

A natural point mutation changes both target selectivity and mechanism of action of sea anemone toxins

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ABSTRACT APETx3, a novel peptide isolated from the sea anemone *Anthopleura elegantissima*, is a naturally occurring mutant from APETx1, only differing by a Thr to Pro substitution at position 3. APETx1 is believed to be a selective modulator of human ether-à-go-go related gene (hERG) potassium channels with a K_d of 34 nM. In this study, APETx1, 2, and 3 have been subjected to an electrophysiological screening on a wide range of 24 ion channels expressed in *Xenopus laevis* oocytes: 10 cloned voltage-gated sodium channels (Na_v 1.2–Na_v1.8, the insect channels DmNa_v1, BgNa_v1–1a, and the arachnid channel VdNa_v1) and 14 cloned voltage-gated potassium channels (K_v1.1–K_v1.6, K_v2.1, K_v3.1, K_v4.2, K_v4.3, K_v7.2, K_v7.4, hERG, and the insect channel *Shaker* IR). Surprisingly, the Thr3Pro substitution results in a complete abolishment of APETx3 modulation on hERG channels and provides this toxin the ability to become a potent (EC₅₀ 276 nM) modulator of voltage-gated sodium channels (Na_vs) because it slows down the inactivation of mammalian and insect Na_v channels. Our study also shows that the homologous toxins APETx1 and APETx2 display promiscuous properties since they are also capable of recognizing Na_v channels with IC₅₀ values of 31 nM and 114 nM, respectively, causing an inhibition of the sodium conductance without affecting the inactivation. Our results provide new insights in key residues that allow these sea anemone toxins to recognize distinct ion channels with similar potency but with different modulatory effects. Furthermore, we describe for the first time the target promiscuity of a family of sea anemone toxins thus far believed to be highly selective.—Peigneur, S., Béress, L., Möller, C., Marí, F., Forssmann, W.-G., Tytgat, J. A natural point mutation changes both target selectivity and mechanism of action of sea anemone toxins. *FASEB J.* 26, 5141–5151 (2012). www.fasebj.org

Key Words: voltage-gated sodium channel • voltage-gated potassium channel • ion channel inhibitor • ion channel modulator

VOLTAGE-GATED SODIUM CHANNELS (Na_v channels) are transmembrane protein complexes that are constituted of an α -subunit of ~260 kDa and up to 4 auxiliary β -subunits (β 1–4) of 30–40 kDa. The pore-forming α -subunit alone is sufficient to obtain sodium current; however, coexpression of β -subunits modifies expression level, kinetics, and voltage dependence of channel gating (1). The α -subunit is organized in 4 homologous domains (DI–IV). Each domain consists of 6 putative transmembrane segments (S1–S6) connected by extracellular or intracellular loops. The S4 segments are the most conserved segments, and they contain a basic residue, either Lys or Arg, in every third position. These positively charged S4 segments are believed to function as voltage sensors. They transport gating charges by moving outward on membrane depolarization, thus initiating the voltage-dependent activation, which results in the opening of the channel. The selectivity filter and pore are formed by the transmembrane segments S5 and S6 along with the reentrant segments that are part of the loop that connects the S5 and S6 of each domain. The short intracellular linker that connects the DIII and DIV contains a highly conserved sequence of 3 hydrophobic residues (Ile, Phe, and Met) or IFM motif. Sodium channel inactivation is mediated by this hydrophobic motif, since it serves as an inactivation gate crucial for causing fast inactivation by binding to a receptor. This inactivation gate receptor is located near or within the intracellular mouth of the sodium channel pore.

Sea anemones have become “medicinal chemists” of unprecedented skills by evolving a unique peptide

Abbreviations: APETx, *Anthopleura elegantissima* toxin; ASIC, acid-sensing ion channel; hERG, human ether-à-go-go related gene; HWTX, huwentoxin; K_v channel, voltage-gated potassium channel; Na_v channel, voltage-gated sodium channel; RP-HPLC, reverse-phase high-performance liquid chromatography; TTX, tetrodotoxin

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biochemistry and neuropharmacology to develop components that ensure complete shutdown of the nervous system of their prey. Although a number of sea anemone toxins have been isolated and characterized, these animals remain understudied in comparison with other venomous animals, such as scorpions, spiders, cone snails, and snakes. Sea anemones are a known pharmacological treasure of biological active compounds acting on a diverse panel of ion channels, such as Na_v and voltage-gated potassium (K_v) channels, TRPV1 channels, or acid-sensing ion channels (ASICs) (2–4). Out of this group, toxins that target sodium channels are the best studied so far, with >100 known toxins (5). Sea anemone sodium channel toxins can be divided into 3 structural classes, based on their structural differences and activity profile (4, 6–8). All toxins belonging to these three classes exert the same pharmacological activity. They are believed to bind to the channel at receptor site 3. On binding at site 3, these toxins trap the voltage-sensing segment S4 of DIV in its inward position: this prevents the normal outward movement of the voltage sensors and herewith the conformational changes necessary for fast inactivation (9).

To date, 7 toxins from *Anthopleura elegantissima* have been isolated and characterized: APE1-1, APE1-2, APE2-2, and ApC, which are type 1 sodium channel toxins; and APETx1, a modifier of the human ether a go-go related gene (hERG) K⁺ channel; APETx2, which specifically inhibits ASIC3; and APEKTx1, a potent and selective K_v1.1 inhibitor (8, 10–13). APETx1 has been well characterized, and it was first reported to be a selective modulator of hERG channels with an EC₅₀ value of 34 nM, although it also exerts some activity over other K_v channels at higher concentrations (Supplemental Fig. S1 and ref. 10). It is thought that APETx1 binds to the outer vestibule of the hERG channel. Toxin binding causes a shift in the voltage dependence of both activation and inactivation curves, resulting in a blockage of the potassium current. Note that full inhibition is not observed, even at high concentrations. Its interaction with hERG channels is voltage dependent, and it suggests that APETx1 exerts a preferential affinity for the closed state of the channel (10, 14). Further structure-function studies have highlighted amino acids on the hERG channel, which are key residues for APETx1 interaction (15).

APETx2 was found to be a potent and selective inhibitor of ASIC3 channels. The current through these ASIC3 channels is reversibly inhibited by APETx2 with an IC₅₀ value of 63 nM (8). ASIC3 channels are mainly expressed in sensory neurons and have been identified to play an important role in acid-induced hyperalgesia and in high-intensity pain stimuli (8, 16–18). As such, APETx2 has been a valuable tool to study these channels. *In vivo* studies have shown that administration of APETx2 prevents postoperative acid-induced inflammatory pain (19), (20). Recently, it was found that APETx2 could substantially inhibit tetrodotoxin (TTX)-resistant currents and to a lesser extend TTX-sensitive currents in rat DRG neurons. It was

shown that the observed inhibition of Na_v1.8 channels resulted from a rightward shift in the voltage dependence of activation and a reduction in the maximal macroscopic conductance (21). Similar to ASIC3 channels, Na_v1.8 channels are also expressed in sensory neurons, and they are involved in inflammatory pain. Na_v1.8 inhibitors can produce analgesic effects *in vivo* (22–24). The knowledge that APETx2 can exhibit target promiscuity indicates that part of the observed analgesic effect of APETx2 *in vivo* may result from an inhibition of the Na_v1.8 conductance (21).

