Na_vβ Subunits Modulate the Inhibition of Na_v1.8 by the Analgesic Gating Modifier μO-Conotoxin MrVIB

Michael J. Wilson, Min-Min Zhang, Layla Azam, Baldomero M. Olivera, Grzegorz Bulaj, and Doju Yoshikami

Departments of Biology (M.J.W., M.-M.Z., L.A., B.M.O., D.Y.) and Medicinal Chemistry (G.B.), University of Utah, Salt Lake City, Utah

Received December 29, 2010; accepted May 16, 2011

ABSTRACT

Voltage-gated sodium channels (VGSCs) consist of a pore-forming α-subunit and regulatory β-subunits. Several families of neuroactive peptides of *Conus* snails target VGSCs, including μO-conotoxins and μ-conotoxins. Unlike μ-conotoxins and the guanidinium alkaloid saxitoxin (STX), which are pore blockers, μO-conotoxins MrVIA and MrVIB inhibit VGSCs by modifying channel gating. μO-MrVIA/B can block Na_v1.8 (a tetrodotoxin-resistant isoform of VGSCs) and have analgesic properties. The effect of Na_vβ-subunit coexpression on susceptibility to block by μO-MrVIA/B and STX has, until now, not been reported. Here, we show that β1-, β2-, β3-, and β4-subunits, when individually coexpressed with Na_v1.8 in *Xenopus laevis* oocytes, increased the *k*_{on} of the block produced by μO-MrVIB (by 3-, 32-, 2-, and 7-fold, respectively) and modestly decreased the apparent *k*_{off}. Strong

depolarizing prepulses markedly accelerated MrVIB washout with rates dependent on β-subunit coexpression. Thus, coexpression of β-subunits with Na_v1.8 can strongly influence the affinity of the conopeptide for the channel. This observation is of particular interest because β-subunit expression can be dynamic, e.g., β2-expression is up-regulated after nerve injury (*J Neurosci*, **25**: 10970–10980, 2005); therefore, the effectiveness of a μO-conotoxin as a channel blocker could be enhanced by the conditions that may call for its use therapeutically. In contrast to MrVIB's action, the STX-induced block of Na_v1.8 was only marginally, if at all, affected by coexpression of any of the β-subunits. Our results raise the possibility that μO-conotoxins and perhaps other gating modifiers may provide a means to functionally assess the β-subunit composition of VGSC complexes in neurons.

Introduction

The characterization of the effects of the guanidinium alkaloids tetrodotoxin (TTX) and saxitoxin (STX) in the 1960s provided critical biochemical corroboration of the Hodgkin-Huxley model for the ionic basis of action potentials (Hille, 2001; Narahashi, 2008); ever since, there has been intense interest in understanding the mechanism of action of other compounds that affect the activity of voltage-gated sodium channels (VGSCs). Many of the more recently discovered ligands that target VGSCs are components of animal venoms (Al-Sabi et al., 2006; Billen et al., 2008). The peptide toxins from cone snail venoms are a particularly rich source (Terlau and Olivera, 2004), and at least four families of conopeptides target VGSCs, each via a different mechanism: μ-conotoxins, like the action of TTX and STX, block the channel's pore (Catterall et al., 2007); μO-conotoxins are gating modifiers that

block channel activation (Zorn et al., 2006; Heinemann and Leipold, 2007; Leipold et al., 2007); ι-conotoxins promote channel activation (Fiedler et al., 2008); and δ-conotoxins inhibit fast inactivation (Leipold et al., 2005; West et al., 2005).

VGSCs are multisubunit complexes, with a single α-subunit and one or more β-subunits (Catterall et al., 2007). In mammals, there are nine known α-subunits (Na_v1.1–Na_v1.9), each of which when expressed alone can form a functional channel with distinctive kinetic and pharmacological properties (Catterall et al., 2005). Four Na_vβ subunits (β1–β4) have been identified thus far, and coexpression of β-subunits with α-subunits can have several functional consequences, including elevated sodium currents, faster inactivation kinetics, and alterations in voltage-sensitivity profiles; furthermore, β-subunits can interact with a variety of other cellular elements and mediate cell-adhesive interactions (Patino and Isom, 2010).

Two of the nine isoforms of VGSCs, Na_v1.8 and Na_v1.9, share two distinctive features: they are expressed in primary sensory neurons that convey nociceptive signals and are highly resistant to TTX (Akopian et al., 1996; Dib-Hajj et al.,

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM 48677].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.110.178343.

