Modulation of neuronal sodium channels by the sea anemone peptide BDS-I

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Liu P, Jo S, Bean BP. Modulation of neuronal sodium channels by the sea anemone peptide BDS-I. J Neurophysiol 107: 3155–3167, 2012. First published March 21, 2012; doi:10.1152/jn.00785.2011.—Blood-depressing substance I (BDS-I), a 43 amino-acid peptide from sea anemone venom, is used as a specific inhibitor of Kv3-family potassium channels. We found that BDS-I acts with even higher potency to modulate specific types of voltage-dependent sodium channels. In rat dorsal root ganglion (DRG) neurons, 3 μM BDS-I strongly enhanced tetrodotoxin (TTX)-sensitive sodium current but weakly inhibited TTX-resistant sodium current. In rat superior cervical ganglion (SCG) neurons, which express only TTX-sensitive sodium current, BDS-I enhanced current elicited by small depolarizations and slowed decay of currents at all voltages (EC50 ~ 300 nM). BDS-I acted with exceptionally high potency and efficacy on cloned human Nav1.7 channels, slowing inactivation by 6-fold, with an EC50 of approximately 3 nM. BDS-I also slowed inactivation of sodium currents in N1E-115 neuroblastoma cells (mainly from Nav1.3 channels), with an EC50 ~ 600 nM. In hippocampal CA3 pyramidal neurons (mouse) and cerebellar Purkinje neurons (mouse and rat), BDS-I had only small effects on current decay (slowing inactivation by 20–50%), suggesting relatively weak sensitivity of Nav1.1 and Nav1.6 channels. The biggest effect of BDS-I in central neurons was to enhance resurgent current in Purkinje neurons, an effect reflected in enhancement of sodium current during the repolarization phase of Purkinje neuron action potentials. Overall, these results show that BDS-I acts to modulate sodium channel gating in a manner similar to previously known neurotoxin receptor site 3 anemone toxins but with different isoform sensitivity. Most notably, BDS-I acts with very high potency on human Nav1.7 channels.

amino toxin; site 3 toxin; sodium channel modulator; inactivation curve; activation curve; resurgent sodium current

SEA ANEMONE VENOM IS A RICH source of toxic peptides and proteins acting on ion channels (reviewed by Honma and Shiomi 2006). A large group of these peptide toxins bind to the neurotoxin receptor site 3 of voltage-gated sodium channels and slow the time course of inactivation (reviewed by Bosmans and Tytgat 2007; Catterall et al. 2007; Moran et al. 2009; Shiomi 2009; Smith and Blumenthal 2007). More than 50 site 3 sodium channel toxins have been identified and classified (Honma and Shiomi 2006; Norton 2009; Smith and Blumenthal 2007; Catterall et al. 2007; Moran et al. 2009; Sacco et al. 2008). Among these are BDS-I and BDS-II from Anemonia sulcata (Diochot et al. 1998), which are closely homologous. BDS-II was initially suggested to be an antagonist for receptor site 3 of voltage-gated sodium channels, because it displaced a radiolabeled site 3 agonist from binding sites in rat brain synaptosomes but did not produce the positive inotropic effect on cardiac contraction typical of site 3 agonists (Llewellyn and Norton 1991). However, subsequent studies showed that BDS-I toxin had little functional effect on sodium channels in mouse neuroblastoma cells, but appeared to be a specific blocker of the fast inactivating Kv3.4-subfamily (Diochot et al. 1998). Based on this, effects of BDS-I were initially considered to identify involvement of Kv3.4 subunits (e.g., Pannaccione et al. 2007; Shevchenko et al. 2004). More recently, however, it was shown that BDS-I toxin blocks not only Kv3.4-containing channels but also other Kv3-family subunits, including both Kv3.1 and Kv3.2, with similar effectiveness (Yeung et al. 2005). Subsequently, BDS-I has been regarded more broadly as an inhibitor of Kv3-family channels (e.g., Dhawan et al. 2010; Martina et al. 2007; Sacco et al. 2006).

We began studies using BDS-I to explore the contribution of Kv3 channels to action potential repolarization in rat small DRG neurons. We unexpectedly found that BDS-I not only produced broadening of the spike, but also accelerated the upstroke of the action potentials. We then found that BDS-I modifies gating and enhances current carried by neuronal voltage-gated tetrodotoxin (TTX)-sensitive sodium channels, with especially potent interaction with Nav1.7 channels.

MATERIALS AND METHODS

Preparation of DRG and superior cervical ganglion (SCG) neurons. Dissociated DRG or SCG neurons were prepared using enzymatic treatment as previously described (Blair and Bean 2002). Briefly, DRG or SCG neurons were removed from Sprague–Dawley rats [postnatal days (P)15 to P29], cut in half, and treated for 20 min at 37°C with 20 μM papain (Worthington Biochemical, Lakewood, NJ) and 5 mM di-cysteine in a calcium- and magnesium-free (CMF) Hank’s buffer containing 137 mM NaCl, 5.36 mM KCl, 0.33 mM Na3HPO4, 0.44 mM KH2PO4, 5 mM HEPES, 5.55 mM glucose, 0.001% phenol red (pH 7.40, adjusted with NaOH); 300–310 mOsm. Ganglia were then treated for 20 min at 37°C with 3 mg/mL collagenase (type I; Roche Diagnostics, Indianapolis, IN) and 4 mg/mL dispase II (Roche Diagnostics) in CMF Hank’s buffer. Cells were dispersed by trituration with a fire-polished glass Pasteur pipette in a solution composed of two media combined in a 1:1 ratio: Leibovitz’s L-15 medium (Invitrogen, Grand Island, NY) supplemented with 5 mM HEPES, and Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen); this solution also contained 100 ng/mL nerve growth factor (NGF; Invitrogen). Cells were then plated on glass coverslips treated with 40 μg/mL poly-D-lysine and then 20 μg/mL laminin (Invitrogen). Then cells were incubated at 37°C (5% CO2).
3 h, after which Neurobasal medium (Invitrogen) containing B-27 supplement (Invitrogen), penicillin, and streptomycin (Sigma, St. Louis, MO), and 100 ng/mL NGF was added to the petri dish. Cells were stored at 4°C and used within 48 h. Small DRG neurons with 15- to 22-μm diameter were chosen and tested for sensitivity to capsaicin in voltage clamp. Only neurons responding to 1 μM capsaicin with activation of current (tested with a voltage-ramp protocol from +100 mV to −110 mV in 50 ms) were included in the analysis.

