in the range from -110 to +60 mV in steps of 10 mV were applied at an interval of 10 s. The peak currents in absence and presence of MrVIA were fit with a Hodgkin-Huxley activation formalism involving m=3 activation gates and a single-channel characteristics according to Goldman, Hodgkin, and Katz:

$$I(V) = P_o(V)\,\Gamma_{max}\,V\,\frac{1-e^{-(V-E_{rev})/25mV}}{1-e^{-V/25mV}} \qquad \text{(Eq. 1)}$$

$$P_o(V) = \frac{1-P_{inh}}{\left(1+e^{-(V-V_m)/k_m}\right)^3}$$

where I is the test pulse current and V is the test pulse voltage. V_m is the voltage of half-maximal gate activation, k_m is the corresponding slope factor, Γ_{max} is the maximal conductance of all channels and E_{rev} the reversal potential. P_{inh} describes the probability of the channels to be inhibited by the toxin under steady-state conditions. P_{inh} depends on the experimental conditions such as the holding voltage, but is largely independent of the test pulse voltage. In control experiments, i.e., in the absence of toxin P_{inh} was set to zero.

Functional competition of MrVIA, GIIIA and Ts1. Functional competition of MrVIA, GIIIA, and Ts1 was measured applying the toxins individually and in combinations of two. Cells were held at a potential of -120 mV and a series of test depolarizations ranging from -125 to +55 mV was applied in steps of 5 mV, 50 ms before and after a 50-ms conditioning pulse to +40 mV (Fig. 5A). The conditioning pulse was used to facilitate activation of the channels before the second test pulse series; the 50-ms segments before and after conditioning ensured recovery of the channels from fast inactivation. The repetition interval was 10 s. For a quantitative description normalized conductances were calculated from current-voltage relationships, plotted versus the test pulse voltage and fit with a double Boltzmann formalism:

$$\Gamma/\Gamma_{max}(V) = \frac{1 - P_{inh}}{\left(1 + e^{-(V-V_m)/k_m}\right)^3} + \frac{P_{shift}}{\left(1 + e^{-(V-V_m-\Delta V)/k_m}\right)^2} \qquad \text{(Eq. 2)}$$

with P_{shift} as the probability of the channels to activate at subthreshold potentials and ΔV as shift in the activation voltage. This formalism was used since Ts1-modified channels are assumed to activate with two activation gates whenever the β-toxin is bound to the channels.[17] In control experiments and in the presence of MrVIA or GIIIA alone P_{shift} was set to zero. This means describing channel activation according to a Hodgkin-Huxley theory, i.e., using three independent activation gates.

Steady-state inactivation. From a holding potential of -120 mV cells were conditioned for 500 ms at voltages ranging from -140 to -30 mV in steps of 10 mV. Subsequently, peak current was determined at 0 mV. The repetition interval was 20 s. The peak current plotted versus the conditioning voltage was described with a Boltzmann function:

$$I(V) = \frac{I_{min}}{1 + e^{-(V-V_h)/k_h}} \qquad \text{(Eq. 3)}$$

with the half-maximal inactivation voltage V_h and the corresponding slope factor k_h that indicates the voltage dependence of steady-state fast inactivation.

Depolarization-induced loss of MrVIA inhibition. From a holding potential of -120 mV the peak current amplitude was measured 40 ms before and 100 ms after conditioning pulses of various lengths. The conditioning voltages were 0, +40 and +80 mV. The repetition interval was 30 s. The probability of the channels to be inhibited by MrVIA (P_{inh}) was calculated from the ratio of the current amplitudes under control conditions and during application of 400 nM MrVIA after the conditioning and plotted as a function of the conditioning duration. The kinetics of the depolarization-induced loss of toxin effect was described by a single-exponential function yielding a time constant, τ, characterizing the relief of MrVIA-induced inhibition. These time constants, plotted as a function of conditioning voltage, were also fit with an exponential function providing an estimate of the effective charge transfer, q, during the voltage-dependent relief of inhibition:

$$\tau(V) = \tau_\infty + \tau_0\, e^{(-Vq/kT)} \qquad \text{(Eq. 4)}$$

with the absolute temperature T, and Boltzmann's constant k.

RESULTS

Inhibition of sodium channels by MrVIA is voltage dependent. In a previous study we have shown that MrVIA efficiently inhibits $Na_V1.4$ channels expressed in HEK 293 cells with an apparent IC_{50} value of approximately 250 nM without marked effects on current kinetics or voltage dependence of channel activation.[16] That study also showed that μO-conotoxins interact with an epitope in the pore loop of domain-3 that is also a target site for scorpion β-toxins,[17] suggesting that both types of toxins may act via similar molecular mechanisms. If this was true, mutations that eliminate the effect of scorpion β-toxins might also affect the action of μO-conotoxins. In $Na_V1.4$ such a mutation is G658N in the S3/S4 linker of domain-2. In the background of $Na_V1.2$ channels the equivalent mutation (G845N) abolishes the activity of CssIV, a β-toxin from *Centruroides suffusus suffusus*, to shift channel activation.[11] For $Na_V1.4$ we have previously shown that G658N impairs this action of Tz1[17] by hindering the trapping of the voltage sensor usually obtained by the action of scorpion β-toxins. Thus, we compared the effect of 400 nM MrVIA on wild-type $Na_V1.4$ channels (Fig. 1, top) and mutant G658N (Fig. 1, bottom) and found that both channel types were inhibited similarly (65 ± 5% for the wild type and 57 ± 7% for G658N) without noticeable effects on current kinetics and voltage dependence of activation.

This result questions if μO-conotoxins and scorpion β-toxins act on Na_V channels according to a similar molecular mechanism. Since there was no clear competition between the pore blocker STX and MrVIA,[15] μO-conotoxins may belong to the class of gating-modifier toxins. Such toxins, for example α-toxins and β-toxins from scorpions, interact with the voltage-sensor elements in Na_V channel domains 4 and 2, respectively.[8,11] Both toxins show some kind of use-dependence: The effect of scorpion α-toxins (example in ref. 20) and δ-conotoxins vanishes when channels are subjected to strong depolarizations.[10] For β-toxins the opposite mechanism seems to hold as the shift in voltage-dependent activation is pronounced after channel activation.[11,17,25] Therefore we examined the ability of MrVIA to inhibit $Na_V1.4$ channels in dependence of prepulse depolarizations. As shown in Figure 2A, the inhibition of Na^+ currents by the toxin can be removed when the test pulse is preceded by a depolarization that opens the channels. Increasing durations of the depolarization progressively removed the effect; the time course at +40 mV conditioning voltage was described with a single-exponential function with a time constant of 16.3 ± 5.3 ms. Such time constants for further conditioning voltages are shown in Figure 2B. The continuous curve is a single-exponential fit (Eq. 4) associating the translocation of 1.8 elementary charges across the transmembrane electric field to the voltage dependence of toxin dissociation. A nearly complete recovery from block occurs with conditioning pulses to +40 and +80 mV (Fig. 2C).