ABBREVIATIONS: TTX, tetrodotoxin; DMSO, dimethyl sulfoxide; *I*_{Na}, sodium current; STX, saxitoxin; VGSC, voltage-gated sodium channel; DRG, dorsal root ganglion.

1998; Tate et al., 1998), unlike all other Na_v s, except $\text{Na}_v1.5$, which is modestly resistant to TTX (Satin et al., 1992). Mutant mice in which the genes for $\text{Na}_v1.8$ or $\text{Na}_v1.9$ were knocked out display a deficit in pain behavior (Akopian et al., 1999; Priest et al., 2005). In view of the unique pharmacological properties and physiological roles of $\text{Na}_v1.8$ and $\text{Na}_v1.9$, it has been hypothesized that the ligands that preferentially target these channels may have therapeutic potential for the alleviation of pain (Cummins et al., 2007; Momin and Wood, 2008).

The μO -conotoxins are one of the few ligands known to block $\text{Na}_v1.8$ and TTX-resistant currents in mammalian DRG neurons (Daly et al., 2004; Bulaj et al., 2006; Ekberg et al., 2006), although the selection is being expanded (Zhu et al., 2010). Two members of the μO family have been identified, MrVIA and MrVIB, which differ in only 3 of their 31 residues (Fainzilber et al., 1995; McIntosh et al., 1995). Indeed, MrVIB has analgesic properties (Bulaj et al., 2006; Ekberg et al., 2006), although its precise target in this regard remains to be established.

As already mentioned, μO -conotoxins are gating modifiers that block channel activation. Other gating modifiers, such as scorpion toxins, can interact with β -subunits; in fact, a scorpion toxin played a role in the initial discovery of β -subunits (Hartshorne and Catterall, 1981). Thus, we were motivated to examine whether coexpression of β -subunits with $\text{Na}_v1.8$ influenced the block by MrVIB. We demonstrate here, using cloned channels exogenously expressed in *Xenopus laevis* oocytes, that the block produced by MrVIB is markedly affected by which β -subunit is coexpressed with $\text{Na}_v1.8$.

TTX-resistant currents in rat DRG neurons and $\text{Na}_v1.8$ are considerably more sensitive to STX than to TTX (Roy and Narahashi, 1992; Sivilotti et al., 1997); thus, we compared the inhibition of $\text{Na}_v1.8$ by MrVIB with that by STX. In contrast to the results with MrVIB, the block by STX was only marginally, if at all, affected by β -subunit coexpression.

Materials and Methods

Clones. The clone for rat $\text{Na}_v1.8$, provided by Prof. John Wood (University College, London, UK) in pRK7, was linearized with HpaI. Clones for rat $\beta1$ and $\beta2$ ($\text{Na}_v\beta1$ and $\text{Na}_v\beta2$), provided by Prof. Alan Goldin (University of California, Irvine, CA), in pBSTA and pLCT1, respectively, were linearized with NotI. Clones for rat $\beta3$ and $\beta4$ ($\text{Na}_v\beta3$ and $\text{Na}_v\beta4$), in pcDNA3.1zeo(+) and pcDNA3.1zeo(−), provided by Prof. Lori Isom (University of Michigan, Ann Arbor, MI), were linearized with XbaI and BamHI, respectively. cRNA for $\text{Na}_v1.8$ was prepared using SP6 RNA polymerase. cRNAs for $\text{Na}_v\beta1$ –4 were constructed using T7 RNA polymerase (mMessage mMachine kit; Ambion, Austin, TX). A poly(A) tail was subsequently added to the cRNAs for $\text{Na}_v\beta3$ and $\text{Na}_v\beta4$ subunits [poly(A) tailing kit; Ambion].

Injection of *X. laevis* Oocytes. Oocytes were prepared essentially as described previously (Fiedler et al., 2008). Each oocyte was injected with 69 nl of $\text{Na}_v1.8$ cRNA without or with β -subunit cRNA (35 ng of each). Oocytes were incubated at 16°C for 6 to 11 days in a solution of ND96⁺ (96 mM NaCl, 2 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, 0.1 mg/ml bovine serum albumin, and 0.01% DMSO) supplemented with the antibiotics penicillin (100 units/ml), streptomycin (0.1 mg/ml), amikacin (0.1 mg/ml), and Septra (0.2 mg/ml).