Human Nav1.7 cell line. Experiments on human Nav1.7 channels were done by generating a cell line with stable expression of human Nav1.7 channels (Klugbauer et al. 1995; GenBank Accession Number X82835.1) in HEK293 cells. HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in modified Eagle’s medium (ATCC) containing 10% fetal bovine serum (FBS, Sigma), penicillin, and streptomycin (Sigma) at 37°C (5% CO2). The Nav1.7 plasmid was kindly provided by Dr. Norbert Klugbauer (Albert-Ludwigs-Universität Freiburg, Freiburg, Germany) via Dr. Geng Kuo Wang (Brigham and Women’s Hospital, Boston, MA). To generate a Nav1.7-stable cell line, the Nav1.7 plasmid was transfected using Lipofectamine LTX with PLUS reagent (Invitrogen) into HEK293 cells according to the manufacturer’s protocol. Cells were selected with G418 (Invitrogen) for 4–6 wk.

Mouse N1E-115 neuroblastoma cells. Mouse N1E-115 neuroblastoma cells were purchased from ATCC. Cells were grown in DMEM (ATCC) containing 10% fetal bovine serum (Sigma), penicillin, and streptomycin (Sigma) at 37°C (5% CO2). For electrophysiological recording, cells were grown on coverslips for 12 to 24 h after plating.

Preparation of cerebellar Purkinje neurons and hippocampal CA3 pyramidal neurons. Cerebellar Purkinje neurons were acutely dissociated from Sprague–Dawley rats or Black Swiss mice (P11–P15) as previously described (Carter and Bean 2009; Raman and Bean 2001). Animals were anesthetized with isoflurane and the cerebellum was quickly removed into ice-cold solution consisting of 110 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 25 mM glucose, 75 mM MgCl2, pH adjusted to 7.4 with NaOH. For isolation of Purkinje neurons, the cerebellum was cut into (~1-mm²) chunks and was treated for 10–20 min at room temperature with 3 mg/mL protease XXIII (Sigma Life Science) dissolved in a dissociation solution consisting of 82 mM Na2SO4, 30 mM K2SO4, 5 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. The protease solution was then replaced by ice-cold dissociation solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL bovine serum albumin and the chunks were kept on ice in this solution until immediately before use. To release individual cells, the tissue was passed through Pasteur pipettes with fire-polished tips. A drop of the suspension was placed in the recording chamber and diluted with a large volume of Tyrode’s solution, consisting of 155 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH adjusted to 7.4 with approximately 5 mM NaOH. Purkinje neurons could be recognized by their large size and a single large dendritic stump.

CA3 pyramidal neurons were isolated using the same enzymatic treatment. The whole brain was removed into ice-cold solution and hippocampal slices were cut on a tissue chopper. The CA3 regions were dissected out, incubated in enzyme, washed with trypsin inhibitor, and dissociated by trituration. Pyramidal neurons were chosen for recording based on their shape.

Electrophysiology. Recordings were performed at room temperature (22–23°C) using an Axon Instruments Multiclamp 700B Amplifier (Molecular Devices). Patch pipettes were pulled on a Sutter P-97 puller (Sutter Instrument) and shanks were wrapped with Parafilm to reduce the pipette capacitance and thereby allow optimal series resistance compensation without oscillation. The resistances of the pipettes were 1–3 MΩ. Seals were formed in Tyrode’s solution containing 151 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 13 mM glucose, 10 mM HEPES, pH 7.4 adjusted with NaOH. Cell capacitance was nulled and series resistance was partially compensated (75–85%) before the recording. The cell was then lifted up and placed in front of an array of quartz-fiber flow pipes and solutions were changed (in ~1 s) by moving the cell from one pipe to another. Voltage or current commands were delivered and signals were recorded using a Digidata 1321A analog-to-digital board (Axon Instruments) controlled by pCLAMP 9.2 software (Molecular Devices). Current and voltage records were filtered at 5 kHz and digitized at 100 kHz. Voltage-clamp current records were corrected for linear capacitive and leak current by subtracting scaled responses to 5-mV hyperpolarizations delivered from the holding potential. In current-clamp experiments, action potentials were evoked with 0.5-ms current injections ranging from 1 to 3 nA, leaving most of the action potential unaffected by the injected current. In action potential clamp experiments in DRG neurons (Blair and Bean 2002; de Haas and Vogel 1989), each cell’s own action potential was recorded and used as the command waveform in voltage clamp. In action potential clamp experiments in rat Purkinje neurons, a prerecorded action potential from a different rat Purkinje neuron (kindly furnished by Brett Carter) was used; this action potential was recorded at room temperature during spontaneous firing from a dissociated rat Purkinje neuron using solutions as in Carter and Bean (2009). Analyses were performed in Igor Pro4.0 (WaveMetrics, Lake Oswego, OR). Data are given as mean ± SD and statistical significance was assessed using Student’s paired t-test or ANOVA. To quantify the slowing of inactivation, we measured the time to half-decay because in most cases the decay of sodium current inactivation was not fit very well by a single exponential.

