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ProTx-II, a Selective Inhibitor of $Na_V1.7$ Sodium Channels, Blocks Action Potential Propagation in Nociceptors

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ABSTRACT

Voltage-gated sodium (Na_V1) channels play a critical role in modulating the excitability of sensory neurons, and human genetic evidence points to $Na_V1.7$ as an essential contributor to pain signaling. Human loss-of-function mutations in SCN9A, the gene encoding $Na_V1.7$, cause channelopathy-associated indifference to pain (CIP), whereas gain-of-function mutations are associated with two inherited painful neuropathies. Although the human genetic data make $Na_V1.7$ an attractive target for the development of analgesics, pharmacological proof-of-concept in experimental pain models requires $Na_V1.7$-selective channel blockers. Here, we show that the tarantula venom peptide ProTx-II selectively interacts with $Na_V1.7$ channels, inhibiting $Na_V1.7$ with an IC_{50} value of 0.3 nM, compared with IC_{50} values of 30 to 150 nM for other heterologously expressed Na_V1 subtypes. This subtype selectivity was abolished by a point mutation in DIIS3. It is interesting that application of ProTx-II to desheathed cutaneous nerves completely blocked the C-fiber compound action potential at concentrations that had little effect on Aβ-fiber conduction. ProTx-II application had little effect on action potential propagation of the intact nerve, which may explain why ProTx-II was not efficacious in rodent models of acute and inflammatory pain. Mono-iodo-ProTx-II (^{125}I-ProTx-II) binds with high affinity (K_d = 0.3 nM) to recombinant $hNa_V1.7$ channels. Binding of ^{125}I-ProTx-II is insensitive to the presence of other well characterized Na_V1 channel modulators, suggesting that ProTx-II binds to a novel site, which may be more conducive to conferring subtype selectivity than the site occupied by traditional local anesthetics and anticonvulsants. Thus, the ^{125}I-ProTx-II binding assay, described here, offers a new tool in the search for novel $Na_V1.7$-selective blockers.

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Pain relief remains an important, currently unmet, medical need. Voltage-gated sodium channels play a critical role in modulating the excitability of most neurons, including nociceptive sensory neurons signaling pain. Despite the clinical use of systemically administered lidocaine to treat chronic pain since the 1950s (Kugelberg and Lindblom, 1959) and the approval of the weak sodium channel blocker carbamazepine for the treatment of trigeminal neuralgia (Campbell et al., 1966), several oral sodium channel blockers have failed to show efficacy in large clinical trials (Wallace et al., 2002; Vinik et al., 2007). The failure of drugs in the clinic may at least partially be attributed to the narrow therapeutic window of non–subtype-selective sodium channel blockers.

Recently, three publications have shown that loss-of-function mutations in the sodium channel subtype $Na_V1.7$ are the cause for channelopathy-associated insensitivity to pain (CIP) (Cox et al., 2006; Ahmad et al., 2007; Goldberg et al., 2007). Furthermore, genetic linkage analysis has identified gain-of-function mutations in $Na_V1.7$ as the cause of inherited erythromelalgia (Yang et al., 2004; Han et al., 2006; Dib-Hajj et al., 2007) and paroxysmal extreme pain disorder (Fertleman et al., 2006). Both are rare autosomal dominant disorders that are associated with spontaneous or evoked bouts of burning pain accompanied by heat and flushing.

$Na_V1.7$ (Klugbauer et al., 1995) is one of seven sodium channel subtypes expressed in the peripheral nervous sys-

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ABBREVIATIONS: CIP, congenital indifference to pain; ANOVA, analysis of variance; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; CFA, Complete Freund's Adjuvant; Na_V, voltage-dependent sodium channel; PCR, polymerase chain reaction; SIF, synthetic interstitial fluid; SNAP, sensory nerve compound action potential; TFA, trifluoroacetic acid; TTX, tetrodotoxin; HEK, human embryonic kidney; ANOVA, analysis of variance; HPLC, high-performance liquid chromatography.

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tem. In dorsal root ganglion neurons from adult rodents, $Na_V1.7$ is the most abundant Na_V1 subtype, based on reverse-transcription polymerase chain reaction experiments (Lopez-Santiago et al., 2006), and the biophysical characteristics of the TTX-sensitive sodium current found in these neurons are similar to those of recombinant $Na_V1.7$ channels (Cummins et al., 1998). Within rodent dorsal root ganglia, $Na_V1.7$ is preferentially expressed in small-diameter nociceptive neurons (Djouhri et al., 2003) and is up-regulated in response to inflammation (Black et al., 2004; Yeomans et al., 2005). A role of $Na_V1.7$ in mediating inflammatory pain signaling in rodents is further supported by the finding that knockdown of $Na_V1.7$ expression, either through herpes vector-mediated application of antisense oligonucleotides or by nociceptor-specific genetic ablation, reduces the development of inflammatory hyperalgesia (Nassar et al., 2004; Yeomans et al., 2005). Further analysis of the function of $Na_V1.7$ in pain signaling would benefit from the discovery of a $Na_V1.7$-selective sodium channel blocker. Historically, truly subtype-selective sodium channel blockers have been rare, and despite significant efforts by the pharmaceutical industry, only a few subtype-selective small-molecule blockers have been published to date (Jarvis et al., 2007; Rosker et al., 2007; Williams et al., 2007).

Peptides found in the venoms of snakes and spiders have been a rich source of ion channel modulators and are often highly selective. Indeed, ProTx-II from the tarantula *Thrixopelma prurient* has been reported as a blocker of $Na_V1.7$ channels (Middleton et al., 2002; Smith et al., 2007). In this manuscript, we show that ProTx-II is at least 100-fold selective for $Na_V1.7$ over other sodium channel subtypes and provides a versatile experimental tool for studying the role of $Na_V1.7$.

Materials and Methods

Materials. HEK-293 cell lines stably expressing $hNa_V1.2$, $hNa_V1.7$, or $hNa_V1.8$ together with the sodium channel $\beta1$ subunit were established in-house. Stable HEK-293 cell lines expressing $hNa_V1.5$ or $hNa_V1.7$ (α subunit only) were obtained from Dr. Hartman and Aurora Biosciences (San Diego, CA), respectively. $hNa_V1.3$ and $hNa_V1.6$, stably expressed in Chinese hamster ovary and HEK-293 cells, respectively, were obtained from Millipore (Billerica, MA). All tissue culture media were from Invitrogen Corporation (Carlsbad, CA). For electrophysiological recordings, cells were plated on 3 × 3-mm glass coverslips coated with poly(D-lysine). ProTx-II was chemically synthesized by Peptides International (Louisville, KY). Microbeads P-6 were a generous gift from Bio-Rad (Hercules, CA). For in vivo experiments, ProTx-II was dissolved in saline. Css II was a kind gift from Dr. Gerardo Corzo (Universidad Nacional Autónoma de México, Cuernavaca Morelos, Mexico).

Animals. All procedures involving animals were carried out in accordance with the United Kingdom Animal (scientific procedures) Act or the National Institutes of Health guidelines for the use of live animals and were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Behavioral studies were carried out by Algos Therapeutics, Inc. (St. Paul, MN) in accordance with the International Association for the Study of Pain guidelines and were approved by the University of Minnesota's Institutional Animal Care and Use Committees. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were maintained in a temperature-controlled (21°C) facility with a 12-h light/dark cycle and had ad libitum access to water and regular rodent chow. At the time of the experiment, rats weighed approximately 240 g.