These results clearly show that the action of MrVIA is voltage dependent, highly suggestive of a voltage-sensor toxin mechanism. In addition, the kinetics and amount of current recovery after prepulses to +40 mV in the presence of MrVIA for mutant G658N were indistinguishable from the wild type (Fig. 2B and C, filled symbols) corroborating that modifications in the domain-2 S3/S4 linker that affect scorpion β-toxin action are not necessarily important for μO-conotoxins.

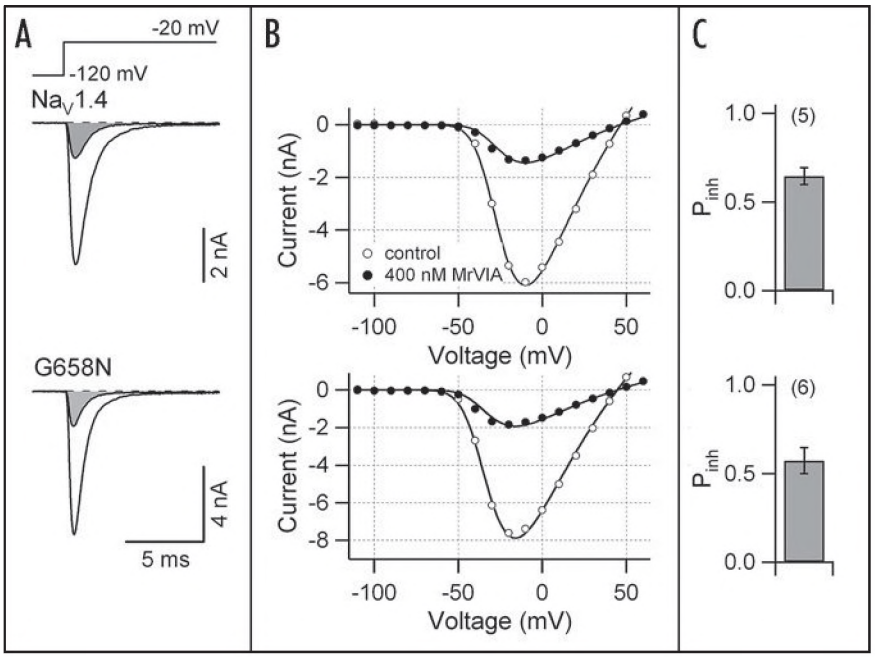

Figure 1. Inhibition of $Na_V1.4$ and mutant G658N channels by MrVIA. (A) Superposition of whole-cell current recordings of HEK 293 cells expressing wild-type $Na_V1.4$ channels (top) and mutant G658N (bottom) for the indicated pulse protocol before and after (grey shading) application of 400 nM MrVIA. (B) Peak currents as a function of voltage before and after application of MrVIA for representative experiments. The continuous curves are data fits according to the Hodgkin-Huxley model (Eq. 1). (C) Mean probability of channels being blocked by 400 nM MrVIA (P_{inh}) calculated from data fits according to Eq. 1. Error bars are S.E.M. values for the number of experiments indicated in parentheses.

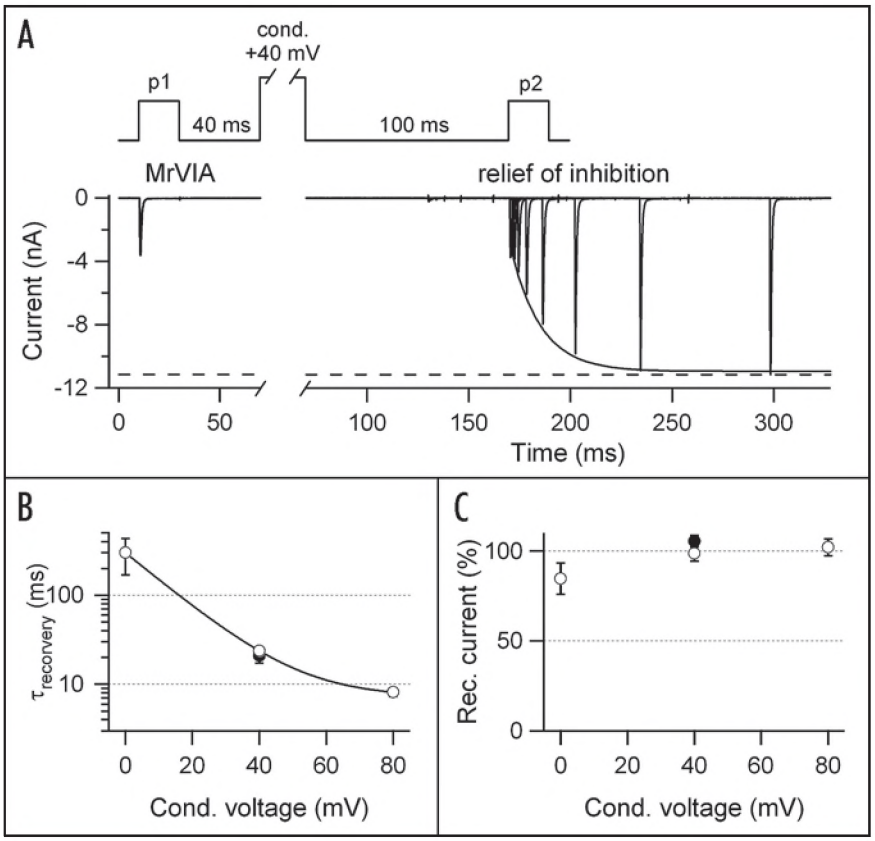

Figure 2. MrVIA-mediated inhibition of $Na_V1.4$ channels is voltage dependent. (A) Pulse protocol, consisting of a test pulse to -20 mV (p1), a conditioning pulse to +40 mV with increasing duration in individual sweeps, and a second test pulse to -20 mV (p2). Current response of $Na_V1.4$ channels expressed in HEK 293 cells after application of 400 nM MrVIA (left). The dashed line indicates the magnitude of peak current before toxin application. The superimposed current traces were recorded following conditioning at +40 mV for various time intervals (relief of inhibition). The continuous curve represents a fit to the recovered peak current as a function of conditioning time at +40 mV with a single-exponential function. (B) Time constants and (C) maximum recovered current derived from fits as shown in (A) plotted against conditioning potential. Error bars are S.E.M. values for n = 5. The continuous curve in (B) indicates a fit to the data points according to Eq. 4, resulting in an estimate of the effectively moved gating charge of 1.8 electron charges. The filled symbols in (B) and (C) indicate values for mutant G658N at +40 mV.