Since these experiments were done in DRG neurons, little information on the Na_v subtype- or phyla-selectivity is available for APETx2.

Here we present the biochemical analysis and electrophysiological characterization of APETx3, a natural occurring mutant of APETx1 differing by a Thr to Pro substitution at position 3. APETx3 is a novel sodium channel toxin, structurally unrelated to the existing classes of sea anemone toxins targeting sodium channels. Furthermore, we re-evaluated the selectivity of APETx1 and APETx2 to show that they are not as selective as previously thought.

MATERIALS AND METHODS

Purification and toxin identification

The toxin APETx3 was isolated as described previously (10, 13). The fraction containing APETx3 was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) with a semipreparative Vydac C18 column (4.6×250 mm; Grace, Columbia, MD, USA). Solvent A was 0.1% TFA in water, and solvent B was 0.085% TFA in acetonitrile. A linear gradient from 0 to 80% solution B was carried out for 80 min at a flow rate of 1 ml/min. A second purification was performed with the same column using a linear gradient from 20 to 40% solution B at a flow rate of 0.2 ml/min. All fractions were dried by speed-vac evaporation and stored at –20°C.

APETx1 was purified as previously described (10). Synthetic APETx2 and huwentoxin (HWTX) IV were purchased from Smartox Biotechnology (Grenoble, France). The purity of these toxins was examined using RP-HPLC as described above.

Biochemical characterization of toxins

The mass of APETx1 and APETx2 was verified by ESI-MS using a LCQ Deca XP spectrometer (Thermo Finnigan, Waltham, MA USA).

Sequence determination

The amino acid sequences of APETx1 and APETx2 were verified by Edman degradation using an automated Protein Sequencer PPSQ-33A (Shimadzu, Kyoto, Japan).

For APETx3, the peptide was reduced and alkylated as described previously and adsorbed onto Biobrene-treated fiberglass filter. Amino acid sequencing was carried out by Edman degradation using a Procise model 491A sequencer (Applied Biosystems, Foster City, CA, USA). The concentration of the peptide was determined by using the calibrated

absorbencies of the first 3 PTH-amino acid residues on samples that were not reduced and alkylated.

Expression of voltage-gated ion channels in *Xenopus laevis* oocytes

For the expression of Na_v channels (rNa_v1.2, rNa_v1.3, rNa_v1.4, hNa_v1.5, mNa_v1.6, rNa_v1.7, rNa_v1.8; the insect channels DmNa_v1 and BgNa_v1; the arachnid channel VdNa_v1; and the auxiliary subunits β 1, β 1, and TipE) and of K_v channels (K_v1.1-K_v1.6, K_v2.1, K_v3.1, K_v4.2, K_v4.3, K_v7.2, K_v7.4, hERG, and the insect channel *Shaker* IR) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMessage-mMachine transcription kit (Ambion, Carlsbad, CA, USA). The harvesting of stage V-VI oocytes from anesthetized female *X. laevis* frogs was previously described (25). Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng/nl using a microinjector (Drummond Scientific, Broomall, PA, USA). The oocytes were incubated in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, and 5 mM HEPES (pH 7.4), supplemented with 50 μ g/ml gentamicin sulfate.

Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed at room temperature (18–22°C) using a Geneclamp 500 amplifier (Molecular Devices, Downingtown, PA, USA) controlled by a pClamp data acquisition system (Axon Instruments, Union City, CA, USA). Whole-cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was the following (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 2 MgCl₂, and 5 HEPES (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.5 and 1.5 M Ω . The elicited currents were filtered at 1 kHz and sampled at 20 kHz using a 4-pole low-pass Bessel filter. Leak subtraction was performed using a $-P/4$ protocol. To avoid overestimation of a potential toxin-induced shift in the current-voltage relationships of inadequate voltage control when measuring large sodium currents in oocytes, only data obtained from cells exhibiting currents with peak amplitude < 2 μ A were considered for analysis. For the electrophysiological analysis of toxins, a number of protocols were applied from a holding potential of -90 mV with a start-to-start interval of 0.2 Hz. Sodium current traces were evoked by 100-ms depolarizations to V_{max} (the voltage corresponding to maximal sodium current in control conditions). The current-voltage relationships were determined by 50-ms step depolarizations between -90 and 70 mV, using 5-mV increments. The sodium conductance (g_{Na}) was calculated from the currents according to Ohm's law: $g_{Na} = I_{Na}/(V - V_{rev})$, where I_{Na} represents the Na⁺ current peak amplitude at a given test potential V , and V_{rev} is the reversal potential. The values of g_{Na} were plotted as a function of voltage and fitted using the Boltzmann equation: $g_{Na}/g_{max} = \{1 + [\exp(V_g - V)/k]\}^{-1}$, where g_{max} represents maximal g_{Na} , V_g is the voltage corresponding to half-maximal conductance, and k is the slope factor. Toxin-induced effects on the steady-state inactivation were investigated using a standard 2-step protocol. In this protocol, 100-ms conditioning 5-mV step prepulses ranging from -90 to 70 mV were followed by a 50-ms test pulse to

-30 or -10 mV. Data were normalized to the maximal Na⁺ current amplitude, plotted against prepulse potential and fitted using the Boltzmann equation: $I_{Na}/I_{max} = \{(1 - C)/[1 + \exp[(V - V_h)/k]]\} + C$, where I_{max} is the maximal I_{Na} , V_h is the voltage corresponding to half-maximal inactivation, V is the test voltage, k is the slope factor, and C is a constant representing a noninactivating persistent fraction (close to 0 in control). The recovery from inactivation was assayed with a double-pulse protocol, where a 100-ms conditioning pulse to -30 or -10 mV was followed by a 50-ms test pulse to the same voltage. Both pulses were interspersed by a repolarization to -90 mV for a gradually increasing time interval (1–40 ms). The I_{Na} obtained in the test pulse was normalized to the I_{Na} obtained in the conditioning pulse, plotted against the corresponding time interval, and fitted with the following exponential equation: $f(t) = Ae^{-t/\tau} + C$, where t is time, A is the amplitude of the current, τ is the time constant for the fast inactivation, and C is a constant representing a noninactivating persistent fraction (close to 0 in control).