Electrophysiology. Two-electrode voltage-clamp recordings were made essentially as described previously (Fiedler et al., 2008). The

recording chamber consisted of a 4-mm-diameter, 30- μl well in a wafer of Sylgard (Dow Corning, Midland, MI). Intracellular electrodes contained 3 M KCl (<0.3 M Ω resistances). Sodium currents (I_{Na}) were recorded by stepping the membrane potential to 20 mV, unless indicated otherwise, for 30 ms from a holding potential of −100 mV once every 20 s. Currents were low-pass-filtered at 2 kHz, sampled at a rate of 10 kHz, and leak-subtracted using a P/8 protocol. Data acquisition and analysis were performed with in-house software constructed with LabVIEW (National Instruments, Austin, TX). The capacitance of a cell was measured following essentially the procedure used by Isom et al. (1995): the membrane potential was stepped to −105 and −95 mV from a holding potential of −100 mV, and the area of the capacitive transients was measured. Conductance values were calculated with the formula $g_{\text{Na}} = I_{\text{Na}} / (V_{\text{step}} - V_{\text{rev}})$, where g_{Na} is the conductance, I_{Na} is the peak current amplitude in response to the potential step, V_{step} is the test potential, and V_{rev} is the estimated reversal potential. Normalized activation and inactivation curves were fit to the Boltzmann equation of the form $Y = 1 / (1 + \exp[(V_{\text{step}} - V_{1/2})/k])$, where Y is the normalized g_{Na} or I_{Na} , V_{step} is the test pulse (for activation curves) or the 500-ms conditioning prepulse (which, for inactivation curves, immediately preceded the test pulse to 20 mV), $V_{1/2}$ is the voltage at half-maximal activation or inactivation, and k is the slope factor.

The influence of strong conditioning pulses on the reversibility of MrVIB was examined by holding the membrane potential at −100 mV and presenting a pair of pulses: a 300-ms depolarizing conditioning pulse followed by the usual 30-ms test pulse to 20 mV. The interval between the end of conditioning pulse and beginning of the test pulse was 3 s. This pair of pulses was presented every 20 s during toxin washout. In a given trial, the amplitude of the conditioning pulse was kept constant, and in separate trials it varied between +40 and +120 mV. The time course of recovery from block was fit to a single-exponential function to yield k'_{off} , and its voltage dependence was determined by plotting k'_{off} as a function of the amplitude of the conditioning pulse; the resulting curve was fit to a single-exponential function, $k'_{\text{off}}(V) = \exp(K \times V_{\text{cond}})$, where V_{cond} is the amplitude of the conditioning pulse in mV and K is the best-fit constant. An estimate was made of the apparent charge transferred in the “gating” of k'_{off} by assuming that $K = (Z_g \times q_e) / (k_B \times T)$, where Z_g is the gating charge, q_e is the elementary charge (i.e., that of a proton), k_B is Boltzmann’s constant, and T is absolute temperature (c.f. Leipold et al., 2007; see also Hille, 2001). The factor $q_e / (k_B \times T)$ has a value close to 1/25 mV^{−1} at room temperature, so $Z_g \approx 25 \text{ mV} \times K$.

It might be noted that the strong depolarizing pulses we used induced a potentiation of the I_{Na} of oocytes expressing $\text{Na}_v1.8$. This “run-up” of I_{Na} was frequently seen regardless of whether and which β -subunit was coexpressed. No differences have been discerned in the biophysical or pharmacological properties of the I_{Na} obtained before versus after run-up, and the mechanism underlying run-up remains to be determined. To minimize drift resulting from run-up, in many cases oocytes were pretreated with conditioning pulses until I_{Na} stabilized before commencing the experiment.

Toxin Solutions and Delivery. MrVIB, synthesized as described previously (Bulaj et al., 2006), was dissolved in ND96⁺ and stored refrigerated. STX was purchased from either Calbiochem (San Diego, CA) or the National Research Council Canada (Halifax, NS, Canada). Two pumps were used to perfuse the oocyte-containing well: control ND96⁺ was delivered with a peristaltic pump, whereas a motorized 2.5-ml syringe (Cavro OEM; Tecan Sytems, San Jose, CA) was used to deliver toxins. The well was perfused with ND96⁺ at a rate of 30 $\mu\text{l}/\text{min}$. To expose the oocyte to toxin, the perfusion with control ND96⁺ was halted, and toxin solution was delivered with the motorized syringe, initially at a rate of 250 $\mu\text{l}/\text{min}$ for 48 s to rapidly replace the fluid in the well, then at a rate of 30 $\mu\text{l}/\text{min}$. The motorized syringe was connected, via Teflon tubing, to a disposable 1-ml pipettor tip, which was mounted on a micromanipulator and delivered toxin directly to the recording well. Toxin was washed out by perfusing the well with ND96⁺ at a rate of 150 $\mu\text{l}/\text{min}$.