Identification of the voltage sensor relevant for MrVIA-induced channel inhibition. The experiments shown thus far do not provide information on the molecular source of the voltage dependence of MrVIA action. It is conceivable that the toxin itself, when charged, has to move partially through the transmembrane electric field to leave its binding site. Alternatively, the voltage dependence may originate from the channel protein. The latter scenario may mean that the activation of at least one of the four voltage sensors lowers the MrVIA affinity to the channel protein, in effect transiently reducing its blocking efficacy. A systematic alteration of the voltage sensor structures might therefore help to identify the relevant voltage sensor. We thus individually replaced the two outermost charged residues of the S4 segments in all four domains with cysteine residues. Expression of all mutants in HEK 293 cells resulted in functional channels with current amplitudes indistinguishable from that of wild-type $Na_V1.4$ (Fig. 3). Channel activation and steady-state

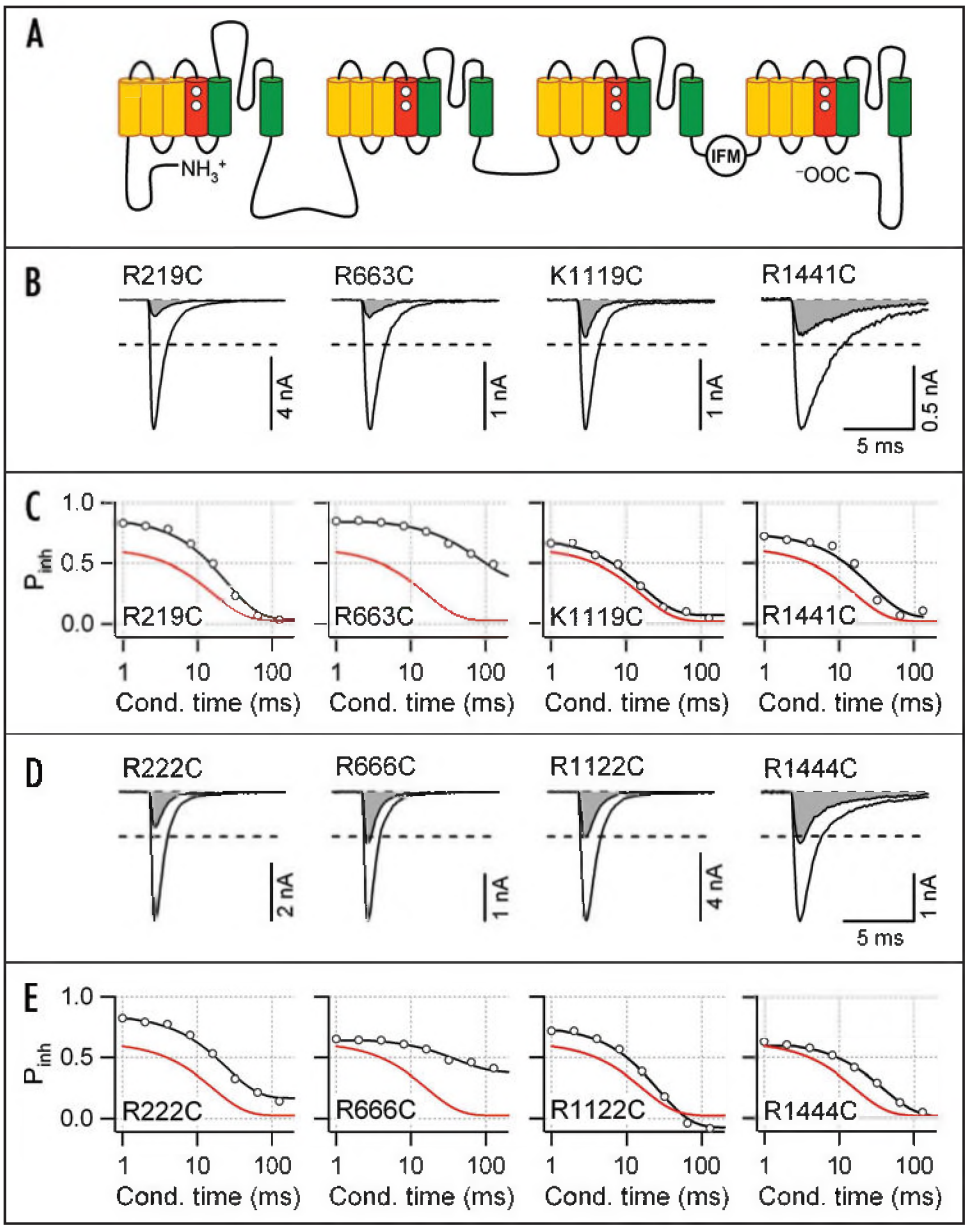

Figure 3. Charge neutralizations in voltage-sensor segments of $Na_V1.4$ channels affects MrVIA inhibition. (A) Topological presentation of a sodium channel α-subunit. The voltage-sensor segments are highlighted in red, the positions of the mutated positively charged residues are shown as circles. (B and D) Current traces in response to depolarization to +20 mV of the indicated mutants before and after application of 400 nM MrVIA (grey shading). The dashed line indicates the mean current block achieved for the wild-type channel. (C and E) Time course of inhibition relief induced by depolarization to +40 mV with superimposed single-exponential fits. The red curves represent the mean wild-type data.

inactivation were evaluated as described in the methods, summarized in Table 1. The half-maximal activation voltage was shifted by more than -10 mV for mutations in domain-1 and the slope factors of all mutants were about 1–2 mV greater than that for the wild type, statistically significant only for R219C, R666C, K1119C, R1441C, and R1444C. Steady-state inactivation was significantly affected by mutating the sensor in domain-4 (R1441C and R1444C) as expected from the coupling of the domain-4 voltage sensor to the inactivation gate.[8,26-28] The other mutations had only negligible effects on channel steady-state inactivation. The time course of inactivation at -20 mV, estimated with single-exponential functions, was slowed down by both charge mutations in domain-4 and the first charge in domain-1 (Fig. 3 and Table 1).

All mutants were subsequently assayed for the block effect of 400 nM MrVIA and the time constant of toxin dissociation at +40 mV. As shown in Figure 4A, alteration of all four voltage sensors increased the time constant for toxin dissociation with respect to the wild type, but only the modification in domain-2 resulted in a statistically significant effect. Neutralization of the outermost charge in this voltage sensor (R663C) increased the dissociation time constant more than 5-fold to 87.7 ± 8.0 ms; elimination of the second charge (R666C) slowed down this process about 2.5-fold (41.2 ± 6.5 ms).

In Figure 4B the absolute blocking potency for 400 nM MrVIA (grey) and the amount of current recovered with +40 mV conditioning pulses (white) are shown for all mutants. Neutralization of the first charged residues in domain-1 and domain-2 resulted in stronger inhibition by MrVIA (77.6 ± 3.2% and 86.2 ± 1.1%, respectively). In both mutants of domain-2 the inhibiting effect could not be completely relieved by depolarizing pulses to +40 mV (56.6 ± 9.5% for R663C and 55.1 ± 5.6% for R666C).

These results highlight the voltage sensor of domain-2 as the major source of the voltage dependence of MrVIA action. A reduced gating charge in that sensor seems to stabilize the toxin at its receptor site during strong depolarizations.