To assess the concentration-response relationships, data were fitted with the Hill equation: $y = 100/[1 + (EC_{50}/[toxin])^h]$, where y is the amplitude of the toxin-induced effect, EC_{50} is the toxin concentration at half maximal efficacy, $[toxin]$ is the toxin concentration, and h is the Hill coefficient. The time constants τ of the Na_v channel fast inactivation were measured directly from the decay phase of the recorded Na⁺ current using a single exponential fit. Comparison of 2 sample means was made using a paired Student's t test ($P < 0.05$). All data are presented as means \pm SE of ≥ 5 independent experiments ($n \geq 5$). All data were analyzed using pClamp Clampfit 10.0 (Molecular Devices) and Origin 7.5 software (Originlab, Northampton, MA, USA).

RESULTS

Purification and toxin identification

The screening of fractions obtained from the sea anemone *A. elegantissima*, after anion and cation exchange and gel filtration, yielded one fraction, APETx3, that was active on Na_v channels. This fraction was further purified in 2 steps using RP-HPLC.

Biochemical characterization

With ESI-MS analysis, a mass of 4544.7 Da APETx3 was observed. The theoretical mass of 4545.0 Da corresponds well with the experimentally determined mass. The intact toxin was subjected to Edman degradation. The following sequence was obtained: GTPCYCGKTIGIYWFGTKTSPNRYGTGSCGYFLGICCCYPVD (Fig. 1).

The molecular mass and primary sequence of APETx1 and APETx2 were determined to verify the purity of both toxins. Both the mass spectrum analysis and amino acid sequence determination were in complete accord with previous reports (8, 10).

APETx3	GT PCY CGKTIGIYWFGTKT CP SNRGYTG SCGYFLGICCCYPVD	Identity (%) 100
APETx1	GT TCY CGKTIGIYWFGTKT CP SNRGYTG SCGYFLGICCCYPVD	98
APETx2	GT ACS CGNSKGIYWFYR SP ETDRGYTG SCRYFLGTCTPAD	64

Figure 1. Amino acid sequence of APETx3 and alignment with APETx1 and APETx2. Amino acid residues identical with APETx3 are shown on gray background, cysteine residues are in red.

The sequence data of APETx3 were deposited in the UniProt Knowledgebase under accession number B3EWF9.

Electrophysiological recordings

APETx3 is a modulator of Na_v channels

APETx3 was screened against a panel of 7 mammalian Na_v channel isoforms and 3 insect Na_v isoforms (Fig. 2). Among the mammalian isoforms, 1 μ M APETx3 slowed the inactivation of Na_v1.2, Na_v1.3, Na_v1.4, and Na_v1.6. Na_v1.7 and the TTX-resistant channels Na_v1.5 and Na_v1.8 were not affected by APETx3. Furthermore, the same concentration of toxin had a profound effect on the inactivation of the insect isoforms DmNa_v1 and BgNa_v1. APETx3 completely inhibited their inactivation, resulting in sustained noninactivating currents. Although APETx3 could also slow down its inactivation, the arachnid channel VdNa_v1 was less sensitive compared with the other insect Na_v channel isoforms. Concentration-response curves were constructed for Na_v1.6 and DmNa_v1. EC₅₀ values yielded 2265 ± 185 and 276 ± 37 nM, respectively (Fig. 2B). The most toxin-sensitive isoform among the mammalian Na_v channels, Na_v1.6, and the insect Na_v channel DmNa_v1 were used to further investigate the characteristics of APETx3 in inducing modulation of channel gating (Fig. 3A, B). No shift in the midpoint of activation was observed (Supplemental Table S1). However, 1 μ M APETx3 could significantly shift the voltage dependence of steady-state inactivation. The $V_{1/2}$ shifted from -69 ± 0.3 to -62 ± 0.2 mV for Na_v1.6 ($n=4$; $P<0.05$).

For DmNa_v1 channels, the $V_{1/2}$ shifted from -51 ± 0.2 to -43 ± 0.4 mV ($n=6$; $P<0.05$). From Fig. 3A, B, it can be seen that the steady-state inactivation became incomplete after toxin addition. For Na_v1.6, a $56 \pm 2\%$ noninactivating current component was observed, while for DmNa_v1, a complete removal of steady-state inactivation can be seen.

To verify that APETx3 does bind to neurotoxin site 3, competitive binding experiments were performed using CgNa, a type I sea anemone toxin known to bind to site 3 of Na_v channels. Application of CgNa at its EC₅₀ value of 250 nM (26) resulted in an $18 \pm 3\%$ noninactivating current component of Na_v1.6 channels. Subsequent addition of EC₅₀ concentrations of APETx3 did not result in further slowing down of inactivation (Fig. 3C).

APETx3 is not a modulator of hERG channels

APETx3 can be considered as a natural mutant of APETx1, as they only differ in one residue (Fig. 1). Therefore, we decided to investigate whether APETx3 displays the same activity profile of APETx1. APETx1 has been reported to be a selective and potent modulator of hERG channels with an EC₅₀ value of 34 nM (8), a result that we have been able to confirm (Fig. 4). Interestingly, even at concentrations up to 50 μ M, APETx3 failed to induce any modulation of hERG channels (Fig. 4).

Since it has been reported that APETx1 exhibits activity for other K_vs, although only in higher concentrations (8), we tested the activity of APETx3 against 13