Solutions. To study the effect of BDS-I on action potentials and on total membrane currents in DRG neurons, recording solutions had quasi-physiological ionic composition, with an internal solution consisting of 140 mM K-aspartate, 13.5 mM NaCl, 1.8 mM MgCl2, 0.09 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 adjusted with KOH) and an external Tyrode’s solution consisting of 150 mM NaCl, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 10 mM glucose (pH 7.40 adjusted with NaOH). To study the effect of BDS-I on voltage-activated sodium current in DRG neurons, SCG neurons, human Nav1.7 cells, and N1E-115 cells, recordings were made using an internal solution containing 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl2, 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH), and an external Tyrode’s solution with tetraethylammonium (TEA) replacing potassium (150 mM NaCl, 3.5 mM TEACl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). In experiments on transient sodium current in CA3 pyramidal neurons and cerebellar Purkinje neurons, the same CsCl-based internal solution was used but a different external solution was necessary to reduce the very large sodium currents to sizes that could be well controlled; this solution contained 25 mM NaCl, 128.5 mM TEA-Cl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose (pH 7.40). To study the effect of BDS-I on resurgent current in CA3 pyramidal neurons and cerebellar Purkinje neurons, recordings were made using the same CsCl-based internal solution and an external solution with full sodium and with TEA replacing potassium (150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). Experiments recording sodium current evoked by action potential waveforms in Purkinje neurons were done using the same CsCl-based internal solution and an external solution with full sodium, TEA replacing potassium, and cobalt replacing calcium (150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CoCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4 with NaOH).

BDS-I (Alomone Labs, Jerusalem, Israel) was made as 100 μM stock solutions in Tyrode’s solution and aliquots were stored at −20°C and diluted for use on the day of the experiment; 1 mg/mL BSA (Sigma) was added to all solutions to minimize binding of the BDS-I peptide to tubing and chamber. TTX (Sankyo, Tokyo, Japan)
RESULTS

**BDS-I enhances TTX-sensitive sodium currents in rat small DRG neurons.** BDS-I, a 43 amino-acid peptide from sea anemone venom, has been regarded and used as a specific inhibitor of Kv3-family channels (Diochot et al. 1998; Martina et al. 2007; Sacco et al. 2006; Yeung et al. 2005). To explore the contribution of Kv3 channels to action potential repolarization in rat small DRG neurons, we used BDS-I to inhibit potassium currents through Kv3 channels. At 3 μM, BDS-I produced broadening of action potentials (increase to 118 ± 20% of control, \(P = 0.026, n = 10\)), suggestive of Kv3 family channels contributing to spike repolarization (Fig. 1A). Surprisingly, however, BDS-I also increased the peak of the action potential (from 31 ± 8 to 39 ± 4 mV, \(P = 0.001, n = 10\)) as well as accelerating the upstroke (from 57 ± 19 to 65 ± 22 V/s, \(P = 0.006, n = 10\)) (Fig. 1A). The effect on upstroke is not expected from block of Kv3 channels, which require relatively large depolarizations to be activated (Rudy and McBain 2001) and would be unlikely to activate significantly during the early rising phase of the action potential.

To better understand the effects of BDS-I on ionic currents during the action potential, we did voltage-clamp experiments in which each cell’s own action potential was recorded and used as the command waveform (Blair and Bean 2002). We found that 3 μM BDS-I dramatically enhanced the inward current during the rising phase of the action potential (increase to 180 ± 69% of control, \(P = 0.037, n = 8\)) as well as reducing the net outward current during the falling phase (Fig. 1B). We then studied the effect of BDS-I on voltage-activated currents using step depolarizations. Cells were held at −80 mV, and voltage-activated currents were evoked by 200-ms pulses to voltages from −60 to +40 mV in 10-mV increments; 3 μM BDS-I reduced voltage-activated outward currents (Fig. 1C), as might be expected from inhibition of Kv3 channels, but the effect on outward current was relatively small (for a step to 0 mV, decrease to 88 ± 13% of control, \(P = 0.005, n = 8\)). Much more strikingly, BDS-I also enhanced the initial inward current (Fig. 1C), mainly carried by the influx of sodium ions (Blair and Bean 2002). This effect was especially large for currents evoked by moderate depolarizations; inward current evoked by a step to −30 mV was increased to 447 ± 379% of control (\(P = 0.004, n = 8\)) and inward current evoked by a step to 0 mV was increased to 171 ± 58% of control (\(P = 0.008\),

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**Fig. 1.** Effects of BDS-I on action potentials and ionic currents in rat small DRG neurons. A: effect of 3 μM BDS-I on the action potential in a small DRG neuron triggered by a short (0.5 ms) current injection. B: effect of 3 μM BDS-I on ionic currents evoked by the action potential waveform in voltage-clamp mode. The cell’s own action potential was recorded and used as the command waveform. Capacitive current was nulled using the capacity compensation circuitry of the amplifier. C: effect of 3 μM BDS-I on ionic currents evoked by step depolarizations to voltages from −30 to +20 mV from a holding potential of −80 mV. Internal solution: 140 mM K aspartate, 13.5 mM NaCl, 1.8 mM MgCl₂, 0.09 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 adjusted with KOH). External solution: 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose (pH 7.40 adjusted with NaOH).
These effects, much more dramatic than the modest reduction of outward current, suggest that BDS-I may affect voltage-activated sodium channels.