Electrophysiology. Sodium currents were examined by whole-cell voltage clamp (Hamill et al., 1981) using an EPC-9 amplifier and Pulse software (HEKA Electronics, Lamprecht, Germany). Electrodes were fire-polished to resistances of 1.5 to 4 MΩ. Voltage errors were minimized by series resistance compensation (75–85%), and the capacitance artifact was canceled using the amplifier's built-in circuitry. Data were acquired at 50 kHz and filtered at 10 kHz. The bath solution typically consisted of 40 mM NaCl, 120 mM *N*-methyl-D-glucamine chloride, 1 mM KCl, 2.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM *N*-methyl-D-glucamine HEPES, pH 7.4, and the internal (pipette) solution contained 110 mM cesium-methanesulfonate, 5 mM NaCl, 20 mM CsCl, 10 mM CsF, 10 mM BAPTA (tetra-cesium salt), and 10 mM cesium-HEPES, pH 7.4. In some cases, *N*-methyl-D-glucamine chloride in the bath solution was replaced by NaCl to increase current amplitudes. Liquid junction potentials were less than 4 mV and were not corrected for. Where applicable, data are presented as mean values and S.E.M.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed on $hNa_V1.7$ and $hNa_V1.2$ by using the QuikChange II kit from Stratagene (La Jolla, CA) and following the manufacturer's protocols. The sense primers used for mutagenesis had the following sequences: 5′-TTAAGTTTAGTGGAGCTCggTCTAGCAGATGTGGAAGG-3′ for the $hNa_V1.7$ F813G mutant, 5′-TTAAGTTTAGTGGAGCTCagcCTAGCAGATGTGGAAGG-3′ for the $hNa_V1.7$ F813S mutant and 5′-GAGCCTTAGTTTAATGGAACTgttcTTGGCAAATGTGGAAGGATTG-3′ for the $hNa_V1.2$ G839F mutant. To rule out rearrangements or secondary amino acid changes, the entire open-reading frames of the mutant constructs were sequenced. Mutant constructs were transiently transfected into TsA-201 cells, together with an expression plasmid for green fluorescent protein, using FuGENE6 from Roche (Basel, Switzerland).

Acute and Inflammatory Pain Tests. For intrathecal administration of drugs, animals were anesthetized with isoflurane, and a catheter was inserted into the subarachnoid space at L5–L6 of the spinal column. The catheter was flushed and filled with artificial cerebrospinal fluid and plugged. The exposed incision was closed with sutures and wound clips, and animals were allowed to recover for 4 days. At the end of an experiment, animals were injected with 10 μl of 2% lidocaine, followed by a 15-μl artificial cerebrospinal fluid flush, and hind limb paralysis was used to verify proper implantation of the intrathecal catheter.

Local inflammation was induced by subcutaneous injection of 50 μl of Complete Freund's Adjuvant (CFA) into the plantar surface of the left hind paw. Withdrawal thresholds to a noxious mechanical stimulus were measured in the inflamed (ipsilateral) and uninjured (contralateral) hind paw using a Ugo Basile apparatus (Stoelting Co., Wood Dale, IL). Paw pressure was applied to the plantar surface of the paw by a dome-shaped plastic tip placed between the third and fourth metatarsus and was increased linearly until the animal vocalized, withdrew the paw, or struggled. To avoid tissue damage, a cut-off pressure was set at 390 g on the ipsilateral paw and 750 g on the contralateral paw. Mechanical threshold was defined as the force in grams at the first pain behavior, and data are presented as mean and S.E.M. for the inflamed and uninjured paw. Hyperalgesia of the inflamed paw was determined by comparing mechanical thresholds in the ipsilateral and contralateral paw using an unpaired *t* test. The effect of ProTx-II on hyperalgesia was analyzed at each time point by using a one-way analysis of variance (ANOVA). Planned comparisons of means (each group compared with saline) were performed using a Dunnett's post hoc test.

Isolated Nerve Preparation. The skin-nerve in vitro preparation was used to record Aβ- and C-fiber sensory nerve compound action potentials (SNAP). Female Sprague-Dawley rats, weighing 300 to 400 g, were killed by cervical dislocation. The skin of the hind limb and the attached saphenous nerve were removed as described previously (Reeh, 1986). The preparation was mounted corium-side up in an organ bath and superfused with oxygenated synthetic interstitial fluid (SIF) consisting of 108 mM NaCl, 3.5 mM KCl, 3.5

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mM $MgSO_4$, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 9.6 mM sodium gluconate, 5.55 mM glucose, 7.6 mM sucrose, and 10 mM HEPES, pH 7.4. The temperature of the bath was maintained at 32°C. The proximal end of the nerve was isolated from SIF by liquid paraffin, desheathed, and teased into filaments suitable for recording from single nerve fibers. Recordings were obtained with a pair of gold-wire electrodes, a custom-made low-noise differential amplifier in common rejection mode, a low-pass filter of 1 kHz, and a high-pass filter of 1 Hz. A self-sealing stainless steel ring (inner diameter, 6 mm; outer diameter, 8 mm) was placed on top of a section of the saphenous nerve and filled with liquid paraffin at 32°C. In some experiments, the nerve section covered by the stainless steel ring was desheathed before the experiment. The whole nerve was stimulated using Ag/AgCl electrodes with a noninsulated tip diameter of 2 mm (EPO5; World Precision Instruments, Hertfordshire, UK) at a frequency of 1 Hz (Aβ-volley) or 0.25 Hz (C-volley), a stimulus width of 0.2 ms (Aβ-volley) or 2 ms (C-volley), and an intensity of 5 to 20 V. The amplitude of the SNAP was measured peak-to-peak and expressed as a percentage of control for different treatments. ProTxII was diluted into SIF from a 100 μM stock solution and warmed to 32°C before the experiment.

Iodination of ProTx-II. Lactoperoxidase and glucose oxidase were coupled to P6 microbeads, small polyacrylamide beads of approximately 1 to 3 μm in size, following the protocol provided by Bio-Rad for production of Enzymobeads (beads). Beads were flash-frozen in liquid N_2 in 100-μl aliquots and stored at −80°C for future use. For iodination with either ^{125}I-Na or ^{127}I-Na, one aliquot of beads was thawed on ice, centrifuged, and washed once with 200 μl of $NaPO_4$, pH 8.0. To the washed beads, 30 μl of ProTx-II solution (3.33 mg/ml in 100 mM $NaPO_4$, pH 8.0) and 14 μl of a solution containing either 5 mCi of ^{125}I-Na (2200 Ci/mmol) or 20 μg/ml ^{127}I-Na in 10 μM NaOH was added, followed by 5 μl of 5% β-D-glucose. After incubating at room temperature for 30 min, the reaction was centrifuged, and the supernatant was loaded onto a 300 Å pore size C_{18} reverse-phase HPLC column (0.46 × 25 cm; Vydac, Hesperia, CA) that had been equilibrated with 10 mM trifluoroacetic acid (TFA). Elution was achieved with a linear gradient of 50% acetonitrile in 5 mM TFA (0–50% for 2 min, 50–70% for 40 min) at a flow rate of 0.5 ml/min. Material eluting from the column was monitored by measuring absorbance at 210 nm. A peak eluting at 54.3% was collected, concentrated to half its original volume, diluted with an equal volume of 10 mM TFA, and purified to homogeneity by HPLC under identical chromatographic conditions. After the iodination of peptide with ^{127}I-Na, chromatographically homogeneous peaks were collected and analyzed by electrospray mass spectroscopy. The peak identified as monoiodotyrosine ProTx-II was lyophilized dry and reconstituted in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. Aliquots were frozen in liquid N_2 and stored at −70°C.