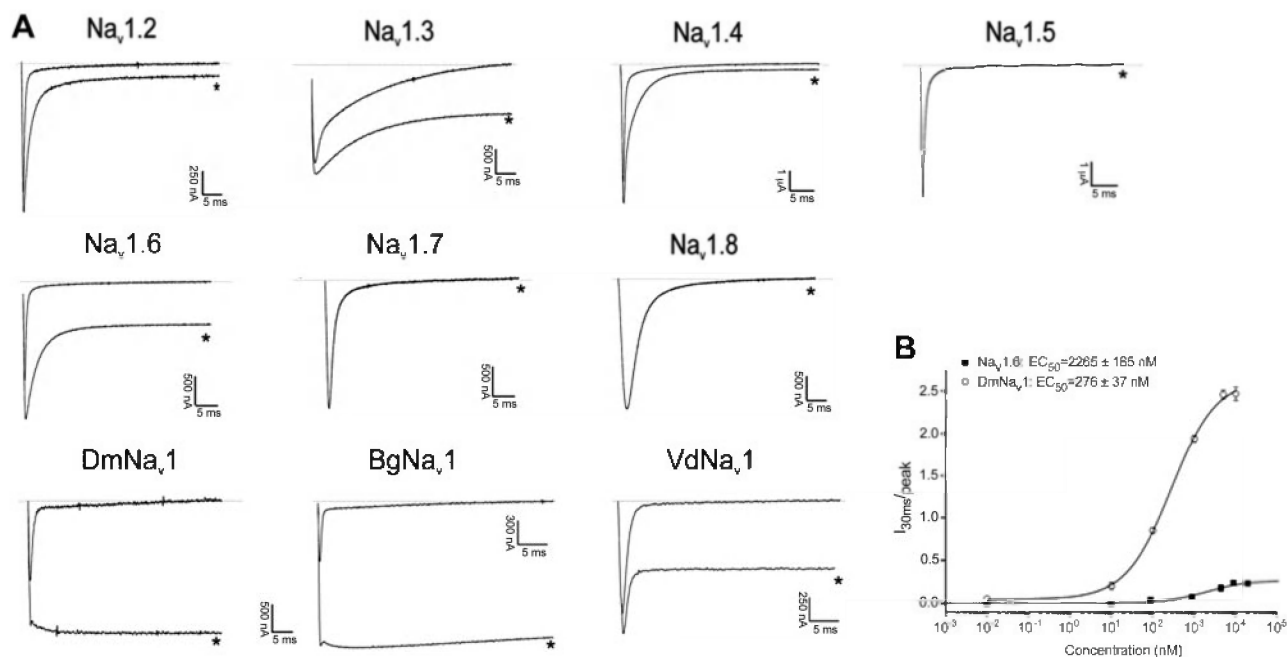


Figure 2. Electrophysiological characterization of APETx3 on Na_v channels. A) Activity profile of APETx3 on several Na_v channel isoforms. Representative whole-cell current traces in control and toxin conditions are shown. Dotted line indicates 0 current level; asterisk indicates steady-state current trace after application of 1 μ M toxin. Traces represent ≥ 3 independent experiments ($n \geq 3$). B) Concentration-response curves for Na_v1.6 and DmNa_v1, indicating the concentration dependence of the APETx3-induced increase of the Na⁺ peak current.

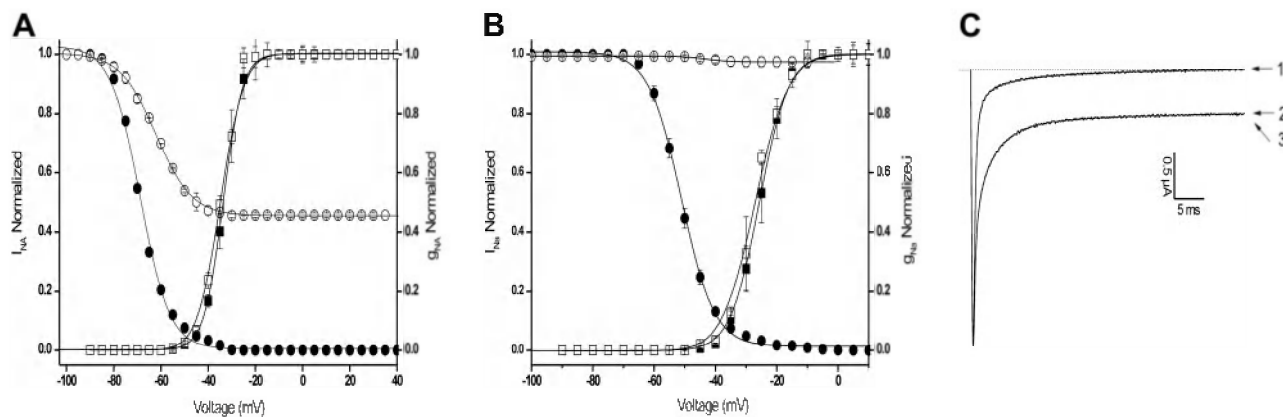


Figure 3. A, B) Electrophysiological characterization of APETx3 on Na_V1.6 (A) and DmNa_V1 (B) channels. Steady-state activation and inactivation curves in control conditions (solid symbols) and toxin conditions (1 μ M APETx3; open symbols). C) Competitive experiment to indicate that APETx3 does bind at site 3. Representative traces are shown: 1) control; 2) after application of 250 nM CgNa (26); and 3) after subsequent addition of 2200 nM APETx3.

different K_V channel isoforms. APETx3 showed no activity against members of the *Shaker* (K_V1.1–K_V1.6 and the insect channel *Shaker* IR), *Shab* (K_V2.1), *Shaw* (K_V3.1), *Shal* (K_V4.2 and K_V4.3) subfamilies, nor did APETx3 exhibit affinity for KCNQ gene family encoded K_V channels, such as K_V7.2 and K_V7.4 (Supplemental Fig. S1).

APETx1 inhibits the conductance through Na_V channels

At a concentration of 1 μ M, APETx1 was tested for its activity against the same panel of Na_V channel isoforms. Remarkably, it was found that APETx1 could inhibit the sodium conductance of all mammalian Na_V channel isoforms tested except Na_V1.7 (Fig. 5A). APETx1 showed the highest affinity for Na_V1.8, as the sodium peak amplitude (I_{Na}) was reduced by $89 \pm 2\%$ ($n=4$; $P<0.05$). Na_V1.2 and Na_V1.6 were equally sensitive for this toxin since 1 μ M APETx1 inhibited the peak amplitude by 61 ± 4 and $59 \pm 1\%$ ($n=3$; $P<0.05$), respectively. The same concentration of toxin could inhibit Na_V1.4 and Na_V1.5 by 56 ± 2 and $50 \pm 2\%$ ($n=3$; $P<0.5$), respectively. Na_V1.3 was the less affected by APETx1 since only $5 \pm 1\%$ ($n=3$; $P<0.5$) inhibition

could be observed. None of the insect Na_V channel isoforms tested were affected by APETx1. To investigate the concentration-response relationships, EC₅₀ values were defined for Na_V1.2 and Na_V1.8 channels. EC₅₀ values yielded 31 ± 6 and 92 ± 4 nM, respectively (Fig. 5B).

Na_V1.2 channels were used to determine whether APETx1 acts as a pore blocker by physically obstructing the ion pathway or rather as a modulator of channel gating. No shift in the voltage dependence of activation or a significant alteration of the steady-state inactivation curves was observed (Supplemental Fig. S2A and Supplemental Table S1). The toxin-induced reduction of peak amplitude was not associated with a shift in the reversal potential, indicating no modification in the ion selectivity after toxin application (Supplemental Fig. S2B). APETx1 did not influence the rate of recovery from inactivation (Supplemental Table S1). The time constant of recovery yielded 3 ± 0.04 ms in control conditions and 3 ± 0.1 ms after application of 50 nM APETx1 ($n=4$; $P<0.05$; Supplemental Fig. S2C). Furthermore, it should be noted that, even after the application of toxin concentrations up to 20 μ M, a residual current of 5–10% remained. For Na_V1.2 channels, time value of APETx1 binding yielded 29 ± 3 s (τ_{on}) while unbinding on washout occurred with a time constant of 18 ± 2 s (τ_{off}) (Supplemental Fig. S2D).