To examine possible effects of BDS-I on voltage-activated sodium currents in the absence of potassium currents, we next did voltage-clamp experiments using a Cs-based intracellular solution (and with replacement of extracellular potassium by TEA) to block potassium currents (Fig. 2). We found that 3 µM BDS-I enhanced voltage-activated sodium currents at voltages between −45 and +40 mV, with particularly dramatic effects for small depolarizations to voltages between −40 and −10 mV (at −30 mV, increase to 436 ± 248% of control, P = 0.0001, n = 9; at 0 mV, increase to 131 ± 31% of control, P = 0.009, n = 9). As has been intensively studied, three different components of total sodium current in small DRG neurons can be distinguished based on voltage dependence, kinetics, and pharmacology: rapidly gating TTX-sensitive current, slowly gating TTX-resistant current, and persistent TTX-resistant current (Cummins et al. 1999; Elliott and Elliott 1993; Roy and Narahashi 1992; Rush et al. 2007). Small DRG neurons express multiple sodium channel isoforms, including TTX-sensitive (Nav1.1, Nav1.2, Nav1.6, and Nav1.7) and TTX-resistant (Nav1.8 and Nav1.9) sodium channels, with predominant expression of Nav1.7, Nav1.8, and Nav1.9 channels (Ho and O’Leary 2011). To explore which type of sodium currents BDS-I enhances, we tested the TTX-sensitivity of the current that is affected by BDS-I (Fig. 2C). We found that 3 µM BDS-I enhanced the TTX-sensitive sodium current (increase to 311 ± 159% of control for current evoked at 0 mV, P = 0.004, n = 7), while weakly inhibiting the TTX-resistant sodium current (decrease to 81 ± 11.9% of control for current evoked at 0 mV, P = 0.01, n = 7). As expected, the TTX-sensitive sodium current showed rapid activation and inactivation compared with the TTX-resistant current.

BDS-I slows sodium current inactivation through Nav1.7 channels. To examine the effects of BDS-I on TTX-sensitive sodium channels in more detail, we next tested the effect of BDS-I on sodium currents in various cell types that express only TTX-sensitive sodium channels of various types. We first examined the effects of BDS-I in SCG neurons, which express only TTX-sensitive sodium current, primarily carried by Nav1.7 channels (Djouhri et al. 2003; Toledo-Aral et al. 1997).

**Fig. 2. BDS-I enhancement of TTX-sensitive sodium currents in rat small DRG neurons.**

**A:** effect of 3 µM BDS-I on voltage-activated inward currents using solutions in which potassium currents were blocked. **B:** effect of 3 µM BDS-I on peak inward current as a function of voltage. **C:** effect of 3 µM BDS-I on TTX-sensitive and TTX-resistant sodium current. Voltage-activated current for a step from −70 mV to 0 mV was recorded successively in four solutions: control, 1 µM TTX, 3 µM BDS-I with 1 µM TTX, and 3 µM BDS-I alone after washing out TTX. TTX-sensitive currents in control and in the presence of 3 µM BDS-I were acquired by subtracting sodium currents in TTX from those in the respective solutions without TTX. Internal solution: 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl₂, 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH). External solution: 150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose (pH 7.40 with NaOH).

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BDS-I produced a dramatic enhancement of sodium currents elicited by small depolarizations, with an increase to 1.045 ± 113% of control at −30 mV (P = 0.037, n = 7) (Fig. 3, A and B). However, BDS-I had little effect on peak sodium current for steps to 0 mV or higher (at 0 mV, decrease to 90 ± 9% of control, P = 0.19, n = 7) (Fig. 3B). Although only enhancing peak current evoked by small depolarizations, BDS-I dramatically slowed the decay of currents evoked by large as well as small depolarizations (Fig. 3, A and C). We quantified the slowing of sodium current inactivation by measuring the time required for half-decay of the sodium current at 0 mV. The half-decay time was 0.39 ± 0.06 ms in control and increased >4-fold in 3 μM BDS-I, to 1.55 ± 0.10 ms (P = 0.004, n = 7). The effect of BDS-I on inactivation was dose dependent (Fig. 3C), with a half-maximal effective concentration of approximately 300 nM (Fig. 3D).

These results suggest potent effects of BDS-I on rat Nav1.7 sodium channels, the dominant isoform in SCG neurons. We next examined the effect of BDS-I on human Nav1.7 channels using a cell line with stable expression of human Nav1.7 alpha subunits. The effects of BDS-I were even more potent on these channels. Concentrations of BDS-I as low as 0.3 nM significantly slowed the inactivation of currents carried by human Nav1.7 channels (Fig. 4, A–D). The time required for half-decay increased from 0.32 ± 0.07 ms in control to 1.21 ± 0.20 ms in 10 nM BDS-I (P = 0.00005, n = 12) and to 1.70 ± 0.32 ms in 3 μM BDS-I (P = 0.001, n = 12). The half-maximal effective concentration for slowing of inactivation in human Nav1.7 channels was 3 nM (Fig. 4D). Despite the dramatic slowing of inactivation, currents for a test step to 0 mV still decayed completely to zero (within 30 ms), even in the largest concentrations of BDS-I (although this is not obvious on the expanded time base used in Fig. 4, A–C to illustrate the changes in decay kinetics).