Competition of MrVIA with Ts1 and GIIIA. The dependence of the action of µO-conotoxins on the voltage sensor in domain-2 (this study) and the pore loop of domain-3[16] is reminiscent of the interaction pattern of scorpion β-toxins with Na_V channels. Such toxins also interact with the pore loop of domain-3[17] and a reduced charge of the voltage sensor of domain-2 results in more efficient voltage-sensor trapping by scorpion β-toxins.[29] To test whether µO-conotoxins functionally interact with scorpion β-toxins we measured the effect of MrVIA and Ts1 (from *Tityus serrulatus,* also referred to as Ts-γ) on $Na_V1.4$ channels, alone and in combination. For these experiments we chose 2 µM MrVIA as this concentration inhibited 87.8 ± 1.8% of all channels, and this block was reduced to 26.0 ± 7.2% by a 50-ms depolarization to +40 mV (Fig. 5B, red area). Application of 100 nM of the scorpion β-toxin Ts1 had a dual effect (Fig. 5C): About 50% of the current disappeared—here termed "inh" channel fraction. Of the remaining 50% of the channels about 40% activated at subthreshold voltages (V_m was shifted by -33.2 ± 0.6 mV

Table 1 **Voltage-dependent gating parameters of wild-type and mutant channels**

	Activation		Inactivation			
Channel	**V_m (mV)**	**k_m (mV)**	**V_h (mV)**	**k_h (mV)**	**τ_{hinf} (ms)**	**n**
$Na_V1.4$	-41.9 ± 1.6	10.3 ± 0.5	-75.5 ± 2.3	4.8 ± 0.1	0.69 ± 0.05	6
G658N	-40.4 ± 1.7	11.1 ± 0.1	-76.3 ± 1.9	4.6 ± 0.1*	0.77 ± 0.05	6
R219C	-56.7 ± 1.5***	12.1 ± 0.3**	-81.9 ± 1.3*	4.9 ± 0.1	1.04 ± 0.11*	5
R222C	-53.1 ± 0.7***	12.4 ± 0.5*	-80.6 ± 1.2	5.3 ± 0.2	0.76 ± 0.03	7
R663C	-37.4 ± 1.3	13.6 ± 1.0*	-76.0 ± 1.3	5.0 ± 0.1	0.82 ± 0.09	5
R666C	-39.0 ± 1.0	12.5 ± 0.3**	-75.7 ± 0.9	4.7 ± 0.1	0.75 ± 0.05	6
K1119C	-40.3 ± 1.5	12.4 ± 0.2**	-76.4 ± 0.9	5.3 ± 0.1*	0.87 ± 0.07	6
R1122C	-43.1 ± 1.3	11.2 ± 0.3	-79.4 ± 1.2	6.0 ± 0.3**	0.69 ± 0.03	7
R1441C	-44.5 ± 1.3	12.7 ± 0.3**	-81.7 ± 1.8	9.9 ± 0.3***	2.92 ± 0.13***	7
R1444C	-36.6 ± 1.3*	12.6 ± 0.4**	-76.5 ± 1.4	6.6 ± 0.2***	1.08 ± 0.08**	5

The indicated gating parameters were determined under control conditions with equations (1) and (3). τ_{hinf}, the time constant of channel inactivation, was obtained from current responses to depolarizing steps to -20 mV. All data were tested for their statistical significance with respect to the wild type using a two-sided Student's t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

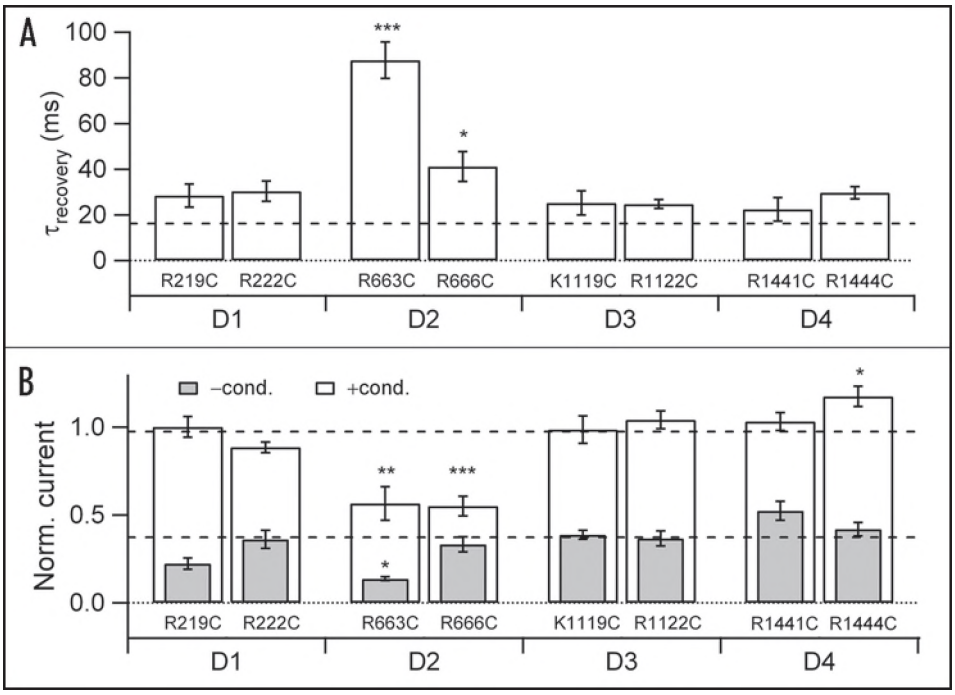

Figure 4. A reduced gating charge in domain-2 stabilizes MrVIA at $Na_V1.4$ channels. (A) Time constants describing the loss of MrVIA effect for $Na_V1.4$ mutant channels during a conditioning pulse to +40 mV (400 nM MrVIA). The bar labeling specifies the site of mutagenesis. The dashed line indicates the mean time constant of removal of MrVIA effect for $Na_V1.4$ wild-type channels. Data are grouped according to the domains that carry the modified voltage sensor (D1–D4). (B) Relative current amplitudes after application of 400 nM MrVIA at a test potential of -20 mV before (grey) and after (white) conditioning at +40 mV. The dashed lines indicate the relative amplitudes for $Na_V1.4$ wild-type channels before (upper line) and after (lower line) conditioning. Labeling and grouping of the data is as described for panel (A). All error bars are S.E.M. values for $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

as compared to toxin-free channels) (Fig. 5C, blue area), while 10% showed normal activation. Since Ts1 is a toxin without much use-dependence,[30] the conditioning pulse did not change much of this situation, as indicated by equal sizes of grey and black bars in the right panels of Figure 5C.