^{125}I-ProTx-II Binding. HEK-293 cells stably expressing $hNa_V1.7/\beta1$ were seeded at a density of 50,000 cells/well in 96-well poly(D-lysine)-coated plates and were allowed to attach for approximately 18 h at 37°C. ^{125}I-ProTx-II was added to wells in an assay volume of 200 μl, and incubation took place under normal growth conditions. For saturation experiments, cells were incubated with increasing concentrations of ^{125}I-ProTx-II for >5 h, and quadruplicate samples were averaged for each experimental point. At the end of the incubation, cells were washed twice with 200 μl of Dulbecco's phosphate-buffered saline plus 10% fetal bovine serum to separate bound from free ligand. Cells were lysed by the addition of 200 μl of 0.2% SDS, and radioactivity associated with the cell lysate was determined using a γ counter. Nonspecific binding was defined as binding in the presence of 200 nM ProTx-II. Specific binding, obtained by subtracting nonspecific from total binding, was analyzed according to the equation $B_{eq} = (B_{max}\,L^*)/(K_d + L^*)$, where B_{eq} is the amount of ^{125}I-ProTx-II bound at equilibrium, B_{max} the maximum receptor concentration, K_d the equilibrium dissociation constant, and L* the free ^{125}I-ProTx-II concentration. Competition binding experiments were carried out in the absence or presence of increasing concentration of a test compound. IC_{50} values for inhibition of ^{125}I-ProTx-II binding by the test compound were determined using the equation $B_{eq} = (B_{max} - B_{min})/[1 + (D/IC_{50})^{nH}] + B_{min}$, where B_{max} represents bound ^{125}I-ProTx-II in the absence of test compound (D), B_{min} is the amount of ^{125}I-ProTx-II bound at a saturating concentration of test compound, D is the concentration of test compound, n_H is the Hill coefficient, and IC_{50} is the compound concentration resulting in half-maximal inhibition. To determine kinetics of ligand association, cells were incubated with ^{125}I-ProTx-II for different periods of time. The association rate constant (k_1) was determined from the equation $k_1 = k_{obs}(B_{eq}/(L^* B_{max}))$, where B_{eq} is the amount of ^{125}I-ProTx-II bound at equilibrium, B_{max} the maximum receptor concentration, and k_{obs} is obtained by fitting the slope of $\ln(B_{eq}/(B_{eq} - B_t))$ plotted as a function of time. Dissociation kinetics were initiated either by addition of 200 nM ProTx-II or by washing the wells twice with media and incubating for different periods of time. The dissociation rate constant k_{-1} was calculated by fitting the data to a single, monoexponential decay.

Results

ProTx-II is a 30-amino acid peptide from the tarantula *T. prurien* and had been identified previously as a sodium channel modulator (Middleton et al., 2002; Smith et al., 2007). ProTx-II belongs to the cystine-knot family of inhibitory peptides and inhibits sodium currents by an effect on conductance combined with a shift of the activation voltage to more positive potentials.

Selectivity of ProTx-II for $Na_V1.7$. Previous results have indicated that ProTx-II interacted more potently with $Na_V1.7$ than with $Na_V1.5$ channels (Smith et al., 2007). To characterize the selectivity of ProTx-II in more detail, we examined its effects on seven of the nine hNa_V1 subunits identified to date. Because ProTx-II acts in part by shifting the voltage-dependence of channel activation to more positive potentials, inhibitory effects are dependent on test pulse potential. To standardize the protocol across Na_V1 subtypes with different activation voltages, the test pulse potential was set to the average $V_{0.5}$ of channel activation for each Na_V1 subtype in the stable cell line used for the ProTx-II experiments ($Na_V1.2$, −13 mV; $Na_V1.3$, −5 mV; $Na_V1.4$, −20 mV; $Na_V1.5$, −28 mV; $Na_V1.6$, −20 mV; $Na_V1.7$, −8 mV; and $Na_V1.8$, +5 mV). Short (6-ms) test pulses were applied at 0.5 Hz from a holding potential at which steady-state inactivation was minimal, ranging from −90 mV for $Na_V1.8$ to −110 mV for $Na_V1.5$. Figure 1A shows block of $hNa_V1.6$ (□) and $hNa_V1.7$ (■) by 10 nM ProTx-II under these conditions, resulting in 26 and 99% block, respectively. Data for all seven Na_V1 subtypes tested are summarized in Fig. 1B and consist of at least three data points for each concentration and Na_V1 subtype. Fitting the data with the Hill equation $Y = 100/[1 + ([ProTx\text{-}II]/IC_{50})]$, and assuming a Hill coefficient of 1, yielded the following IC_{50} values: $Na_V1.2$, 41 nM; $Na_V1.3$, 102 nM; $Na_V1.4$, 39 nM; $Na_V1.5$, 79 nM; $Na_V1.6$, 26 nM; $Na_V1.7$, 0.3 nM; and $Na_V1.8$, 146 nM. A more accurate determination of the IC_{50} for block of $Na_V1.7$ by ProTx-II, achieved by testing lower concentrations of the peptide, would be desirable but was precluded by the exceedingly long application times required to reach steady-state inhibition levels. Nevertheless, the data show that ProTx-II is at least 100-fold selective for block of $Na_V1.7$ over the six other Na_V1 subtypes tested.

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Site of Action of ProTx-II on $Na_V1.7$. Assuming that the binding site for ProTx-II resides within the transmembrane helices or in the extracellular domains of sodium channels, we examined the sequences of all hNa_V1 subtypes for amino acid residues in these regions that are conserved among hNa_V1 subtypes other than $hNa_V1.7$ and subject to a non-conservative substitution in $hNa_V1.7$. One such amino acid is found at position 839 (based on the $Na_V1.2$ sequence) in the S3b helix of domain II, where a phenylalanine in $hNa_V1.7$ (Phe813) replaces a glycine residue in the other hNa_V1 subtypes, except for a serine in $hNa_V1.6$ (Fig. 2A). Mutating phenylalanine 813 in $hNa_V1.7$ to glycine (F813G) resulted in channels with similar voltage-dependence of activation ($V_{0.5}$ of -12 ± 1 mV, $n = 7$, versus -8 ± 1 mV, $n = 7$) and inactivation ($V_{0.5}$ of -62 ± 2 mV, $n = 7$, versus -68 ± 1 mV, $n = 107$) compared with wild-type $hNa_V1.7$ channels. Channels with a serine in position 813 (F813S) displayed a similar voltage-dependence of activation ($V_{0.5}$ of -9 ± 2 mV, $n = 4$) compared with wild-type $hNa_V1.7$ channels but inactivated at more depolarized potentials ($V_{0.5}$ of -34 ± 1 mV, $n = 3$, $p < 0.01$). It is remarkable that both mutant constructs, $hNa_V1.7$ F813G and F813S, were approximately 100-fold less sensitive to block by ProTx-II than wild-type channels (Fig. 2, B and C). Indeed, the IC_{50} values for block of $hNa_V1.7$ F813G and $hNa_V1.7$ F813S channels by ProTx-II was 27 and 31 nM, respectively, similar to the IC_{50} values of ProTx-II for the other hNa_V1 subtypes. Based on these results, we introduced the opposite mutation into recombinant $hNa_V1.2$ channels ($hNa_V1.2$ G839F) in an attempt to increase the sensitivity to ProTx-II. Human $Na_V1.2$ G839F channels, expressed transiently in TsA-201 cells, activated at slightly more depolarized voltages than the stably expressed wild-type $hNa_V1.2$ channels ($V_{0.5}$ of -7.8 ± 1.5 mV, $n = 8$, versus -12.5 ± 0.9 mV, $n = 8$, $p = 0.017$). This difference is consistent with an effect of the $\beta 1$ subunit, coexpressed in the stable cell line but absent in the transiently expressed mutant channels (Isom et al., 1995). Block of $Na_V1.2$ G839F by ProTx-II was not significantly different from block of $Na_V1.2$ wild-type channels ($p = 0.1$) with IC_{50} values of 25 and 41 nM for $Na_V1.2$ G839F and $Na_V1.2$ wild-type channels, respectively.