These results show potent effects of BDS-I on the gating of Nav1.7 channels. To explore the effects on gating in more detail, we examined the effect on the voltage dependence of activation and inactivation of various concentrations of BDS-I (Fig. 4E). BDS-I altered the voltage dependence of activation, shifting the midpoint in the depolarizing direction from −30 ± 7 mV in control to −22 ± 3 mV in 3 μM BDS-I (P = 0.016, n = 12) and making the activation curve much less steep, from a slope factor of 2.6 ± 1.6 mV in control to 8.5 ± 1.4 mV in 3 μM BDS-I (P = 0.0005, n = 12). Accompanying the shallower slope, there was an enhancement of relative activation for small depolarizations (between −60 and −30 mV) despite the shift of the midpoint in the depolarizing direction. BDS-I also altered the voltage dependence of inactivation in a dose-dependent manner, shifting the midpoint of the inactivation curve in the depolarizing direction from −67 ± 2 mV in control to −61 ± 2 mV in 3 μM BDS-I (P = 0.009, n = 12). In contrast to the shallower steepness of the activation curve, the voltage dependence of steady-state inactivation was equally steep in BDS-I as that in control (slope factor of

**Rat SCG neurons**

![Rat SCG neurons](image)

**Fig. 3.** Effect of BDS-I on sodium currents in rat SCG neurons. **A:** effect of 3 μM BDS-I on voltage-activated sodium currents evoked by steps from −80 mV to −30, −20, and −10 mV in a rat SCG neuron. **B:** peak sodium current as a function of voltage in control (black) and in the presence of 3 μM BDS-I (red). **C:** dose-dependent slowing of inactivation by BDS-I for sodium current evoked by a step from −80 mV to 0 mV. Each BDS-I concentration was applied until steady state was reached (>5 min for lower concentrations; >2 min for higher concentrations). **D:** dose-dependent effect of BDS-I on relative peak sodium current at 0 mV (red symbols, normalized in each cell to the half-decay time in control) and on relative peak sodium current at 0 mV (gray symbols, normalized to peak sodium current in control). Data represent mean ± SD for measurements in 7 SCG neurons. Internal solution: 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl2, 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH). External solution: 150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose (pH 7.40 with NaOH).
Interestingly, the magnitude of peak human Nav1.7 sodium currents evoked by a step from −100 mV to 0 mV increased slightly for BDS-I concentrations from 1 to 10 nM, but then progressively decreased with BDS-I concentrations >30 nM (Fig. 4C), with half-inhibition by about 1 μM BDS-I (decrease to 51% of control, \( P = 0.001, n = 12 \)) and reduction to approximately 43% by 3 μM BDS-I (\( P = 0.0004, n = 12 \)). This is a much larger reduction than can be accounted for by

![Graphs showing the effect of BDS-I on voltage-activated sodium current](image)

Fig. 4. Effect of BDS-I on cloned human Nav1.7 channels. A: effect of 0.1 to 1 nM BDS-I on voltage-activated sodium current evoked by a step from −100 mV to 0 mV. B: effect of 1–10 nM BDS-I (different cell from A). C: effect of 10 nM to 3 μM BDS-I (same cell as B). D: dose-dependent effect of BDS-I on half-decay time at 0 mV (red symbols, normalized in each cell to the half-decay time in control) and relative peak sodium current at 0 mV (gray symbols, normalized to peak sodium current in control). Data represent mean ± SD for measurements in 12 hNav1.7 cells. E: concentration-dependent effect of BDS-I on voltage dependence of activation and inactivation in a hNav1.7 cell. Activation was determined by peak sodium conductance normalized to maximal peak conductance in each condition, assuming a reversal potential of +50 mV. Inactivation was determined by peak sodium current during a test pulse to 0 mV normalized to peak sodium current during a 200-ms prepulse from a holding potential of −100 mV. Solid curves: best fits to activation data of Boltzmann functions given by \( \frac{1}{1 + \exp\left(-\left(V - V_h\right) / k \right)} \), where \( V_h \) is voltage of half-maximal activation, \( V \) is test potential, and \( k \) is the slope factor. Dashed curves: best fits to inactivation data of Boltzmann functions given by \( \frac{1}{1 + \exp\left(\left(V - V_h\right) / k \right)} \), where \( V_h \) is voltage of half-maximal inactivation, \( V \) is test potential, and \( k \) is the slope factor. Internal solution: 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl\(_2\), 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH). External solution: 150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose (pH 7.40 with NaOH).

8.6 ± 0.9 mV in control and 7.8 ± 0.4 mV in 3 μM BDS-I, \( P = 0.24, n = 12 \).
the shift of activation. The reduction of peak current at 0 mV by concentrations of BDS-I > 30 nM suggests the possibility of a second, lower-affinity binding site than that responsible for the slowing of inactivation.

**BDS-I slows sodium current inactivation in N1E-115 neuroblastoma cells.** Mouse-derived N1E-115 neuroblastoma cells are a widely used preparation for studying the pharmacology of neuronal voltage-gated sodium currents (e.g., Benzinger et al. 1999). An earlier study showed that 100 nM BDS-I had only small effects on TTX-sensitive sodium currents in N1E-115 cells (Diochot et al. 1998), slowing inactivation slightly. Figure 5 shows an experiment examining the dose-dependent effect of BDS-I on sodium currents in N1E-115 cells. In agreement with Diochot and colleagues, a concentration of 100 nM BDS-I had only a modest effect of slowing of inactivation. However, more dramatic effects were seen at higher concentrations (Fig. 5A). The half-maximal effective concentration for slowing of inactivation was approximately 600 nM (Fig. 5B), and the maximal effect by 3 μM BDS-I was to increase the time for half-decay of the current at 0 mV from 0.28 ± 0.04 ms to 0.60 ± 0.13 ms in 3 μM BDS-I (P = 0.009, n = 7). The predominant sodium channels expressed in N1E-115 cells are Nav1.3 channels (Jo and Bean 2011). These results show that BDS-I can slow sodium current inactivation in Nav1.3 channels, but acting with less potency than on Nav1.7 channels. On average, BDS-I had no clear effect on the magnitude of sodium current evoked by a step to 0 mV in N1E-115 cells (Fig. 5B); although there was a small enhancement of current during the successive application of solutions with increasing BDS-I concentrations in some cells (e.g., Fig. 5A), in other cells there was a small reduction, and small amounts of run-up or run-down also sometimes occurred in cells even under control conditions.