What do we expect if the toxins were acting on the sodium channels independently? The current signal obtained in the presence of Ts1 should be blocked by about 90% if 2 μM MrVIA would be added to the solution. This is indicated by the histogram bars labeled "inh" in Figure 5; they indicate about 90% inhibition of MrVIA (Fig. 5B) and 50% inhibition for Ts1 (Fig. 5C). The results for a combination of MrVIA and Ts1 as shown in Figure 5D, however, strongly deviate from the expectation of independence since the fraction of the conductance showing "inh" is far smaller than that for MrVIA. Compared to the sole application of Ts1, the proportion of channels activating at subthreshold potentials—indicative of a Ts1 activity—was reduced by about 5% (compare Figs. 5C and D, blue areas and "shift" bars). On the other hand, at high voltages a small fraction of current (10%) was blocked in a conditioning-dependent manner, indicative of an MrVIA activity (Fig. 5D, red area). Thus, the phenotype of the scorpion β-toxin effect is clearly dominant showing that μO-conotoxins and scorpion β-toxins functionally compete for modifying voltage-gated sodium channels.

The results shown above could indicate that there is a competition between any Na_V channel specific conotoxin and scorpion β-toxins. To rule this out, we also investigated the effect of the μ-conotoxin GIIIA from *Conus geographus* on $Na_V1.4$ channels. As shown in Figure 5E, 300 nM GIIIA blocked 82 ± 2% of the current in a voltage-independent manner. In addition, the GIIIA-induced block could not be reversed with conditioning prepulses already showing a strong functional difference to μO-conotoxins. If both toxins act independently on the channels, a combination of 2 μM MrVIA and 300 nM GIIIA should yield almost complete channel block with very little prepulse dependency. This is exactly what was observed (Fig. 5F), thus showing

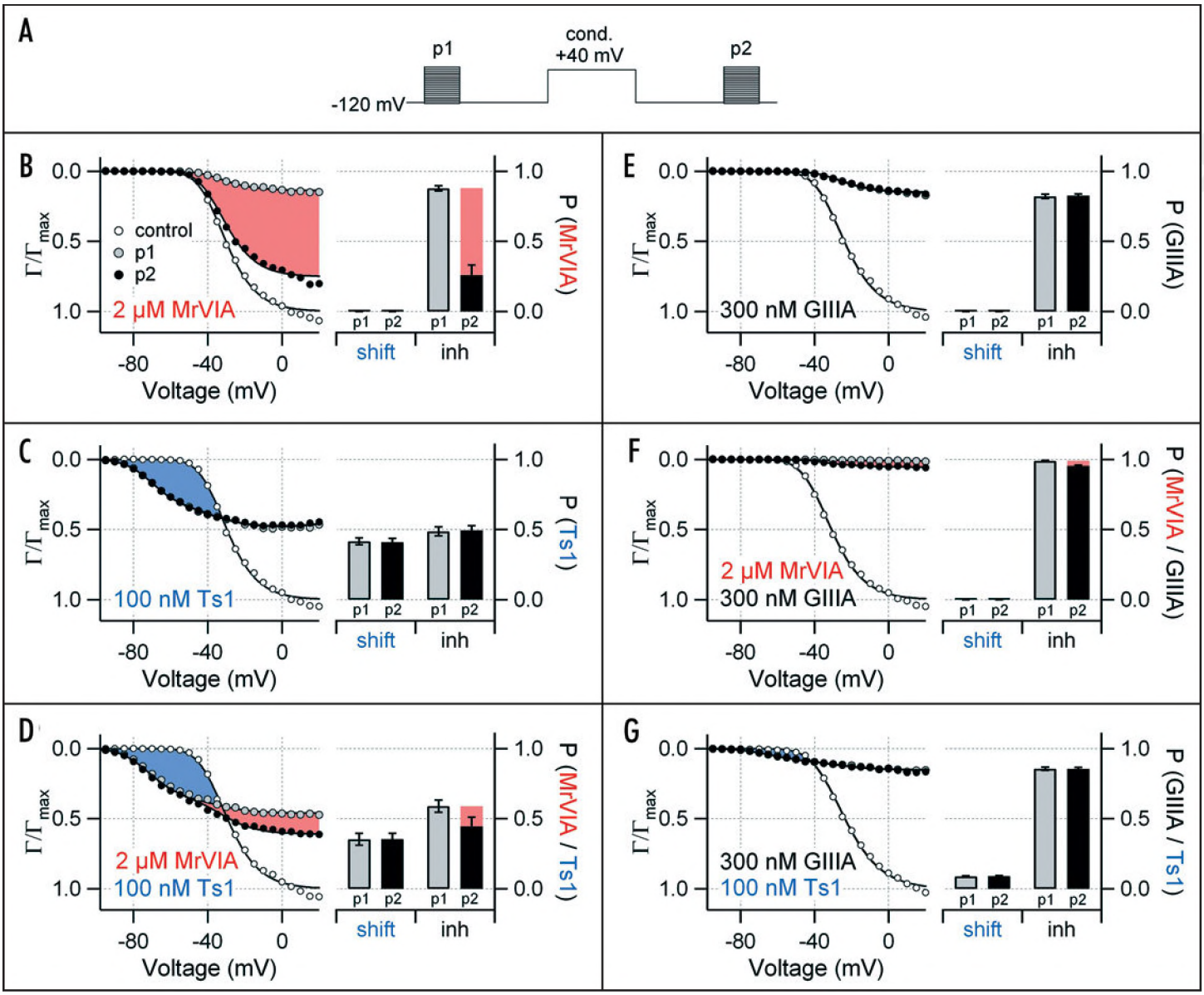

Figure 5. MrVIA functionally competes with scorpion β-toxin Ts1 but not with μ-GIIIA on $Na_V1.4$ channels. (A) Pulse protocol used to elicit Na^+ currents. "p1" denotes a first series of depolarizations ranging from -125 to +55 mV; "p2" is a second series of depolarizations applied after a 50-ms conditioning pulse to +40 mV. The holding potential was -120 mV. (B–G) Superposition of representative normalized conductances (Γ/Γ_{max}, left) calculated from current-voltage relationships and plotted as a function of voltage in the absence (control) or presence of the indicated concentrations of toxins or toxin combinations without (p1) or with (p2) a conditioning prepulse to +40 mV. The mean values ± S.E.M. of the channel fractions that activate at subthreshold potentials (shift) and that are inhibited (inh) during application of toxin without (p1) or with (p2) a conditioning pulse to +40 mV were calculated according to Eq. 2 from five independent experiments and plotted as histograms (*right*). (B) 2 μM MrVIA; (C) 100 nM Ts1; (D) MrVIA plus Ts1; (E) 300 nM GIIIA; (F) MrVIA plus GIIIA; (G) GIIIA plus Ts1. The red areas (B, D and F) mark the fractions of MrVIA-inhibited channels that were recovered after conditioning to +40 mV; the blue areas (C, D and G) mark the fractions of channels that activate at subthreshold potentials, indicative of the action of scorpion β-toxin Ts1.

that μO-conotoxins and μ-conotoxins do not compete for the same binding site. Likewise, in a combination of 300 nM GIIIA with 100 nM Ts1, the β-toxin-induced subthreshold current vanished almost completely (Fig. 5G, blue area) showing that GIIIA is fully active in the presence of Ts1.