Data Analysis. Rate constants of toxin action were determined by fit of peak I_{Na} values to a single-exponential function with Prism (GraphPad Software Inc., San Diego, CA) or KaleidaGraph (Synergy Software, Reading, PA). Rates of fast inactivation were obtained by fitting the falling phases of I_{Na} to a single exponential with home-made software written with LabVIEW. Prism software was used to fit the activation and inactivation curves to the Boltzman equation. Statistical comparisons were made with two-tailed unpaired t tests. All data are presented as mean $\pm$ S.E.M., with n values representing the number of oocytes tested.

Results

Functional Consequences of Coexpression of Each of Four Na_vβ-Subunits with Na_v1.8. Sodium currents (I_{Na}) of Na_v1.8 expressed in *X. laevis* oocytes either alone or coexpressed with β-subunits are illustrated in Fig. 1. Coexpres-

Fig. 1. Current traces and activation and inactivation curves of Na_v1.8 without and with β-subunit coexpression. *X. laevis* oocytes were voltage-clamped as described under *Materials and Methods*. A, representative current traces in response to voltage steps from -60 to 60 mV in 5 -mV increments. For clarity, every other trace is gray. B, activation (left) and inactivation (right) curves were acquired as described under *Materials and Methods*. Parameters of both sets of curves are presented in Table 1. Data points represent mean $\pm$ S.E.M., with n values as tabulated in Table 1.

TABLE 1

Activation and inactivation parameters and time constants of fast inactivation of Na_v1.8 without and with β-subunit coexpression

Values represent mean $\pm$ S.E.M. with n values as tabulated.

Na _v 1.8	Activation ^a			Inactivation ^b			Inactivation ^c	
	$V_{1/2}$	k	n	$V_{1/2}$	k	n	τ	n
	mV			mV			ms	
Alone	13 ± 0.2	8 ± 0.1	44	-30.3 ± 0.6	-12.1 ± 0.5	9	7.6 ± 0.3	21
+ β1	$6.5 \pm 0.1^*$	$7.2 \pm 0.1^*$	47	$-47.2 \pm 0.5^*$	$-10.5 \pm 0.4^*$	30	$4.2 \pm 0.2^*$	13
+ β2	$0.9 \pm 0.1^*$	$6.4 \pm 0.1^*$	47	$-37.7 \pm 1.0^*$	$-14.5 \pm 1^*$	14	$4.8 \pm 0.2^*$	22
+ β3	$4.1 \pm 0.1^*$	$7.3 \pm 0.1^*$	18	$-48.1 \pm 0.3^*$	$-7.9 \pm 0.3^*$	16	$6.4 \pm 0.2^*$	18
+ β4	$9.9 \pm 0.1^*$	8.1 ± 0.1	35	$-34.7 \pm 0.5^*$	$-14.0 \pm 0.5^*$	15	$5.5 \pm 0.2^*$	13

^a Data from Fig. 1B, left.

^b Data from Fig. 1B, right.

^c Time constant of fast inactivation from falling phase of I_{Na} , in response to V_{step} to 20 mV, fit to a single exponential.

* Statistically different from Na_v1.8 expressed alone ($p < 0.007$).

sion with β-subunits significantly affected the time course of fast inactivation as well as the voltage sensitivities of activation and inactivation of Na_v1.8 (Table 1).

Coexpression of Each of the Four β-Subunits with Na_v1.8 Increased the Rate of Block by MrVIB. The effects of MrVIB on Na_v1.8 expressed either alone or with each of the β-subunits are illustrated in Fig. 2. The time course of block in each condition was fit to a single exponential to obtain the observed rate constant, k_{obs} . For single-site binding, the relationship, $k_{obs} = k_{on}[MrVIB] + k_{off}$, is expected (Hille, 2001). The plots of k_{obs} versus MrVIB concentration were linear (Fig. 2B), and k_{on} values were obtained from the slopes (Table 2).