**BDS-I slows sodium current inactivation in CA3 pyramidal neurons and cerebellar Purkinje neurons.** Expression of Nav1.7 is confined to the peripheral nervous system (Toledo-Aral et al. 1997), and Nav1.3 channels are expressed in the brain only transiently during development (Schaller and Caldwell 2003). To explore effects on sodium channels present in mammalian central neurons, we tested the effect of BDS-I on sodium currents in acutely dissociated hippocampal pyramidal neurons and cerebellar Purkinje neurons, in which Nav1.1 and Nav1.6 are likely to be the primary sodium channels (Kalume et al. 2007; Raman and Bean 1997; Schaller and Caldwell 2003; Vacher et al. 2008). The effects of BDS-I on the TTX-sensitive sodium current in Purkinje neurons and CA3 pyramidal neurons were far smaller than on Nav1.7- or Nav1.3-expressing cells. At 3 μM, BDS-I produced a modest slowing of inactivation when applied to mouse CA3 pyramidal neurons (Fig. 6A). The time for half-decay at 0 mV increased from 0.29 ± 0.08 ms in control to 0.36 ± 0.09 ms in 3 μM BDS-I (P = 0.01, n = 4), a much smaller effect than seen in Nav1.7- or Nav1.3-expressing cells. BDS-I also had little or no effect on peak sodium current at any voltage (Fig. 6B). These results suggest that BDS-I has substantially weaker effects on Nav1.1 or Nav1.6 channels than on Nav1.7 or Nav1.3 channels.

We also tested BDS-I on TTX-sensitive sodium currents in cerebellar Purkinje neurons isolated from both mouse and rat. Similar to the effects on sodium currents in CA3 neurons, BDS-I produced a small slowing of inactivation of sodium current in Purkinje neurons (Fig. 7). The time for half-decay at 0 mV increased from 0.21 ± 0.03 ms in control to 0.25 ± 0.02 ms in 3 μM BDS-I (P = 0.002, n = 6) in mouse Purkinje neurons, and from 0.25 ± 0.05 ms in control to 0.36 ± 0.08 ms in 3 μM BDS-I (P = 0.01, n = 6) in rat Purkinje neurons.

**BDS-I enhances resurgent sodium current in cerebellar Purkinje neurons.** TTX-sensitive sodium channels in cerebellar Purkinje neurons produce a component of “resurgent” sodium current, which is evoked during repolarization following depolarization (Raman and Bean 1997). Resurgent current appears to originate from a mechanism of inactivation involving voltage-dependent open channel block by an intracellular blocking particle (Raman and Bean 2001). Unlike recovery from “conventional” inactivation involving an intracellular loop formed by the III-IV linker (Vassilev et al. 1988), during which almost no ionic current flows (Kuo and Bean 1994), recovery from binding of the “resurgent” blocker is accompa-

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Effect of BDS-I on sodium current in N1E-115 neuroblastoma cells. **A:** concentration-dependent effect of BDS-I on inactivation kinetics of voltage-activated sodium current at 0 mV. **B:** dose-dependent effect of BDS-I on half-decay time at 0 mV (red symbols, normalized in each cell to the half-decay time in control) and on relative peak sodium current at 0 mV (gray symbols, normalized to peak sodium current in control). Data are mean ± SD in 7 N1E-115 cells. Internal solution: 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl₂, 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH). External solution: 150 mM NaCl, 3.5 mM TEA-Cl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose (pH 7.40 with NaOH).
CA3 pyramidal neurons showed no evidence of resurgent sodium current when tested with the same protocols that elicited resurgent sodium current in Purkinje neurons (Fig. 8B), consistent with previous reports (Castelli et al. 2007; Lewis and Raman 2011; Raman and Bean 1997). In addition, the application of 3 μM BDS-I also did not produce any resurgent current in CA3 neurons \((n = 5; \text{Fig. 8B})\). This is consistent with previous conclusions that CA3 pyramidal neurons simply lack the molecular machinery for generating resurgent current even when conventional inactivation is slowed (Grieco and Raman 2004; Lewis and Raman 2011).

**BDS-I affects sodium current during action potentials in cerebellar Purkinje neurons.** Previously, BDS-I was found to broaden the action potential in rat Purkinje neurons (Martina et al. 2007), an effect interpreted as exclusively reflecting inhibition of Kv3 channels. We tested whether the effects of BDS-I on action potential shape of Purkinje neurons might also involve effects on sodium current during the action potential.

In the action potential clamp experiment shown in Fig. 8B, an action potential waveform recorded from a rat Purkinje neuron was used as a command waveform and TTX-sensitive sodium current elicited by the action potential waveform was recorded in the presence of 3 μM BDS-I. BDS-I had no effect on TTX-sensitive sodium current during the rising phase of the action potential. However, there was an enhancement by BDS-I of TTX-sensitive sodium current flowing during the repolarization phase of the action potential. On average, the TTX-sensitive sodium current during the falling phase of the action potential increased to 169 ± 21% of control or, in absolute magnitude, from 1.1 ± 0.3 to 1.8 ± 0.4 nA \((P = 0.002, n = 5; \text{measured at} −21 \text{mV}, \text{about the midpoint of the falling phase})\). When TTX-sensitive sodium current was measured during the falling phase at −40 mV, the voltage where resurgent current is maximal when studied with step voltage protocols, BDS-I increased sodium current to 204 ± 24% of control or, in absolute magnitude, from 0.8 ± 0.2 to 1.6 ± 0.4 nA \((P = 0.0006, n = 5)\). Presumably the effect of BDS-I reflects a combination of modest slowing of the decay of the sodium current together with enhancement of the resurgent sodium current.