Efficacy in Rat Models of Short-Term Nociception and Inflammation-Induced Hyperalgesia. ProTx-II was examined for effects on short-term nociception and inflammation-induced hyperalgesia by testing the response to noxious mechanical stimuli in the ipsilateral and contralateral paw of rats injected with CFA to produce a local inflammation. Twenty-four hours after the CFA injection, withdrawal thresholds to a noxious mechanical stimulus (paw pressure) were assessed in both hind paws and were compared with baseline measurements taken 24 h before CFA injection. Consistent with data reported in the literature, all animals developed mechanical hyperalgesia in the inflamed hind paw, resulting in withdrawal thresholds of 131 ± 6 g in the inflamed ipsilateral paw compared with 406 ± 9 g in the contralateral paw ($p < 0.001$ unpaired t test, $n = 40$). Intravenous administration of saline had no effect on withdrawal thresholds in the ipsilateral or contralateral paw ($p = 0.12$ and 0.07, respectively, repeated-measures ANOVA, $n = 10$). Intravenous administration of morphine at 3 mg/kg increased paw withdrawal thresholds in both the ipsilateral and the contralateral paw ($p < 0.001$ versus saline, unpaired t test, $n = 10$), consistent with an analgesic effect of morphine (data not shown). In a rat tolerability assay, 0.01 and 0.1 mg/kg i.v. ProTx-II were well tolerated; however, 1 mg/kg ProTx-II was lethal in two rats. Plasma was collected from one rat approximately 10 min after the 1 mg/kg i.v. dose, after the rat had died, and the plasma concentration of ProTx-II was determined in a competition binding assay with ^{125}I-ProTx-II, as detailed under *Materials and Methods*. The plasma concentration of ProTx-II was 3 μM, well above the IC_{50} value for the cardiac sodium channel subtype. Based on these results, ProTx-II was given intravenously at either 0.01 mg/kg or at 0.1 mg/kg in the hyperalgesia study. Thirty minutes after dose, 0.1 mg/kg i.v. ProTx-II produced a slight reversal of mechanical hyperalgesia (Fig. 3), but this effect failed to reach statistical significance ($p = 0.09$, one-way

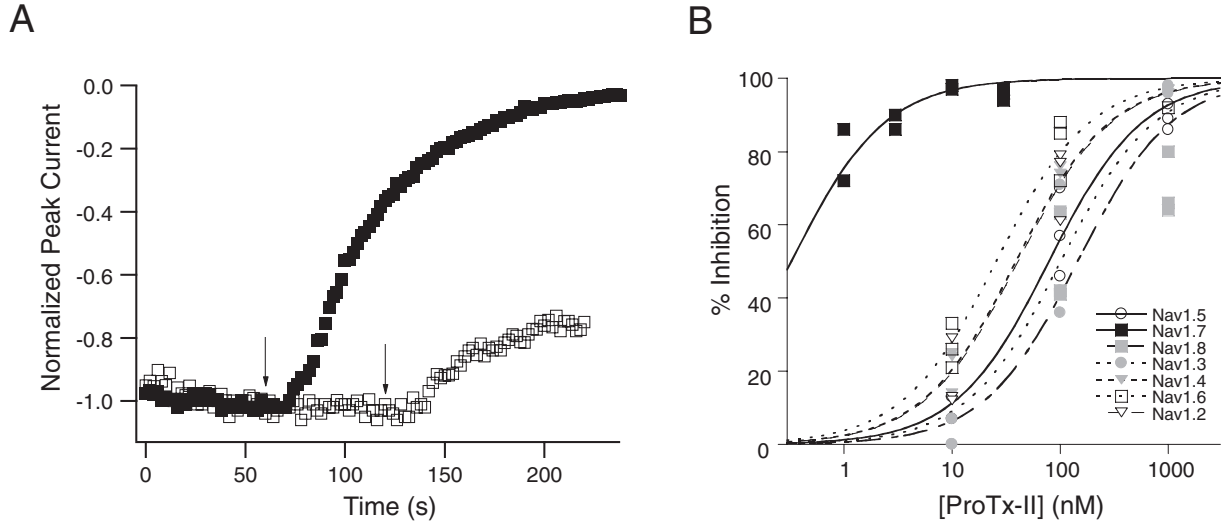

Fig. 1. Block of hNa_V1 subtypes by ProTx-II. A, block of $hNa_V1.7$ (■) and $hNa_V1.6$ (□) by 10 nM ProTx-II was examined by whole-cell voltage clamp. Peak current elicited by steps to the voltage of half-maximal activation (-8 and -20 mV for $hNa_V1.7$ and $hNa_V1.6$, respectively) are shown in control and after the addition of 10 nM ProTx-II indicated by the arrows. B, inhibition of hNa_V1 currents was measured as shown in A and plotted as a function of ProTx-II concentration. Fitting the Hill equation ($n_H = 1$) to the data yielded the following IC_{50} values: $Na_V1.2$, 41 nM; $Na_V1.3$, 102 nM; $Na_V1.4$, 39 nM; $Na_V1.5$, 79 nM; $Na_V1.6$, 26 nM; $Na_V1.7$, 0.3 nM; $Na_V1.8$, 146 nM.

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ANOVA, $n = 10$). Average plasma levels, 4 h after dose, were 8.5 ± 2.3 nM in these animals. Neither the 0.01 mg/kg i.v. nor the 0.1 mg/kg i.v. dose had any effect on withdrawal thresholds in the contralateral paw ($p = 0.87$ at 0.5 h after dose, one-way ANOVA, $n = 10$).

Intrathecal administration of 0.1 mg/kg ProTx-II was lethal in two rats, with death occurring approximately 5 min after the dose. Animals administered 0.01 mg/kg i.t. ProTx-II transiently displayed reduced muscle tone and impaired locomotor function. In the hyperalgesia study, 0.001 mg/kg i.t. and 0.01 mg/kg i.t. ProTx-II failed to show either antihyperalgesic effects in the ipsilateral paw or analgesic effects in the contralateral paw. Systemic levels at 4 h after the 0.01 mg/kg i.t. dose were lower than the limit of detection (approximately 3 nM).