APETx2 inhibits the conductance through Na_V channels

As APETx1 and APETx2 have 64% sequence identity, we examined whether APETx2 also could exert promiscuous activity. This screening revealed that APETx2 exhibits also activity on Na_V channels. However, at a concentration of 1 μ M, APETx2 seems to be more active toward Na_V1.2, Na_V1.6, and Na_V1.8 (Fig. 6A). The I_{Na} through Na_V1.2 channels was reduced by $57 \pm 3\%$ ($n=5$; $P<0.05$), while the same concentration of toxin inhibited the conductance of Na_V1.6 channels by $17 \pm 2\%$ ($n=4$; $P<0.05$) and Na_V1.8 channels by $68 \pm 2\%$ ($n=6$; $P<0.05$), respectively. A slowing of inactiva-

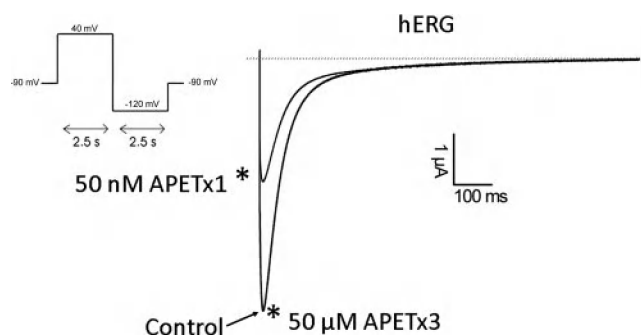


Figure 4. Activity of APETx1 and APETx3 on the hERG expressed in *X. laevis* oocytes. Dotted line indicates 0 current level; asterisk indicates steady-state current trace after application of 1 μ M toxin.

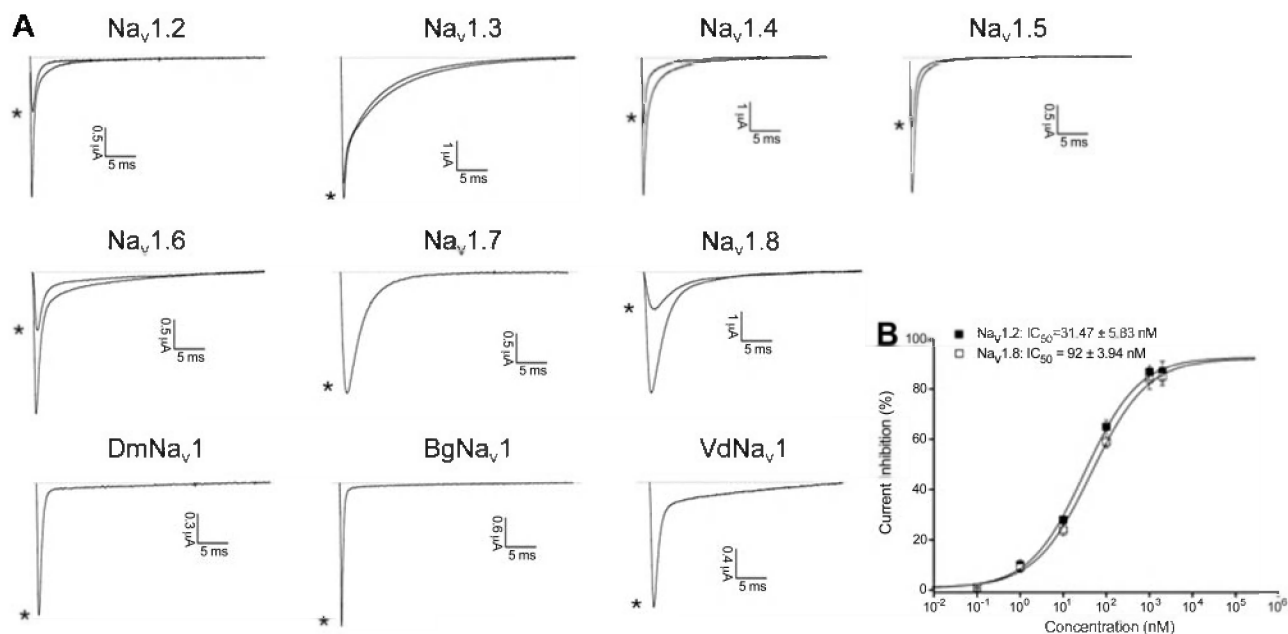


Figure 5. Electrophysiological characterization of APETx1 on Na_v channels. *A*) Activity profile of APETx1 on several Na_v channel isoforms. Representative whole-cell current traces in control and toxin conditions are shown. Dotted line indicates 0 current level; asterisk indicates steady-state current trace after application of 1 μM toxin. *B*) Concentration-response curves for Na_v1.2 and Na_v1.8, indicating the concentration dependence of the APETx1-induced decrease of the Na⁺ peak current.

tion in real time was observed for Na_v1.8 channels. The concentration-response curves were constructed, and EC₅₀ values yielded 114 ± 25 and 55 ± 10 nM for Na_v1.2 and Na_v1.8 channels, respectively (Fig. 6B).

Similar to APETx1, APETx2 did not alter the voltage dependence of activation or steady-state inactivation (Supplemental Fig. S3A and Supplemental Table S1). Toxin

application did not influence the current-voltage relationship or the ion selectivity (Supplemental Fig. S3B). The rate of recovery from inactivation was not different from control conditions (Supplemental Fig. S3C and Supplemental Table S1). APETx2 inhibited Na_v1.2 channels with a τ_{on} of 31 ± 4 s, and inhibition was reversible on washout (τ_{off} = 8 ± 2 s; Supplemental Fig. S3D).