**DISCUSSION**

Our results show that in addition to inhibiting Kv3-family channels, BDS-I potently modulates particular subtypes of neuronal sodium channels. The slowing of sodium channel inactivation by BDS-I is broadly similar to other peptides from anemone venom acting at the neurotoxin receptor site 3 of sodium channels, including ATX-II, anthopleurin B, AFT-II, and Bc-III (reviewed by Catterall et al. 2007; Wanke et al. 2009). The similar functional effects are consistent with the structure of BDS-I, which has substantial similarities with ATX peptides (Driscoll et al. 1989).

**Isoform selectivity.** The effects of BDS-I on neuronal sodium channels in different types of cells varied greatly (summarized in Fig. 10A). Sodium currents in CA3 pyramidal neurons and cerebellar Purkinje cells, likely from a combination of Nav1.6 and Nav1.1 channels (Gong et al. 1999; Kalume et al. 2007; Krzemien et al. 2000; Raman et al. 1997; Westenburg et al. 1989), were much less affected by BDS-I than the cell types expressing Nav1.3 and Nav1.7 channels. In

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 Purkinje neurons, the relatively small effect on decay of sodium current (slowing by approximately 20% in mouse Purkinje neurons and approximately 45% in rat Purkinje neurons) might be partly explained by the presence of the second mechanism of inactivation associated with resurgent current, which could still produce rapid inactivation even if conventional inactivation were slowed substantially. Indeed, resurgent current was enhanced by BDS-I, suggesting that more channels do inactivate by the resurgent mechanism after BDS-I treatment. Moreover, in CA3 neurons, which had no resurgent current either before or after BDS-I treatment, BDS-I still had only a modest effect on the decay of sodium current (slowing by approximately 24%). As the somata of CA3 pyramidal neurons likely express a mixture of Nav1.6 channels (Blumenfeld et al. 2009; Castelli et al. 2007; Krzemien et al. 2000;) and Nav1.1 channels (Gong et al. 1999; Vacher et al. 2008; Van Gelder et al. 2009; Castelli et al. 2007; Krzemien et al. 2000;) containing mouse and rat Nav1.7 have a positively charged lysine at position 1590 in the IVS3–S4 linker. All other channels including mouse and rat Nav1.7 have a positively charged lysine at this residue. Using Nav1,2, Rogers et al. (1996) found that mutating this lysine to alanine enhanced the affinity of ATX-II. It therefore seems plausible that a threonine in this position in human Nav1.7 might enhance binding of BDS-I, a cationic peptide that contains four basic residues and a single acidic residue.

An early characterization of BDS-I found that it displaced the sodium channel site 3 ligand anthopleurin A from binding to rat brain synaptosomes (Llewellyn and Norton 1991), which contain predominantly Nav1.2 (~80%) and Nav1.1 (~20%) (Gordon et al. 1987), suggesting that BDS-I binds to one or both of these channels. Unlike ATX-II and other known anemonine site 3 toxins, BDS-I has little or no functional effect on cardiac muscle (Diochot et al. 1998; Llewellyn and Norton 1991) and also no effect on action potentials in skeletal muscle myotubes (Diochot et al. 1998), suggesting lack of functional interaction with Nav1.4 or Nav1.5 channels. Taken together with our results, it is clear that BDS-I has very strong isoform selectivity among different types of voltage-dependent sodium channels, at least for its ability to produce functional effects, and that the isoform selectivity of BDS-I is very different from that of other site 3 toxins. For example, ATX-II potently modulates Nav1.5 as well as neuronal Nav1.1 and Nav1.6 channels (el-Sherif et al. 1992; Oliveira et al. 2004; Spencer 2009; Wanke et al. 2009), which all appear to be resistant or only weakly sensitive to modulation by BDS-I, whereas anthopleurin A and anthopleurin B preferentially modulate functional effects of Nav1.7 on TTX-sensitive sodium current in mouse and rat cerebellar Purkinje neurons. A: effect of 3 μM BDS-I on TTX-sensitive sodium current in a mouse Purkinje neuron. B: peak TTX-sensitive sodium current as a function of voltage in control (gray) and in the presence of 3 μM BDS-I (black) in a mouse Purkinje neuron (same cell as in A). C: effect of 3 μM BDS-I on TTX-sensitive sodium current in a rat Purkinje neuron. D: peak TTX-sensitive sodium current as a function of voltage in control (gray) and in the presence of 3 μM BDS-I (black) in a rat Purkinje neuron (same cell as in C). TTX-sensitive sodium current was defined by subtracting currents with and without 1 μM TTX using a sequence of four solutions: control, 1 μM TTX, 3 μM BDS-I with 1 μM TTX, and 3 μM BDS-I alone after washing out TTX. Internal solution: 132 mM CsCl, 9 mM NaCl, 1.8 mM MgCl2, 9 mM EGTA, 9 mM HEPES, 14 mM creatine phosphate (Tris salt), 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.20 with CsOH). External solution: 25 mM NaCl, 129.5 mM TEA-Cl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose (pH 7.40).

**Fig. 7.** Effect of BDS-I on TTX-sensitive sodium currents in mouse and rat cerebellar Purkinje neurons.
tion of cardiac channels over neuronal sodium channels (Benzinger et al. 1998; Khra et al. 1995; Sheets and Hanck 1995), apparently opposite to BDS-I. The particularly high potency of BDS-I for modulating Nav1.7 channels over other neuronal isoforms is also very different from three other site 3 anemone toxins (ATX-II, AFT-II, and Bc-III) previously studied in detail, which are all more potent in modulating Nav1.1 and Nav1.6 than Nav1.7 (Oliveira et al. 2004; Wanke et al. 2009).