DISCUSSION

μO-conotoxins are voltage-sensor toxins. μO-conotoxins have attracted attention because they potently inhibit current flow through $Na_V1.8$ channels that are expressed in dorsal root ganglia and, hence, that are potential targets for pain treatment.[4,5] The molecular mechanism by which μO-conotoxins modify Na_V channels, however, has been elusive. Previous competition experiments with labeled STX indicated that μO-conotoxins block Na_V channels similarly to μ-conotoxins, but not through plugging the channel pore.[15] Here we showed that the block of $Na_V1.4$ channels by MrVIA is strongly voltage-dependent unlike the μ-conotoxin GIIIA (Fig. 5C and ref. 31), i.e., the blocking potency of MrVIA decreased when the channels were preconditioned with depolarizing pulses, which opened the channels. This voltage dependent relief of MrVIA block can be interpreted as state dependent toxin-channel interaction. The

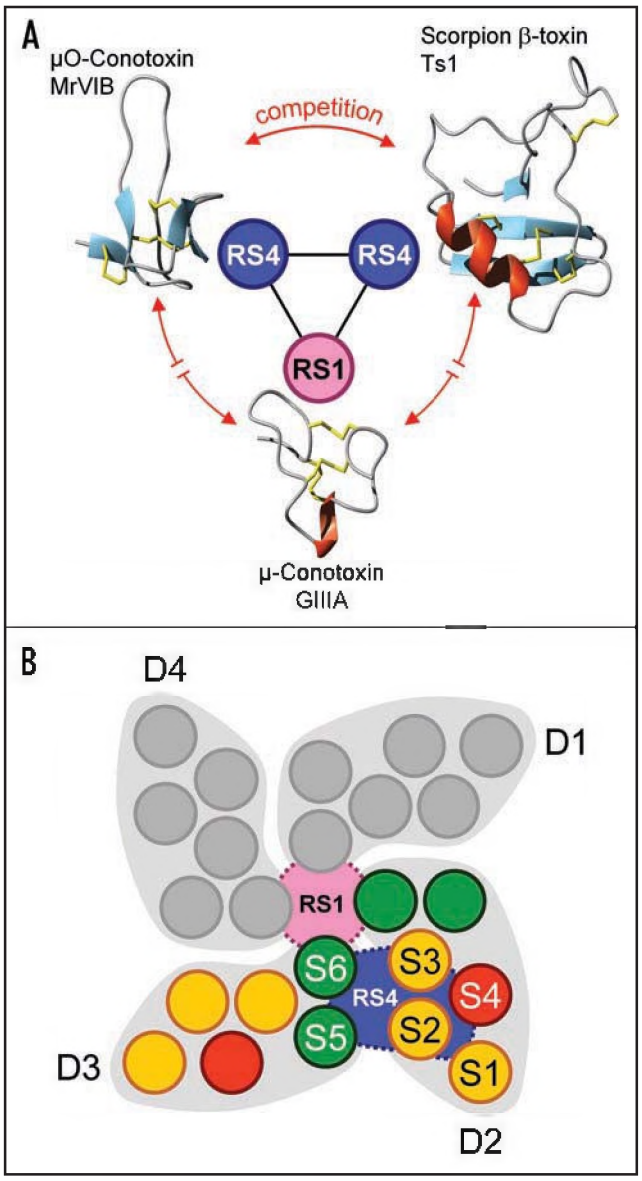

Figure 6. Molecular interpretation of MrVIA action. (A) Structures of the indicated toxins and their association to receptors sites 1 or 4 (RS1 and RS4). The arrows indicate functional competition between μO-conotoxins and scorpion β-toxins and lack of competition between μO-conotoxins and μ-conotoxins as well as μ-conotoxins and scorpion β-toxins. Structures were generated with MolMol[40] according to the following data base entries: MrVIB (1RMK.pdb[13]); GIIIA (1TCG.pdb[41]); Ts1 (1B7D.pdb[42]). Disulfide bridges are indicated as yellow sticks. (B) Cartoon of a Na_V channel protein, top view. The blue area indicates how the μO-conotoxin MrVIA might make contact with segments of domain-2 and domain-3 of Na_V channels to induce channel block by means of an interference with the voltage sensor in domain-2. Since scorpion β-toxins share this interaction pattern, this area can be referred to as receptor site-4. The pink area indicates receptor site-1 at which μ-conotoxins bind to block the channel pore.

most likely explanation is that the affinity of the channels for MrVIA decreases during prolonged depolarizations subsequently causing dissociation of MrVIA.

Our approach of assaying $Na_V1.4$ mutants with reduced gating charges in all four domains identified the voltage sensor in domain-2 as the main source of this voltage-dependent dissociation. Moreover, neutralizing the positions of either of the two outermost arginines in this sensor (R663C and R666C) enhanced the MrVIA block and remarkably slowed down toxin dissociation under depolarizing conditions. A charge reduction in the domain-2 sensor therefore stabilizes MrVIA on the channels. These data favor a model in which MrVIA specifically prevents the activation of the voltage sensor in domain-2 of Na_V channels and hence hinders channels from opening. Depolarizations strong enough to activate the sensor may cause the toxin to dissociate from the channel. It should be noted, though, that theoretically activation of the respective voltage sensor could also be inhibited by rather complex allosteric mechanisms not involving a direct contact of the S4 segment with the toxin.

Interestingly, neutralization of the outermost residue (R663C) was more effective in stabilizing the toxin on the channel than neutralization of the next to outermost residue (R666C). This could provide information on the details of how the voltage sensor interacts with the μO-conotoxins. Since the position of the S4 gating charge is biased towards the cytosolic side, the outermost charges may contribute to the initial gating charge transfer across the transmembrane electric field upon membrane depolarization.[32-35] When the outermost charge of a sensor is removed, e.g., R663C in domain-2, the initial activation of this sensor (outward movement) should be less voltage-dependent. If this initial movement of the voltage sensor already weakens the stability of the MrVIA-channel interaction, neutralization of the outermost charged residue is expected to have a strong impact on the kinetics with which MrVIA is repelled from the channel. Consistent with this notion, neutralization of amino acids located more intracellularly (e.g., R666C at the second position) is observed to influence the function of the toxin less effectively.