Effects of ProTx-II on A- and C-Fiber Conduction. To examine the effect of ProTx-II on action potential firing in intact and desheathed sensory nerves, rat saphenous nerves were isolated and placed into a perfused organ bath (Fig. 4A). Using standard recording techniques, Aβ- and C-fiber compound action potentials were recorded. In a first set of experiments ($n = 3$), 20 μM ProTxII was applied to the intact (nondesheathed) saphenous nerve, and the amplitude of the C-fiber SNAP was monitored for 10 min. There was no significant reduction in the amplitude of the C-fiber SNAP (100 ± 1 and 105 ± 9% of control after 5 and 10 min, respectively). When the same concentration of ProTx-II was applied to the desheathed nerve, the C-fiber SNAP was blocked completely within 20 s, and the amplitude of the Aβ-fiber SNAP was reduced significantly, indicating that the connective tissue or epineurium represented a significant diffusion barrier. All subsequent experiments ($n = 9$) were therefore conducted on desheathed nerves. ProTx-II was applied in ascending concentrations from 0.1 to 30 μM for 5 min, with application of oxygenated SIF for an intervening 5 min. ProTx-II differentially affected the amplitude of Aβ- and C-fiber SNAPs (Fig. 4, B and C): C-fiber SNAPs were significantly reduced at 0.3 μM ProTx-II and completely blocked at 3 to 10 μM, whereas the first significant reduction of the Aβ-SNAP was observed at 1 μM ProTx-II, and even the highest concentration tested (30 μM) did not completely block action potential propagation in large myelinated fibers. IC_{50} values for inhibition of the C-fiber and Aβ-fiber SNAPs were 0.6 and 13.6 μM, respectively. The high concentrations of ProTx-II required to inhibit C-fiber SNAPS in this preparation, relative to the in vitro potency of ProTx-II for block of $hNa_V1.7$, was comparable with the >10 nM concentration of TTX required to inhibit A-fiber and C-fiber SNAPS in the same preparation (data not shown).

Iodination of ProTx-II. The N-terminal amino acid in ProTx-II is the only tyrosine residue found in the peptide, making it a promising candidate for radiolabeling by incorporation of ^{125}I. ProTx-II was reacted with ^{127}I-Na under the conditions described, and resulting products were separated by C_{18} reverse-phase chromatography. Two peaks, presumably corresponding to native and monoiodotyrosine ProTx-II, were identified by measuring the absorbance at 210 nm (Fig. 5A). Peak B was further separated by a second round of C_{18} reverse-phase chromatography (Fig. 5B). Electrospray mass spectroscopy identified the major component, labeled by the asterisk in Fig. 5B, as the monoiodotyrosine-ProTx-II (^{127}I-ProTx-II). The biological activity of ^{127}I-ProTx-II was comparable with that of ProTx-II in electrophysiological recordings of $hNa_V1.7$ channel activity (data not shown). As expected, reaction of ProTx-II with ^{125}I-Na yielded a chromatographic profile identical with that shown in Fig. 5.

Binding of ^{125}I-ProTx-II to $Na_V1.7$. Specific binding of ^{125}I-ProTx-II was observed in intact HEK-293 cells stably

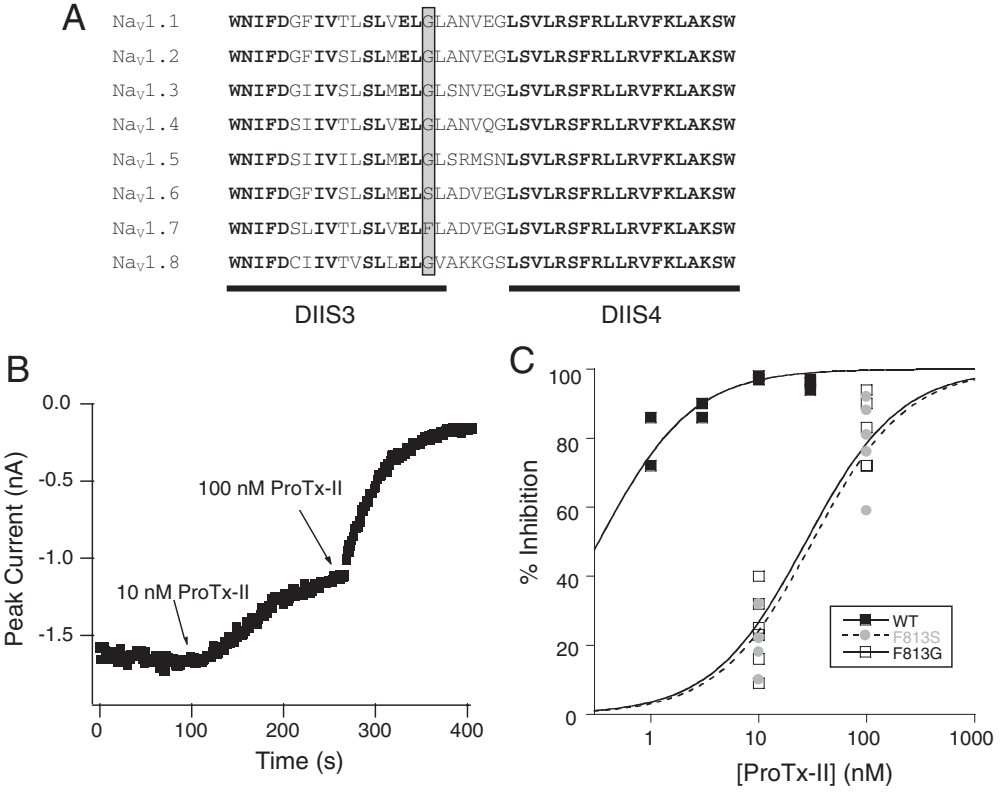

Fig. 2. Block of $hNa_V1.7$ mutants F813G and F813S by ProTx-II. A, sequence alignment for hNa_V1 subtypes of the domain II S3 to S4 segments. B, block of whole-cell sodium current by application of ProTx-II as indicated in cells transiently transfected with $hNa_V1.7$ F813G. C, inhibition of $hNa_V1.7$ F813G and $hNa_V1.7$ F813S plotted as a function of ProTx-II concentration, wild-type $hNa_V1.7$ is shown for comparison. Fitting the Hill equation to the data yielded IC_{50} values of 27 and 31 nM for $hNa_V1.7$ F813G and $hNa_V1.7$ F813S, respectively.

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expressing $hNa_V1.7/\beta1$ and was absent in cells not expressing this channel (data not shown). To characterize the binding affinity of ^{125}I-ProTx-II for $hNa_V1.7/\beta1$, poly(D-lysine)-coated 96-well plates were seeded with ~50,000 $hNa_V1.7/\beta1$ cells and incubated with increasing concentrations of ^{125}I-ProTx-II until equilibrium was achieved in the absence or presence of 200 nM ProTx-II (Fig. 6A). Nonspecific binding, defined as binding in the presence of 200 nM unlabeled ProTx-II, varied linearly with ^{125}I-ProTx-II concentration. In contrast, specific binding, calculated as the difference between total and nonspecific binding, was a saturable function of ^{125}I-ProTx-II concentration. Fitting to a single-site model yielded a K_d value of 295 ± 13 pM (n = 5). These data are in good agreement with the IC_{50} value estimated in electrophysiological recordings. The maximum density of cell surface channels, B_{max}, equaled 72,908 ± 7322 channels/cell (n = 5). In contrast, we observed little specific binding to HEK-293 cells stably expressing $hNa_V1.5$ channels at ligand concentrations up to 1 nM, despite a >2-fold larger current density in the $hNa_V1.5$ expressing cell line compared with the $hNa_V1.7$ cell line, in agreement with the lower potency for block of $hNa_V1.5$ by ProTx-II. ^{125}I-ProTx-II binding to $hNa_V1.7/\beta1$ was completely inhibited by unlabeled ProTx-II with a K_i value of 389 ± 45 pM and a Hill coefficient of 0.76 ± 0.06 (n = 13) (Fig. 6D), consistent with a single class of binding sites.