The different isoform sensitivity of the different anemone toxins suggests that there must be regions on the sodium channel in addition to the IVS3–S4 linker that influence binding (or at least functional effects) of the site 3 toxins (see also Benzinger et al. 1997; Catterall et al. 2007; Moran et al. 2009).

In fact, this is suggested by our results alone, because mouse Nav1.1, which is apparently only weakly modulated by BDS-I, has the same sequence in the IVS3–S4 loop as Nav1.3, which is strongly modulated.

We found that BDS-I dramatically enhances and slows inactivation of the TTX-sensitive sodium current in DRG neurons while having little effect on TTX-resistant sodium current, mediated mainly by Nav1.8 channels under our recording conditions (Blair and Bean 2002). The action on TTX-sensitive but not TTX-resistant sodium current is similar to that of the anemone toxins BgII, BgIII, and ATX-II (Salceda et al. 2002; Snape et al. 2010).

**Effects on channel gating.** We examined the effect of BDS-I on the voltage dependence of channel gating using the human Nav1.7 cell line, where the effects of toxin were greatest. BDS-I shifted the midpoint of both activation and inactivation of human Nav1.7 channels in the depolarizing direction. The steepness of the inactivation curve was unaffected by toxin, whereas the activation curve became less steep, with an enhancement of relative activation for small depolarizations (between $-60$ and $-30$ mV) but an inhibition for large depolarizations (between $-30$ and $+30$ mV).

The enhanced activation for small depolarizations (also evident in the peak current–voltage curves for rat DRG and rat sympathetic neurons; Figs. 2B and 3B) could result from the slowing of inactivation. If channels have microscopic rate constants for activation at small depolarizations that are slow compared with microscopic rate constants for inactivation, agents that slow inactivation increase peak sodium current for small but not large depolarizations (Gonoi and Hille 1987). However, such a mechanism does not account for the shift of the midpoint in the depolarizing direction (an effect that was not evident for sodium current in rat sympathetic neurons). This effect may be mediated by a different binding site, because it required somewhat higher concentrations of BDS-I compared with those affecting inactivation (Fig. 4E).

Interestingly, the effect of BDS-I to alter the voltage dependence of both activation and inactivation (at least for human Nav1.7 channels) differs from the effect of ATX-II, which alters the voltage dependence of inactivation but not the voltage dependence of activation (Chahine et al. 1996; Ulbricht
It is unclear how to account mechanistically for the 3-fold enhancement of TTX-sensitive sodium current seen in DRG neurons, where sodium current was dramatically enhanced for steps to all voltages (Fig. 2B). In principle, the enhancement could reflect partial removal of resting inactivation (i.e., a shift of the inactivation curve in the depolarization direction), such as was seen for human Nav1.7 channels. However, BDS-I produced negligible effects on the inactivation curve of sodium current in rat sympathetic neurons (midpoint of \(-51 \pm 2\) mV, \(k = 7.5 \pm 1\) mV in control and \(-52 \pm 2\) mV, \(k = 7.6 \pm 1\) mV with 1–3 \(\mu\)M BDS-I, \(n = 6\), so this mechanism is unlikely if the main component of TTX-sensitive current in rat DRG neurons is also from Nav1.7 channels.

Whatever its mechanistic origin, the enhancement of sodium current in DRG neurons by BDS-I, together with the slowing of inactivation kinetics, can likely account for broadening of the action potential in DRG neurons, independent of any additional effects on Kv3 channels. Also, the especially dramatic enhancement of TTX-sensitive current for small depolarizations is expected to decrease the action potential threshold, because TTX-sensitive current activates at smaller depolarizations (and also more rapidly) than TTX-resistant current and likely plays the major role in determining action potential threshold (Blair and Bean 2002).

Most small DRG neurons are nociceptors involved in pain sensation, and the extremely potent effect of BDS-I on human Nav1.7 channels, which are expressed in small DRG neurons (Ho and O’Leary 2011), suggests that BDS-I exposed to...
human nociceptors at concentrations near 10 nM could be a highly effective pain-inducing agent. Thus it seems very likely that the action of BDS-I on sodium channels contributes to the painfulness of sea anemone stings in humans.

Two mechanisms of action potential broadening. BDS-I produces broadening of action potentials in both cerebellar Purkinje neurons (Martina et al. 2007) and in DRG neurons (this study), but most likely by different predominant mechanisms. In Purkinje neurons, BDS-I inhibits action potential-evoked potassium current very dramatically, with 1 μM BDS-I blocking the current by approximately 90% (Martina et al. 2007), while enhancing sodium current during the falling phase of the action potential by approximately 70% (Fig. 9). Because the outward potassium current is approximately twice the magnitude of the inward sodium current during the falling phase of the action potential in Purkinje neurons (Carter and Bean 2009), the effect of BDS-I on the potassium current likely accounts for the majority of its effect on action potential shape. In DRG neurons, the effect of BDS-I on the sodium current (3-fold enhancement of TTX-sensitive sodium current together with slowing of inactivation) is much more dramatic and could plausibly account by itself for the broadening of the action potential, especially since there was only a small effect (12% reduction) on the voltage-activated potassium current evoked by voltage steps (Fig. 1C). Thus in DRG neurons, the effects on sodium channels seem likely to be the most important for producing overall effects on neuronal excitability.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.L. and B.P.B. conception and design of research; P.L. and S.J. performed experiments; P.L. analyzed data; P.L. and B.P.B. interpreted results of experiments; P.L. and B.P.B. prepared figures; P.L. and S.J. drafted manuscript; P.L., S.J., and B.P.B. approved final version of manuscript.

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