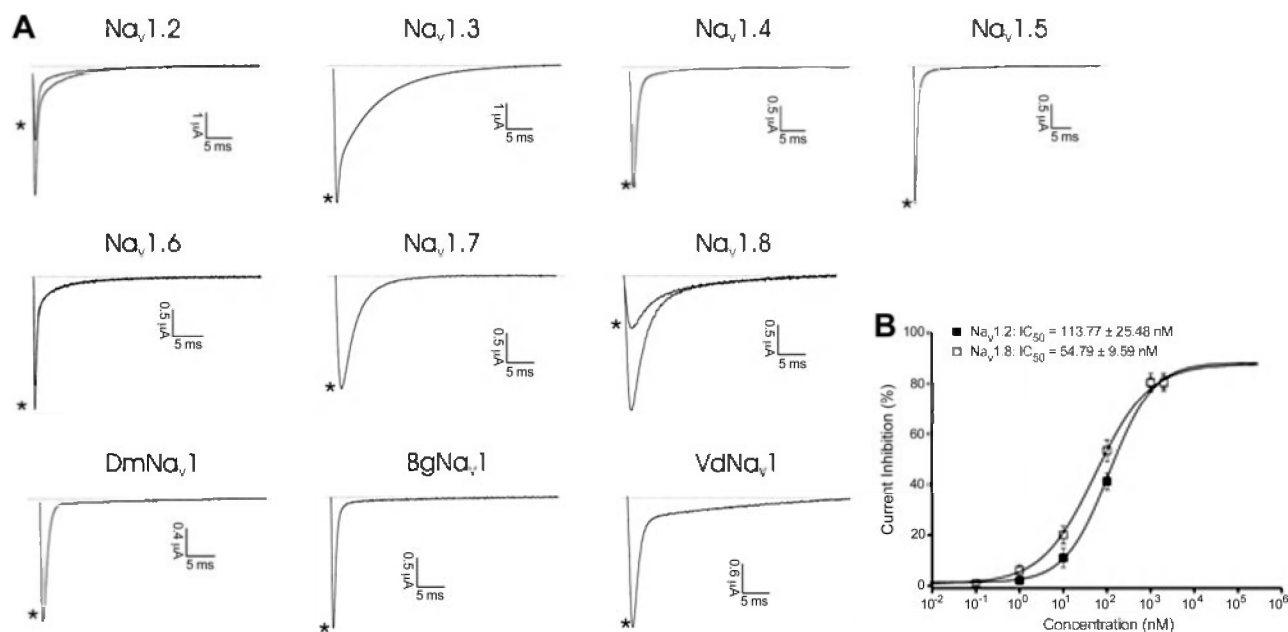


Figure 6. Electrophysiological characterization of APETx2 on Na_v channels. *A*) Activity profile of APETx2 on several Na_v channel isoforms. Representative whole-cell current traces in control and toxin conditions are shown. Dotted line indicates 0 current level; asterisk indicates steady-state current trace after application of 1 μM toxin. *B*) Concentration-response curves for Na_v1.2 and Na_v1.8, indicating the concentration dependence of the APETx2-induced decrease of the Na⁺ peak current.

To investigate whether the inhibition of the sodium conductance depends on toxin interaction with the inactivated state of the α -subunit, the activity of APETx1 and APETx2 was tested on Na_v1.2 channels in which the fast inactivation has been removed (27). Removal of fast inactivation is obtained by mutating the highly conserved hydrophobic residues that contribute to the inactivation gate of Na_v channels (26). Fast inactivation-deficient Na_v1.2 channels have the Ile1488, Phe1489, and Met1490 residues replaced by Gln residues (Na_v1.2 IFM/QQQ channels). A concentration of 1 μ M APETx1 could reduced the I_{Na} by $59 \pm 3\%$ ($n=6$; $P<0.05$), while the same concentration of APETx2 inhibited the current through the Na_v1.2 IFM/QQQ channels by $22 \pm 3\%$ ($n=4$; $P<0.05$; Fig. 7). For both toxins, a shift in the time to peak ($T_{peak Na}$) could be on their application. Addition of APETx1 shifted the $T_{peak Na}$ from 0.4 ms in control conditions to 2 ms ($n=6$; $P<0.01$); 1 μ M APETx2 caused a more modest but still significant shift from 0.3 to 0.7 ms ($n=4$; $P<0.01$). For comparison purposes, identical experiments were done using 50 and 100 nM of TTX; no shift in $T_{peak Na}$ was noticed ($n=3$; $P<0.01$).

To determine whether inhibition occurs during the closed or open state of the channel, 1 μ M toxin was applied to the bath solution with oocytes clamped at -90 mV, allowing interaction with the membrane for 2 min without depolarizing the membrane. After 2 min, currents were elicited by a depolarizing pulse to 0 mV. The obtained currents in presence of toxin were normalized to the currents obtained in the same cells in control conditions. No inhibition was observed when APETx1 or APETx2 was applied to the oocytes without applying depolarizing pulses (Fig. 8A). This indicates that there is no toxin interaction with the channel in

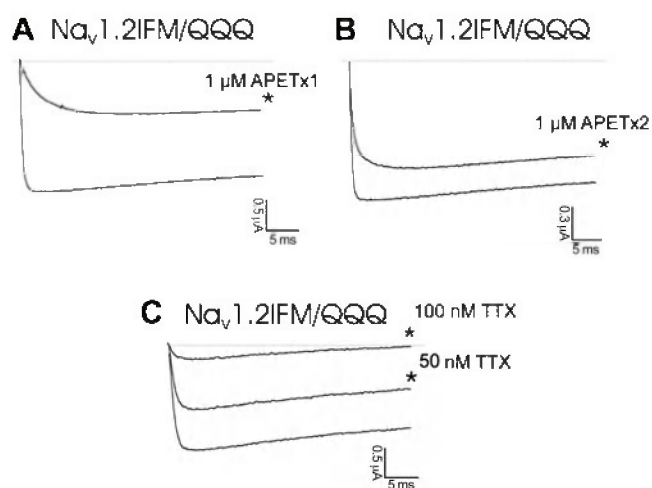


Figure 7. A, B) Effect of APETx1 (A) and APETx2 (B) on fast inactivation-deficient Na_v1.2 channels. C) Effects of 50 and 100 nM TTX, shown for comparison. Dotted line indicates 0 current level; asterisk indicates steady-state current trace after application of 1 μ M toxin.

the closed state and suggests that membrane depolarization and hence most likely also channel opening is required to allow the toxin to bind.

To investigate whether inhibition was use dependent, oocytes expressing Na_v1.2 were submitted to depolarizing pulses at differing frequencies, both in control conditions and after application of 1 μ M toxin (Fig. 8B). No difference in the degree of inhibition was observed at differing frequencies, suggesting that the toxin-induced inhibition is not use dependent.

To determine a possible binding site of APETx1 and APETx2, we investigated whether both toxins share a binding site with HWTX IV, a tarantula toxin that exerts the same characteristics of channel modulation and the binding site of which has been studied in depth (28). Application of HWTX IV at its EC₅₀ concentration of 55 nM reduced the sodium conductance of Na_v1.2 channels by $48 \pm 2\%$ (experimentally determined by constructing a concentration-response curve with for each point $n \geq 3$). Interestingly, subsequent addition of EC₅₀ concentrations of APETx1 or APETx2 resulted in further reduction of the sodium conductance (Fig. 8C). However, the observed reduction was, for both APETx1 and APETx2, $<50\%$ of the remaining current. Subsequent addition of EC₅₀ concentrations of APETx1 inhibited the remaining Na_v channels by $43 \pm 2\%$ ($n=4$) while for APETx2, an inhibition of $37 \pm 1\%$ ($n=3$) was observed after preceding HWTX IV application (Fig. 8D). Since APETx1 and APETx2 failed to compete for the HWTX IV binding site, we next investigated whether both toxins interact with neurotoxin receptor site 1. TTX, a well-known pore blocker of Na_v channels (29), was used for competitive binding experiments (Fig. 8E). Application of TTX at its IC₅₀ value resulted in a blockage of half the expressed Na_v1.2 channels, as a 50% decrease of the sodium current peak amplitude was observed. Subsequent and additional application of the EC₅₀ value of APETx1 or APETx2 did not result in an additional reduction of the peak amplitude. Similar experiments were conducted using the μ -conotoxin KIIIA. This short peptide, isolated from *Conus kinoshitai*, is known to bind a microsite within the pore different from the TTX binding site 1 (30, 31). Application of KIIIA at its IC₅₀ concentration of 60 nM (30) halved the sodium conductance of Na_v1.2 channels. Subsequently, addition of EC₅₀ concentrations of APETx1 or APETx2 failed to introduce any further reduction of the sodium conductance (Fig. 8E). These results suggest that both APETx1 and APETx2 do interact with neurotoxin binding site 1.