Competition of μO-conotoxins and scorpion β-toxins. The μO-conotoxin MrVIA blocks neuronal $Na_V1.2$ channels with an affinity approximately 5-fold higher than that of skeletal muscle $Na_V1.4$ channels; this mild subtype specificity originates in a small epitope in domain-3 of the channels.[16] Here we showed that the voltage sensor in domain-2 is also relevant for the function of MrVIA. This interaction pattern of μO-conotoxins with epitopes in domain-2 and domain-3 of Na_V channels is reminiscent of scorpion β-toxins for which these binding epitopes were originally termed receptor site-4 (RS4).[36] However, in contrast to μO-conotoxins, the major functional impact of β-toxins is to cause subthreshold channel openings by stabilizing an activated conformation of the voltage sensor in domain-2, termed voltage-sensor trapping.[11] Here we utilized the different modes of action of the μO-conotoxin MrVIA ("channel block") and of the scorpion β-toxin Ts1 ("activation shift") to test if both toxins compete for modifying the channel function. A quantitative comparison of current responses recorded in the presence of either of the individual toxins or a mixture of MrVIA and Ts1 indicated that both toxins indeed compete functionally. Phenotypically, the β-toxin appeared to dominate, almost quantitatively preventing the action of MrVIA. Although these data do not rule out a possible allosteric effect such that both toxins can bind to the channel simultaneously but mutually affect their impact on channel function, they strongly suggest that MrVIA and Ts1 share at least part of RS4 as binding site.

In contrast to these results, μO-MrVIA did not compete with the site-1 toxin μ-GIIIA, neither could we detect any competition between Ts1 and μ-GIIIA. This clearly demonstrates that μO-MrVIA and Ts1, although structurally very different (Fig. 6A), functionally belong to the same class of Na_V channel gating modifying toxins

(acting via RS4), while the pore-blocking μ-conotoxins act via RS1. Interestingly, residues in the pore loop of domain-3 have been reported to be relevant for the action of μ-GIIIA (e.g., D1241).[37] The interaction sites reported to Tz1 and MrVIA are very close, but located at the extracellular connecting loop between the pore and S6 (E1251-H1257).[16,17]

Based on structural models of the four subunits of a K_V channel[35] one can envision the interaction of μO-conotoxins with Na_V channels as illustrated in Figure 6B: Within a Na_V channel domain the voltage sensor structure, composed of S1-S4 is expected to form one unit loosely connected to the core segments (S5/S6) in a way that allows the voltage sensor to come into close proximity to the S5/S6 segments of the neighboring domain. In such an arrangement the distance between the voltage sensor in domain-2 and the pore loop in domain-3 is about 2 nm, which is less than the diameter of a toxin molecule. Thus, μO-conotoxins may bind to the pore loop of domain-3 and by this interaction will acquire some degree of channel specificity. Simultaneously they interfere with the voltage sensor in domain-2 with the effect of hindering its outward movement on depolarization (indicated by the blue area in Fig. 6B). Up to this point the mechanism of μO-conotoxin action is identical to that of scorpion β-toxins, which also tend to "block" Na_V channels to some degree (Fig. 5D). However, while β-toxins can arrest the voltage sensor in its activated position (voltage-sensor trapping), μO-conotoxins obviously lack this feature, as they are only capable of "resting state voltage sensor trapping".

Classification of μO-conotoxins. The data presented in this study clearly demonstrate that μO-conotoxins do not share the mode of action with μ-conotoxins. While the latter are pore blockers acting on RS1, μO-conotoxins are voltage-sensor toxins acting on RS4. Thus, it appears as if the association of μO-conotoxins to the O-superfamily of conotoxins is not only justified according to the cysteine knot pattern, but also according to the molecular mechanism, by which Na_V channels are modified. The other members of O-superfamily toxins active on Na_V channels are δ-conotoxins. They remove inactivation by interfering with conserved residues in the voltage sensor in domain-4 (S3/S4 segment).[10] Given the mild subtype specificity of μO-conotoxins one can speculate that conserved residues in domain-2 are also important for their action. Since mutation G658N, which has a strong impact on the sensor trapping by β-toxins, does not affect Na_V channel modification by MrVIA (Fig. 1), it is also conceivable that there is no specific structure of the S3/S4 element in domain-2 needed since the toxins merely have to sterically block the movement of the voltage sensor. Important interaction residues could, however, be located inside S3 like for some voltage-sensor toxins acting on potassium channels.[38,39]