In each case, the rate of recovery from block (k_{off}) was slow, and full recovery from block was not achieved in the time-frame of the experiments (Fig. 2A inset). Thus, the recovery from block was assumed to follow a single exponential time course, and the k_{off} was estimated from the level of recovery obtained after 20 min of washing (Table 2). Longer wash times were not used to minimize errors because of baseline drift. In principle, k_{off} values could also be determined from the y-intercepts of the plots in Fig. 2B, whose values were (with 95% confidence intervals in parentheses): 0.002 (-0.281 to 0.285), 0.009 (-0.178 to 0.195), 0.013 (-0.150 to 0.177), 0.012 (-0.015 to 0.039), and 0.012 (-0.048 to 0.071) min^{-1} , for Na_v1.8 alone and coexpressed with β1, β2, β3, and β4, respectively. These 95% confidence intervals straddled zero; therefore, the k_{off} values obtained in this fashion were not considered further. The k_{on} and k_{off} values were used to determine K_d values (Table 2). IC_{50} values, however, were not obtained because with toxin concentrations near the expected nanomolar IC_{50} values, the times to reach steady-state block can be predicted to take a few hours, given the results in Fig. 2 and Table 2, which were outside the time window of our experiments.

Coexpression of Each of the Four β-Subunits Minimally Affected the Block of Na_v1.8 by STX. In contrast to the block produced by MrVIB, the block by STX was readily reversible, and steady-state dose-response curves were readily obtained within the time window of our experiments (Fig. 3 and Table 3). In all cases, IC_{50} and k_{off} values were minimally affected by β-subunit coexpression. The values of k_{on} were not obtained because the rates of block were too fast to accurately determine k_{obs} at the STX concentrations used.

Effects of Membrane Potential (V_m) on the Interaction of MrVIB with the Channel. To examine whether a modest change of V_m in the clamp protocol would alter the

Fig. 2. Coexpression of β -subunits with $\text{Na}_v1.8$ accelerates the observed rate of block by MrVIB. Oocytes were voltage-clamped as in Fig. 1. A, time course of block of peak I_{Na} by $0.3 \mu\text{M}$ MrVIB is shown for each expression condition (arrow indicates when peptide was added). Data points represent mean $\pm$ S.E.M. ($n \geq 4$ oocytes for each condition), and solid lines are best-fit single exponential curves from which k_{obs} values were obtained for use in B. Inset, recovery from block after toxin washout of oocytes expressing α -subunit alone (bold) or with $\beta 3$ (gray), which yielded the fastest and slowest apparent k_{off} values, respectively (see Table 2); axes units are the same as those in larger plot. B, coexpression of $\text{Na}_v1.8$ with β -subunits increased the k_{on} of block by MrVIB. Data points represent values of k_{obs} obtained as in A but with different concentrations of MrVIB. Each point represents mean $\pm$ S.E.M. ($n \geq 4$ oocytes). Solid lines are linear regression fits; k_{on} values obtained from slopes of these lines are shown in Table 2.

interaction of MrVIB with the channel, the block by MrVIB of $\text{Na}_v1.8$, either alone or coexpressed with $\beta 1$ -subunits, was also tested with a holding potential of -110 mV and a test pulse to 10 mV. No statistically significant differences were observed between the resulting k_{obs} values and their counterparts in Fig. 2B (i.e., those obtained with a holding potential of -100 mV and a test pulse to 20 mV) (data not shown), indicating that to readily observe possible effects of V_m on the interaction of MrVIB with the channel, larger perturbations of V_m should be used. Indeed, readily observable effects on the washout rate of MrVIB can be induced by strong depolarizations, as described immediately below.

The block by MrVIA of $\text{Na}_v1.4$ (expressed in human embryonic kidney cells) was reported to be relieved by strong depolarizing conditioning pulses (Leipold et al., 2007). Thus, we examined whether MrVIB's slow washout from $\text{Na}_v1.8$ could be accelerated by strong depolarizations and, if so, what influence coexpression with β -subunits might have. In a typical experiment, MrVIB was applied, and once steady-state block was achieved, the peptide was washed out. After approximately 10 min of washing (to establish the normal rate of recovery from block), 300 -ms conditioning pulses to $+120$ mV were applied as described under *Materials and Methods*. Oocytes with $\text{Na}_v1.8$ alone or coexpressed with either $\beta 1$ or $\beta 2$ were examined in this fashion, and representative examples of peak I_{Na} versus time are presented in Fig. 4A. The conditioning pulses greatly hastened the recovery from block, and the recovered traces seemed essentially identical to those of control (Fig. 4A insets). The time course of recovery followed an exponential function characterized by the rate constant, k'_{off} , whose values are plotted in the bar graph in the inset of Fig. 4B. To examine the voltage dependence of the relief from block, oocytes expressing $\text{Na}_v1.8 + \beta 1$ and $\text{Na}_v1.8 + \beta 2$ were subjected to conditioning pulses with different amplitudes, and k'_{off} was determined for each amplitude. Over the range of amplitudes tested, voltage dependence of k'_{off} was well fit by a single exponential function with an e-fold change in k'_{off} per ~ 22 mV for both sets of oocytes (Fig. 4C).