DISCUSSION

APETx3

Sea anemone venom is a known source of unique peptide toxins, which can be used as invaluable tools for studying structure and function of ion channels and receptors. Here we report the characterization of a

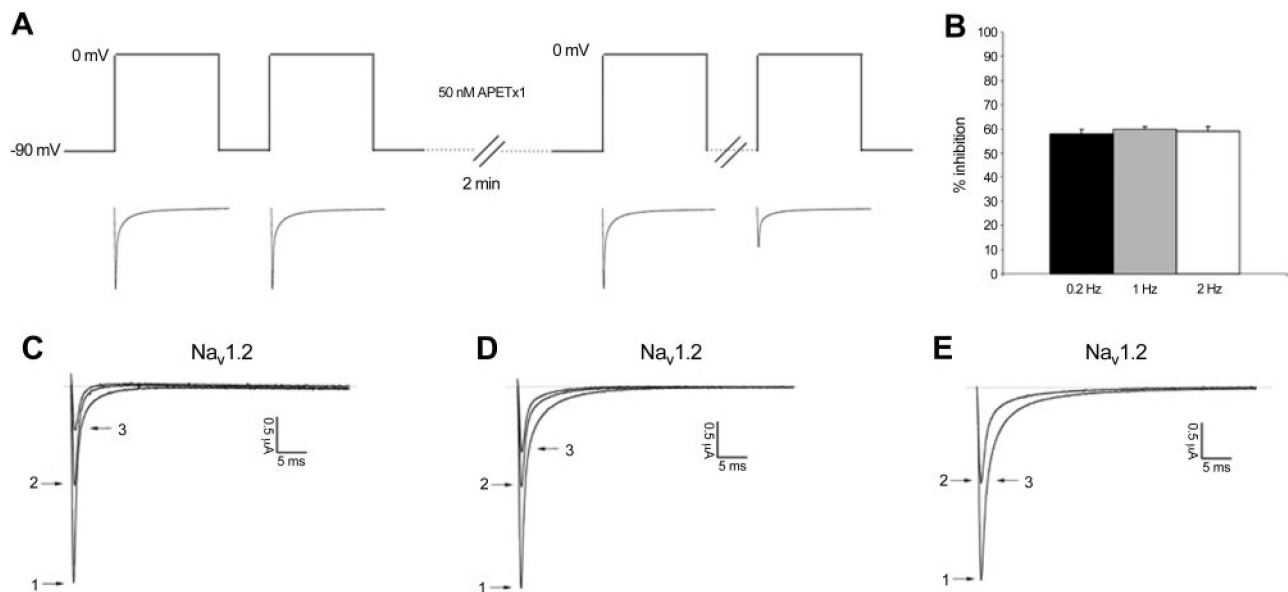


Figure 8. Electrophysiological determination of possible binding sites. *A*) To investigate the state dependence of inhibition, the following protocol was used. As control, a series of depolarizing pulses was applied to an oocyte expressing Na_v1.2 channels. Thereafter, 30 nM APETx1 was added, and no pulsing was performed for 2 min. Next, a similar series of pulses was executed. No inhibition was observed after the 2-min incubation. Inhibition only occurred after membrane depolarization. *B*) Possible use dependence of toxin inhibition was studied by applying 50 nM APETx1 to oocytes subjected to depolarizing pulses at different frequencies. *C*) Competitive experiment to indicate that APETx1 does not compete with HWTX IV. Representative traces are shown: 1) control; 2) after application of 55 nM HWTX IV (46); and 3) after subsequent addition of 30 nM APETx1 (46). *D*) Competitive experiment to indicate that APETx2 does not compete with HWTX IV. Representative traces are shown: 1) control; 2) after application of 55 nM HWTX IV (46); and 3) after subsequent addition of 115 nM APETx2 (46). *E*) Competitive experiment to indicate that APETx1 or APETx2 does bind at site 1. Representative traces are shown: 1) control; 2) after application of 1 nM TTX or 60 nM KIIIA (46); and 3) after subsequent addition of 30 nM APETx1 or 115 nM APETx2.

novel peptide from the sea anemone *A. elegantissima*, APETx3. This toxin can be seen as a natural occurring mutant from APETx1, only differing in 1 aa at position 3 (Thr3Pro). Remarkably, this point mutation dramatically alters the pharmacology profile of the toxin, with APETx3 being inactive and APETx1 being highly active on hERG channels. Furthermore it allows these toxins to exert a differing pharmacology on the same target, namely Na_v channels: APETx3 slows down the inactivation while APETx1 inhibits the conductance. In other words, the point mutation flips the toxin from an inhibitor to a drug that locks Na_v channels open. The APETx3 induced alteration in steady-state inactivation most likely results from the toxin binding to site 3. Many α -scorpion toxins and sea anemone toxins are known to bind to this site and on binding they trap the voltage-sensing S4 of DIV in its inward or deactivated position, hereby preventing the structural movements required for fast inactivation (32, 33). Several toxins capable of binding site 3 have been isolated from sea anemones. However, APETx3 is unrelated to any of the 3 existing sea anemone classes known to act on Na_v channels and it represents the first member of a fourth class of site 3 binding toxins.

The insect-specificity of APETx3 is demonstrated by the complete inhibition of the inactivation of the insect Na_v channels DmNa_v1 and BgNa_v1.1, and it is furthermore emphasized by the 10-fold difference in EC₅₀ values between mammalian (Na_v1.6) and insect

(DmNa_v1) channels. This preference for insect Na_v channels suggests that APETx3 can be used as an insecticide (34, 35).

The functional differences between APETx1 and APETx3 raise an intriguing structure-function conundrum. APETx1 and APETx3 only differ from each other by a Thr to Pro substitution at position 3. It is well known that prolines can induce a structural kink in a peptide structure. It is possible that the Thr3Pro substitution in APETx3 results in a conformational change that allows APETx3 to expose a contact surface differing from that of APETx1, which might explain the functional differences between these peptides. However, detailed structural studies will be required to verify this hypothesis.

APETx1

APETx1 and APETx2 are the first sea anemone peptides shown to inhibit the conductance of Na_v channels. Here we show that APETx1 is a promiscuous toxin, capable of inhibiting Na_v channels in the same concentration range as it is active on hERG channels. APETx1 inhibits the mammalian isoforms Na_v1.2-Na_v1.6 and Na_v1.8. Remarkably, no effect was observed on the insect Na_v channel isoforms. Since APETx3 is an insect-specific modulator of Na_v, it can be discerned that the point mutation at position 3 influences the phyla-specificity of APETx-like peptides.