The association and dissociation kinetics of ^{125}I-ProTx-II are presented in Fig. 6, B and C, respectively. Incubation of stably expressed $hNa_V1.7/\beta1$ channels with 75 pM ^{125}I-ProTx-II resulted in a time-dependent association of the peptide with cell surface-expressed channels, which, at 37°C, reached equilibrium in approximately 5 h. Semilogarithmic transformation of these data, shown in the inset, yielded a linear relationship, indicating a pseudo-first-order reaction (Fig. 6B). The slope of this linear relationship yielded a k_{obs} of 1.27 × 10^{-4}/s, corresponding to an association rate constant, k_1, of 5.3 × 10^5/M/s. Average k_1

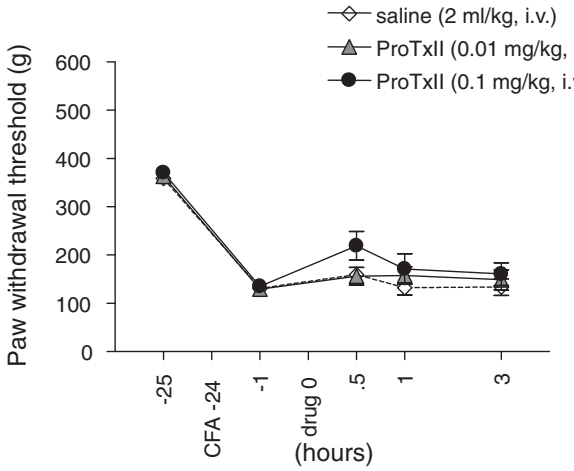

Fig. 3. Effect of ProTx-II in a rat model of inflammatory pain. One day after a subcutaneous injection of CFA, mechanical allodynia was assessed with calibrated von Frey filaments before and after intravenous administration of saline or ProTx-II. Shown are the mean ± S.E.M. of paw withdrawal thresholds for the experimental groups indicated (n = 10).

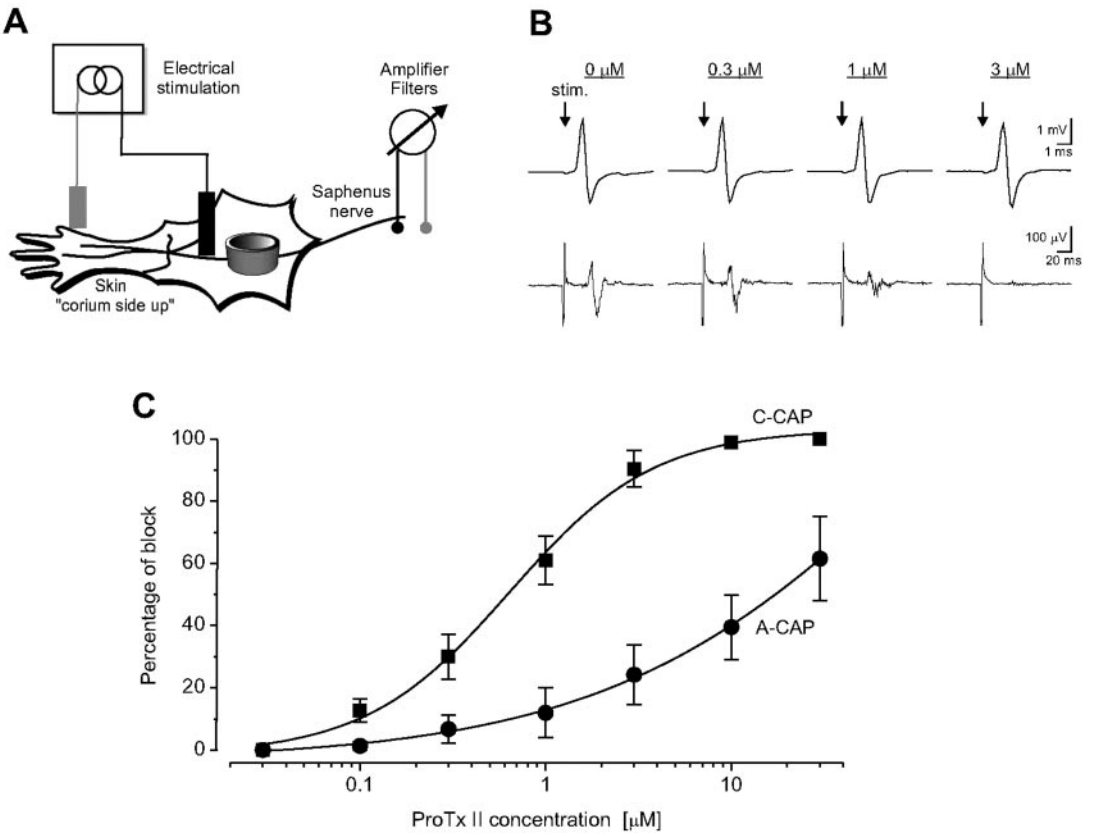

Fig. 4. Block of compound action potentials in an isolated nerve preparation. A, schematic representation of the experimental set-up. B, representative traces of the Aβ-fiber (top) and C-fiber (bottom) compound action potential in response to increasing concentrations of ProTx-II. Arrows indicate the onset of electrical stimulus. C, block of saphenous nerve Aβ-fiber (●) and C-fiber (■) compound action potentials as a function of ProTx-II concentration. Data are shown as mean ± S.E.M. (n = 9).

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values were $5.3 \times 10^5 \pm 0.3 \times 10^5$/M/s ($n = 3$). Dissociation of ^{125}I-ProTx-II followed a single, monoexponential decay, and the dissociation rate constant, k_{-1}, was independent of whether dissociation was initiated by the addition of an excess of unlabeled ProTx-II, $k_{-1} = 7.1 \times 10^{-5} \pm 0.2 \times 10^{-5}$/s ($n = 3$) or by dilution of ^{125}I-ProTx-II, $k_{-1} = 8.1 \times 10^{-5} \pm 0.9 \times 10^{-5}$/s ($n = 3$), (Fig. 6C). The K_d value calculated from the association and dissociation rate constants was 143 pM, a value slightly lower than the K_d determined under equilibrium binding conditions.