References

1. Terlau H, Olivera BM. Conus venoms: A rich source of novel ion channel-targeted peptides. Physiol Rev 2004; 84:41-68.
2. Terlau H, Shon KJ, Grilley M, Stocker M, Stühmer W, Olivera BM. Strategy for rapid immobilization of prey by a fish-hunting marine snail. Nature 1996; 381:148-51.
3. McIntosh JM, Hasson A, Spira ME, Gray WR, Li W, Marsh M, Hillyard DR, Olivera BM. A new family of conotoxins that blocks voltage-gated sodium channels. J Biol Chem 1995; 270:16796-802.
4. Bulaj G, Zhang MM, Green BR, Fiedler B, Layer RT, Wei S, Nielsen JS, Low SJ, Klein BD, Wagstaff JD, Chicoine L, Harty TP, Terlau H, Yoshikami D, Olivera BM. Synthetic μO-conotoxin MrVIB blocks TTX-resistant sodium channel $Na_V1.8$ and has a long-lasting analgesic activity. Biochemistry 2006; 45:7404-14.
5. Ekberg J, Jayamanne A, Vaughan CW, Aslan S, Thomas L, Mould J, Drinkwater R, Baker MD, Abrahamsen B, Wood JN, Adams DJ, Christie MJ, Lewis RJ. 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 2006; 103:17030-5.
6. Goldin AL. Evolution of voltage-gated Na^+ channels. J Exp Biol 2002; 205:575-84.
7. Zlotkin E. The insect voltage-gated sodium channel as target of insecticides. Annu Rev Entomol 1999; 44:429-55.
8. Rogers JC, Qu Y, Tanada TN, Scheuer T, Catterall WA. 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 alpha subunit. J Biol Chem 1996; 271:15950-15962.
9. Leipold E, Lu S, Gordon D, Hansel A, Heinemann SH. Combinatorial interaction of scorpion toxins Lqh-2, Lqh-3, and LqhαIT with sodium channel receptor sites-3. Mol Pharmacol 2004; 65:685-91.
10. Leipold E, Hansel A, Olivera BM, Terlau H, Heinemann SH. Molecular interaction of δ-conotoxins with voltage-gated sodium channels. FEBS Lett 2005; 579:3881-4.
11. Cestèle S, Qu Y, Rogers JC, Rochat H, Scheuer T, Catterall WA. Voltage sensor-trapping: Enhanced activation of sodium channels by β-scorpion toxin bound to the S3-S4 loop in domain II. Neuron 1998; 21:919-31.
12. Cestèle S, Yarov-Yarovoy V, Qu Y, Sampieri F, Scheuer T, Catterall WA. Structure and function of the voltage sensor of sodium channels probed by a β-scorpion toxin. J Biol Chem 2006; 281:21332-44.
13. Daly NL, Ekberg JA, Thomas L, Adams DJ, Lewis RJ, Craik DJ. Structures of μO-conotoxins from *Conus marmoreus*: Inhibitors of tetrodotoxin (TTX)-sensitive and TTX-resistant sodium channels in mammalian sensory neurons. J Biol Chem 2004; 279:25774-82.
14. Li RA, Tomaselli GF. Using the deadly μ-conotoxins as probes of voltage-gated sodium channels. Toxicon 2004; 44:117-22.
15. Terlau H, Stocker M, Shon KJ, McIntosh JM, Olivera BM. μO-conotoxin MrVIA inhibits mammalian sodium channels, but not through site I. J Neurophysiol 1996; 76:1423-9.
16. Zorn S, Leipold E, Hansel A, Bulaj G, Olivera BM, Terlau H, Heinemann SH. The μO-conotoxin MrVIA inhibits voltage-gated sodium channels by associating with domain-3. FEBS Lett 2006; 580:1360-4.
17. Leipold E, Hansel A, Borges A, Heinemann SH. Subtype specificity of scorpion β-toxin Tz1 interaction with voltage-gated sodium channels is determined by the pore loop of domain 3. Mol Pharmacol 2006; 70:340-7.
18. Trimmer JS, Cooperman SS, Tomiko SA, Zhou JY, Crean SM, Boyle MB, Kallen RG, Sheng ZH, Barchi RL, Sigworth FJ, et al. Primary structure and functional expression of a mammalian skeletal muscle sodium channel. Neuron 1989; 3:33-49.
19. Jurman ME, Boland LM, Liu Y, Yellen G. Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. Biotechniques 1994; 17:876-81.
20. Chen H, Gordon D, Heinemann SH. Modulation of cloned skeletal muscle sodium channels by the scorpion toxins Lqh II, Lqh III, and LqhαIT. Pflügers Arch 2000; 439:423-32.
21. Cruz LJ, Kupryszewski G, LeCheminant GW, Gray WR, Olivera BM, Rivier J. μ-conotoxin GIIIA, a peptide ligand for muscle sodium channels: Chemical synthesis, radiolabeling, and receptor characterization. Biochemistry 1989; 28:3437-42.
22. Miranda F, Kupeyan C, Rochat H, Rochat C, Lissitzky S. Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis* hector, *Buthus occitanus tunetanus* and *Leiurus quinquestriatus quinquestriatus*. Eur J Biochem 1970; 16:514-23.
23. Alvarenga LM, Machado de Avila RA, Amim PR, Martins MS, Kalapothakis E, de Lima ME, Santos RG, Granier C, Chávez-Olórtegui C. Molecular characterization of a neutralizing murine monoclonal antibody against *Tityus serrulatus* scorpion venom. Toxicon 2005; 46:664-71.
24. Chávez-Olórtegui C, Moreira Ferreira AM, do Nascimento Cordeiro M, Maria WS, Richardson M, Diniz CR. Immunological and chemical properties of a non-toxic protein purified from the venom of the scorpion *Tityus serrulatus* (Lutz & Mello Campos, 1922). In: Bon C, Goyffon M, eds. Envenomings and their Treatments. Lyon: Fondation Marcel Mérieux, 1996:183-95.
25. Borges A, Alfonzo MJ, García CC, Winand NJ, Leipold E, Heinemann SH. Isolation, molecular cloning and functional characterization of a novel β-toxin from the Venezuelan scorpion, *Tityus zulianus*. Toxicon 2004; 43:671-84.
26. Chahine M, George Jr AL, Zhou M, Ji S, Sun W, Barchi RL, Horn R. Sodium channel mutations in paramyotonia congenita uncouple inactivation from activation. Neuron 1994; 12:281-94.
27. Ji S, George Jr AL, Horn R, Barchi RL. Paramyotonia congenita mutations reveal different roles for segments S3 and S4 of domain D4 in hSkM1 sodium channel gating. J Gen Physiol 1996; 107:183-94.
28. Chen LQ, Santarelli V, Horn R, Kallen RG. A unique role for the S4 segment of domain 4 in the inactivation of sodium channels. J Gen Physiol 1996; 108:549-56.
29. Cestèle S, Scheuer T, Mantegazza M, Rochat H, Catterall WA. Neutralization of gating charges in domain II of the sodium channel alpha subunit enhances voltage-sensor trapping by a β-scorpion toxin. J Gen Physiol 2001; 118:291-302.
30. Jonas P, Vogel W, Arantes EC, Giglio JR. Toxin gamma of the scorpion *Tityus serrulatus* modifies both activation and inactivation of sodium permeability of nerve membrane. Pflügers Arch 1986; 407:92-9.
31. Heinemann SH, Leipold E. Conotoxins of the O-superfamily affecting voltage-gated sodium channels. Cell Mol Life Sci 2007; 64:1329-40.
32. Kontis KJ, Rounaghi A, Goldin AL. Sodium channel activation gating is affected by substitutions of voltage sensor positive charges in all four domains. J Gen Physiol 1997; 110:391-401.

33. Groome JR, Fujimoto E, George AL, Ruben PC. Differential effects of homologous S4 mutations in human skeletal muscle sodium channels on deactivation gating from open and inactivated states. J Physiol 1999; 516:687-98.
34. Bezanilla F. The voltage sensor in voltage-dependent ion channels. Physiol Rev 2000; 80:555-92.
35. Long SB, Campbell EB, Mackinnon R. Voltage sensor of Kv1.2: Structural basis of electromechanical coupling. Science 2005; 309:903-8.
36. Bablito J, Jover E, Couraud F. Activation of the voltage-sensitive sodium channel by a β-scorpion toxin in rat brain nerve-ending particles. J Neurochem 1986; 46:1763-70.
37. Li RA, Tsushima RG, Kallen RG, Backx PH. Pore residues critical for μ-CTX binding to rat skeletal muscle Na^+ channel revealed by cysteine mutagenesis. Biophys J 1997; 73:1874-84.
38. Phillips LR, Milescu M, Li-Smerin Y, Mindell JA, Kim JI, Swartz KJ. Voltage-sensor activation with a tarantula toxin as cargo. Nature 2005; 436:857-60.
39. Yeung SY, Thompson D, Wang Z, Fedida D, Robertson B. Modulation of Kv3 subfamily potassium currents by the sea anemone toxin BDS: Significance for CNS and biophysical studies. J Neurosci 2005; 25:8735-45.
40. Koradi R, Billeter M, Wüthrich K. MOLMOL: A program for display and analysis of macromolecular structures. J Mol Graph 1996; 14:51-5.
41. Wakamatsu K, Kohda D, Hatanaka H, Lancelin JM, Ishida Y, Oya M, Nakamura H, Inagaki F, Sato K. Structure-activity relationships of μ-conotoxin GIIIA: Structure determination of active and inactive sodium channel blocker peptides by NMR and simulated annealing calculations. Biochemistry 1992; 31:12577-84.
42. Polikarpov I, Junior MS, Marangoni S, Toyama MH, Teplyakov A. Crystal structure of neurotoxin Ts1 from *Tityus serrulatus* provides insights into the specificity and toxicity of scorpion toxins. J Mol Biol 1999; 290:175-84.