Similar to the inhibition of potassium currents through hERG channels, no complete inhibition of the sodium currents could be achieved, even at high concentrations of APETx1. However, there are differences in the current inhibition effected by APETx1 for Na_v and hERG channels. Contrary to what is reported for hERG modulation, no modulation in the voltage dependence of activation or steady-state inactivation was observed. APETx1 does not seem to interact with the closed state of the sodium channel. It was also noted that APETx1 inhibition is not use dependent. Competitive experiments with TTX and the μ -conotoxin KIIIA, two known pore-blocker toxins, led to the assumption that APETx1 does inhibit Na_v channels by binding at site 1 or close by. Furthermore, with the use of fast inactivation-deficient Na_v1.2 channels, it was shown that APETx1 does not rely on the inactivated state to exhibit its conductance inhibiting activity. In these experiments, it could be seen that APETx1 causes a significant shift in the time to peak, possibly indicating a modified transition from closed to open state on depolarization.

APETx2

With the use of the oocyte expression system, APETx2 was submitted to a screening on several mammalian and insect Na_v channel isoforms. It was found that APETx2 could also inhibit Na_v1.2 channels with the same potency as Na_v1.8 channels. To a lesser extent, the toxin also inhibited the Na_v1.6 channel. Further electrophysiological investigations demonstrated that APETx2 has the mechanism of interaction with the channels as APETx1. APETx2 did not alter the voltage dependence of activation or inactivation nor did it influence the reversal potential or the recovery from inactivation. No use-dependency was noted, and APETx2 did not seem to rely on the inactivated state to inhibit currents. Furthermore, APETx2 does compete with TTX and μ -conotoxins for their binding site. However, APETx2 did cause a shift in the time to peak of Na_v1.2 IFM/QQQ channels.


Studies of interaction of APETx2 with Na_v1.8 channels in DRG neurons indicated that this toxin acts as a modulator rather than a pore blocker (21). This finding was substantiated by the small (± 5 mV) but significant shift of the voltage dependence of activation toward more depolarized values. Moreover, the toxin-induced slowing down of fast inactivation but not the steady-state inactivation, together with a slight acceleration of the initial phase of recovery suggests that APETx2 destabilizes the inactivated state. The results obtained for Na_v1.2 channels in oocytes are in accord with the results for Na_v1.8 channels in DRG neurons, except for the 5-mV shift in activation and the small acceleration in recovery from inactivation. However, since both results were obtained in different expression systems (patch clamp *vs.* voltage clamp), it is possible that these small differences can be explained as such. Since both systems use different cells to express channels, potential differences in post-translational modifi-

cations of channels and differences in expression of auxiliary subunits might influence kinetics of channel gating. Furthermore, the need for multiple pharmacological manipulations to isolate a specific channel current in neurons, which are unnecessary in *Xenopus* oocytes, and differences in membrane composition together with inherent differences in osmolarity of the recording solutions can provide an explanation for the differences observed between both expression systems.

For both APETx1 and APETx2, it can be concluded that the exact mechanism of action still needs to be determined. Peptide toxins with the ability to inhibit >1 ion channel or receptor are mostly found in spider venoms, more particular tarantula venoms. For example, Hanatoxin I from the tarantula *Grammostola spatulata*, is a gating modifier of Ca_v2 channels as well K_v2 and K_v4 (36, 37). Another example can be found in toxins isolated from the venom of *Grammostola rosea*, which act as gating modifiers of both Na_v and hERG, but not K_v1 channels, by shifting the voltage dependence of activation toward more positive potentials (38). From the Chinese tarantula *Ornithoconus huwena*, two toxins, HWTX-I and HWTX-IV, have been isolated and characterized in depth (39, 40). Both HWTX-I and HWTX-IV are capable of inhibiting, in nanomolar concentrations, TTX-sensitive Na_v channels (28, 41). Interestingly, similar to APETx1 and APETx2, this inhibition occurs without altering the voltage dependence of activation or inactivation. Using site-directed mutagenesis analysis, it was found that HWTX-IV binds to site 4, which is mainly located at the S3–S4 linker of DII. It is proposed that the voltage sensor of DII is trapped in its inward position as a result of HWTX-IV binding, thereby hampering the sodium conductance (28). Competition experiments show that APETx1 and APETx2 do not compete with the HWTX IV binding site. Promiscuous tarantula toxins adopt the ICK motif and share little to no identity with the APETx-like peptides, which are members of the cysteine-stabilized $\alpha\beta$ (Cs $\alpha\beta$) structural family. Nevertheless, it is noteworthy that all promiscuous spider toxins known up to date are believed to interact with the voltage-sensing modules of their ion channel targets. Therefore, one can question whether other toxins that overstep the boundaries of targeting a single ion channel, such as APETx1 and APETx2, are not also expected to interact with the voltage sensor. The electrophysiological data available at present for APETx1 and APETx2 do not prove such an interaction. However, it does also not exclude this. Further structure-function analyses involving site-directed mutagenesis studies, docking, and radiolabeled binding experiments are required to elucidate the site of binding of these peptides to gain further understanding of their mechanism of action.

From the *Anemonia viridis* EST dataset, 12 APETx-like peptides could be identified (42). Recently, using a 454 pyrosequencing approach, it was shown that two other sea anemones, *Stichodactyla helianthus* and *Bunodosoma granulefera*, also express peptides belonging to the APETx family, suggesting that APETx-like peptides

are conserved throughout sea anemone species (43). Interestingly, several APETx-like peptides possess a proline at position 3, similar to APETx3. Moreover, two other members of this family, BDS-I and BDS-II, characterized as $K_v3.4$ blockers, also contain a proline at position 3 (44). Recently, it was shown that BDS-I, similar to APETx3, could slow the inactivation of TTX-sensitive Na_v currents (45). This indicates that the presence of a proline at position 3 is one of the key determinants for Na_v channel inactivation modulation. However, the target promiscuity of APETx-like peptides, as underlined in this work, necessitates functional characterization to identify the different targets of peptides belonging to the APETx family.

APETx1 and APETx2 are two toxins that are synthetically produced, commercially available, and have been used in *in vivo* experiments. We have demonstrated that caution is required in interpretation of these *in vivo* experiments, since APETx1 and APETx2 are not selective modulators of hERG and ASIC3 channels, respectively, as thus far believed. Our data show that these toxins are promiscuous, targeting Na_v channels with an equal potency. Moreover, they are the first Na_v channel inhibitors from sea anemones reported. Overall, the target promiscuity of APETx1 and APETx2 unravels the family of APETx-like peptides as a yet unexplored treasure of promiscuous toxins. APETx1–3 represent valuable probes for further structure-function studies and hereby provide new leads in the development of therapeutic agents for channel related diseases. 

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