To gain insight into the features of the ProTx-II binding site on $Na_V1.7$ channels, we examined the effects of several known sodium channel blockers on binding of ^{125}I-ProTx-II (Cestele and Catterall, 2000). Tetrodotoxin (100 nM, site 1), veratridine (100 μM, site 2), the α-scorpion toxins LqhαIT and Ltx-004 and the sea anemone toxin Anthopleurin B (100 nM, site 3), the β-scorpion toxins Css II and Css IV (100 nM, site 4), brevetoxin (100 nM, site 5), deltamethrin (10 μM, site 7), and tetracaine and mexiletine (100 μM, site 9) all had no effect on ^{125}I-ProTx-II binding. ProTx-I, another peptide found in *T. prurien* venom, displaced ^{125}I-ProTx-II binding with a K_i value of 680 ± 100 pM ($n = 3$), similar to that of unlabeled ProTx-II. Like ProTx-II, ProTx-I conforms to the inhibitory cystine knot motif but, except for the spacing of the six cysteine residues, shares little sequence homology with ProTx-II. Guangxitoxin-1 (GxTX-1), another gating modifier peptide that contains the inhibitory cystine knot motif (Herrington, 2007), is a selective inhibitor of Kv2.1 channels. Consistent with its selectivity, GxTX-1 had no effect on ^{125}I-ProTx-II binding at concentrations of up to 300 nM.

Discussion

We have shown that ProTx-II inhibits $Na_V1.7$ with an IC_{50} value of 0.3 nM and is at least 100-fold selective for $Na_V1.7$ over other sodium channel subtypes. This subtype-selectivity was abolished by a point mutation in the S3b segment of domain II. In desheathed sensory nerves, ProTx-II completely inhibited evoked C-fiber compound action potential at concentrations that had little effect on Aβ-fiber conduction.

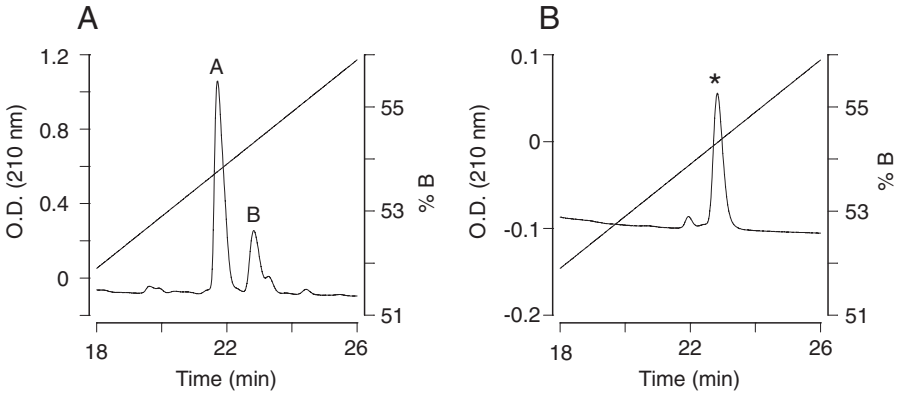

Fig. 5. Iodination of ProTx-II. A, HPLC separation of iodinated derivatives. ProTx-II was iodinated as described under *Materials and Methods*. The reaction mixture was loaded onto an HPLC C_{18} reverse-phase column equilibrated with 10 mM TFA, and elution was achieved with a linear gradient of 50% acetonitrile in 5 mM TFA (0–50% for 2 min, 50–70% for 40 min) at a flow rate of 0.5 ml/min. Material eluting from the column was monitored by measuring absorbance at 210 nm. B, Peak B (A) was further separated by a second round of C_{18} reverse-phase chromatography under identical conditions.

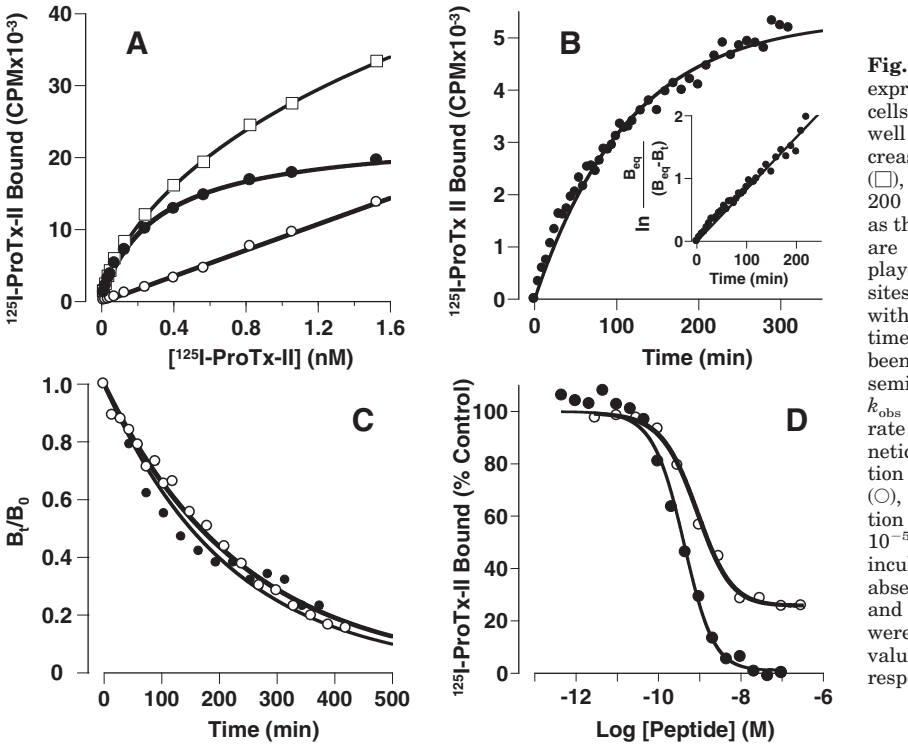

Fig. 6. Binding of ^{125}I-ProTx-II to HEK-293 cells stably expressing $hNa_V1.7/\beta1$. A, saturation studies. HEK-293 cells stably expressing $hNa_V1.7/\beta1$ were seeded in 96-well poly(D-lysine)-coated plates and incubated with increasing concentrations of ^{125}I-ProTx-II. Total binding (□), nonspecific binding determined in the presence of 200 nM ProTx-II (○), and specific binding (●), defined as the difference between total and nonspecific binding, are presented. Specific binding of ^{125}I-ProTx-II displayed a K_d of 309 pM and a B_{max} of 96,300 binding sites/cell. B, association kinetics. Cells were incubated with 75 pM ^{125}I-ProTx-II for the indicated amounts of time at 37°C. Nonspecific binding was constant and has been subtracted from the experimental points. Inset, a semilogarithmic representation of the data, yielding a k_{obs} of 1.27×10^{-4}/s, corresponding to an association rate constant, k_1, of 5.3×10^5/M/s. C, dissociation kinetics. Dissociation of ^{125}I-ProTx-II, initiated by addition of an excess of unlabeled ProTx-II (●) or by dilution (○), followed monoexponential kinetics with dissociation rate constants, k_{-1}, of 7.1×10^{-5}/s and 7.5×10^{-5}/s, respectively. D, competition binding. Cells were incubated with 60 pM ^{125}I-ProTx-II in the presence or absence of increasing concentrations of ProTx-I (○) and ProTx-II (●) for 4 h at 37°C. Specific binding data were fit to a single-site inhibition model, yielding IC_{50} values of 800 and 350 pM for ProTx-I and ProTx-II, respectively.

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Radiolabeled ProTx-II was shown to bind with high affinity to cells expressing $hNa_V1.7$ channels and may serve as a tool to find $Na_V1.7$ selective small-molecule blockers that may be considered for clinical development.

Recently, compelling human genetic evidence has identified $Na_V1.7$ as an essential contributor to pain signaling (Dib-Hajj et al., 2007). Human loss-of-function mutations cause CIP, whereas gain-of-function mutations underlie two inherited painful neuropathies: erythromelalgia (Yang et al., 2004; Han et al., 2006) and paroxysmal extreme pain disorder (Fertleman et al., 2006). The absence, in patients with CIP, of cognitive, sensory, or motor deficits, not related to the inability to sense pain, make $Na_V1.7$ an attractive target for the development of novel analgesics. However, pharmacological intervention with $Na_V1.7$ blockers may not reproduce the human loss-of-function phenotype, if either the lack of pain or the absence of a sympathetic defect in patients with CIP is attributable to developmental changes. Therefore, proof-of-concept experiments in experimental pain models require the development of $Na_V1.7$-selective channel modulators.

ProTx-II, a 30 amino acid peptide found in tarantula venom, is a potent inhibitor of $Na_V1.7$ channels, and we show here that ProTx-II is at least 100-fold selective for $Na_V1.7$ over other Na_V1 subtypes. ProTx-II inhibits sodium channels by decreasing channel conductance and shifting activation to more positive potentials. Based on the biophysical properties of $Na_V1.7$, this mechanism of action may be ideally suited to confer analgesic properties. Relative to other Na_V1 subtypes, $Na_V1.7$ transitions slowly from the resting to the inactivated state and conducts significant current in response to ramp depolarizations, properties that suggest that $Na_V1.7$ may modulate firing thresholds by amplifying generator potentials (Cummins et al., 1998). If $Na_V1.7$ indeed acts predominantly as a threshold channel in nociceptors, shifting the voltage dependence of activation to more positive potentials may decrease neuronal excitability and pain sensitivity while preserving the ability to sense acute painful stimuli. Intravenous or intrathecal administration of ProTx-II at tolerated doses in rats was not associated with significant inhibition of short-term or inflammatory pain responses. A likely explanation for this lack of efficacy is an inability of ProTx-II to cross the blood-nerve barrier. This hypothesis was supported by the observation that ProTx-II inhibited C-fiber action potential propagation in desheathed but not in intact nerve preparations. Although its biophysical properties may limit the usefulness of ProTx-II for in vivo proof-of-concept experiments, the peptide provides an important tool that can be used to find novel selective inhibitors of $Na_V1.7$.

Furthermore, ProTx-II can be used to investigate the contribution of different sodium channel subtypes to excitability control of sensory neurons. It has been suggested that action potential generation in small-diameter (C-fiber) nociceptors is initiated by TTX-sensitive conductances (presumably mediated by $Na_V1.7$), whereas the majority of the inward current during the action potential upstroke is mediated by $Na_V1.8$ (Renganathan et al., 2001; Blair and Bean, 2002). The results presented here show that $Na_V1.7$ is required for action potential propagation in rat nociceptors. A potential explanation for this finding is that amplification of subthreshold depolarizations by activation of $Na_V1.7$ channels may be required to reach the more depolarized threshold of activation of $Na_V1.8$ channels. This hypothesis is supported by the discovery that a gain-of-function mutation in $Na_V1.7$ depolarizes the resting membrane potential and causes hyperexcitability in sensory neurons expressing $Na_V1.8$ channels but renders sympathetic ganglion neurons, devoid of $Na_V1.8$ expression, hypoexcitable (Rush et al., 2006). At high concentrations, ProTx-II resulted in a block of action potential propagation in many Aβ-fibers. One possibility is that most Aβ-fibers express $Na_V1.7$, albeit at lower levels. However, if this were the case, the stimulus-response function should not be right-shifted relative to the C-fiber stimulus response. A more likely explanation therefore is that, at higher concentrations, blockade of $Na_V1.6$ produces failure of action potential propagation in Aβ-fibers.

ProTx-II and a related tarantula venom peptide, ProTx-I, inhibit sodium channels by a mechanism similar to that of the potassium channel gating modifiers hanatoxin and SGTx1 (Swartz and MacKinnon, 1997; Lee et al., 2004). Indeed, ProTx-I shares significant sequence homology with hanatoxin. ProTx-II also contains six disulfide bonded cysteine residues and conforms to the inhibitory cystine knot motif seen in other gating modifiers but otherwise shares no sequence homology with ProTx-I or hanatoxin. Extensive mutagenesis studies have shown that residues in S3b and in the N-terminal portion of S4 of $K_V2.1$ play crucial roles in channel modulation by hanatoxin (Li-Smerin and Swartz, 2000). Similar to hanatoxin (Takahashi et al., 2000), the structure of ProTx-II is amphipathic, with mostly hydrophobic residues on one face of the toxin. Alanine-scanning mutagenesis has shown that toxin residues involved in channel interactions are concentrated on the face of the toxin containing mostly hydrophobic and a few positively charged residues (Smith et al., 2007). Membrane partitioning is believed to be involved in the mechanism of potassium channel inhibition by gating modifiers such as hanatoxin (Milescu et al., 2007). In sodium channels, S3, S4, and the short linker segment are highly conserved between subtypes. $Na_V1.7$ is unique in that residue 813 in the C-terminal portion of domain II S3 is a phenylalanine, whereas all other Na_V1 subtypes carry a glycine residue in this position except for $Na_V1.6$, which has a serine residue. Phe813 seems to be crucial for conferring high sensitivity to ProTx-II, because mutation of Phe813 to glycine or serine in $Na_V1.7$ resulted in mutant channels with ProTx-II sensitivity reduced by ~100-fold and in the range of sensitivities of other Na_V1 subtypes. Mutating the equivalent glycine residue in $Na_V1.2$ (Gly839) to phenylalanine did not significantly increase sensitivity to ProTx-II, suggesting that phenylalanine in this position is necessary but not sufficient to confer high affinity for ProTx-II. It is interesting that Phe813 in $Na_V1.7$ is located six amino acids from a glycine residue (Gly845 in $Na_V1.2$) that is essential for high-affinity interaction of the β-scorpion toxin Css IV with $Na_V1.2$ (Cestele et al., 1998). Similar to ProTx-II, β-scorpion toxins act by reducing channel conductance and interacting with the voltage sensor; however, in contrast to ProTx-II, β-scorpion toxins enhance channel activation by causing a hyperpolarizing shift in the voltage dependence of activation (Cestele et al., 1998). It is believed that β-scorpion toxins trap the voltage sensor in its activated conformation, leading to the speculation that ProTx-II may trap the voltage sensor in the resting conformation or prevent voltage sensor movement into the activated conformation. It is interesting that Css IV and the related peptide Css II did not displace ^{125}I-ProTx-II binding to recombinant $hNa_V1.7/\beta1$, suggesting that they may bind to nonoverlapping sites within the

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voltage sensor. On the other hand β-scorpion toxin may have low affinity for $Na_V1.7$ channels.

Taken together, our results suggest that ProTx-II interacts with a previously unidentified site on $Na_V1.7$ and that binding to this site may be more conducive to conferring subtype selectivity than the site occupied by traditional local anesthetics and anticonvulsants. Given the crucial role of $Na_V1.7$ in human pain perception, this makes ProTx-II an attractive tool in the search for novel analgesics.

Acknowledgments

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