The 300 -ms conditioning pulse was applied every 20 s and therefore occupied only $0.3/20$ (or 1.5%) of the time; so, the true values of the off-rate constants were expected to be many times larger. An experiment to examine the effect of pulse duration was performed with oocytes expressing $\text{Na}_v1.8 + \beta 1$, where the $+120$ -mV condition pulses had a 100 -ms duration instead of the standard 300 -ms duration, but all other parameters remained the same. The values of k'_{off} with the 100 - versus 300 -ms pulses were, respectively, $0.1 \pm 0.02 \text{ min}^{-1}$ ($n = 7$) versus $0.6 \pm 0.1 \text{ min}^{-1}$ ($n = 9$); i.e., a 6-fold smaller k'_{off} was produced with a 3-fold shorter pulse duration, which clearly indicates a nonlinear dependence of k'_{off} on pulse duration.

Discussion

Effects of β -Subunit Coexpression on the Biophysical Parameters of $\text{Na}_v1.8$. In our previous report concerning MrVIB and $\text{Na}_v1.8$, the latter was coexpressed in oocytes with $\beta 1$ (Bulaj et al., 2006). In the present experiments, we examined five conditions: $\text{Na}_v1.8$ expressed alone or coexpressed with one of the four β -subunits. In each case, coexpression of β -subunit increased the rate of fast inactivation and shifted the $V_{1/2}$ values of both the activation and inactivation curves to a less depolarized level (Fig. 1 and Table 1). The changes in all three parameters produced by $\beta 1$ coexpression are qualitatively consistent with those reported by Chahine's group (Vijayaragavan et al., 2001); however, the changes induced by $\beta 2$ or $\beta 3$ coexpression that we observed differ, for the most part, from those reported by the Chahine group (Vijayaragavan et al., 2004). We have no explanation for the differences except to note that our oocytes were injected with lower levels of cRNA and incubated for longer periods after injection than were oocytes used by the Chahine group, and the $\text{Na}_v1.8$ and β -subunit clones we used were

TABLE 2

Effects of β-subunit coexpression on block of Na_v1.8 by MrVIB and electrical capacitance of oocytesValues are mean ± S.E.M., with *n* values as tabulated.

Na _v 1.8	k_{on}^a	k_{off}^b	K_d^c	<i>n</i>	Cell Capacitance ^d	
	$min^{-1}μM^{-1}$	min^{-1}	<i>nM</i>		$μF$	<i>n</i>
Alone	0.25 ± 0.01	0.0110 ± 0.0009	44 ± 6.4	22	0.27 ± 0.00	12
+ β1	0.75 ± 0.02**	0.0064 ± 0.0011*	8.5 ± 2.9	16	0.26 ± 0.01	27
+ β2	7.93 ± 0.21**	0.0076 ± 0.0007*	1.0 ± 0.2	22	0.89 ± 0.04**	30
+ β3	0.57 ± 0.00**	0.0037 ± 0.0009*	6.5 ± 1.8	5	0.30 ± 0.01*	14
+ β4	1.74 ± 0.03**	0.0062 ± 0.0021*	3.5 ± 1.2	4	0.26 ± 0.01	17

^a Values of k_{on} were calculated from slopes of linear regression curves illustrated in Fig 2B.^b Recovery from block after MrVIB washout was extremely slow, so exponential recovery was assumed and k_{off} was calculated from the fraction of sodium current recovered after 20 min of wash.^c K_d values were calculated from k_{off}/k_{on} ratios.^d Membrane capacitances of oocytes were determined as described under *Materials and Methods*.* Statistically different from Na_v1.8 expressed alone (*p* < 0.05).** Statistically different from Na_v1.8 expressed alone (*p* < 0.0001).

Fig. 3. Steady-state dose-response curves for STX block of Na_v1.8 without and with β-subunit coexpression. Oocytes were voltage-clamped as in Fig. 1 and exposed to different concentrations of STX. Solid curves represent fits of data to the equation for the Langmuir adsorption isotherm, $Y = 100 \times 1/(1 + (IC_{50}/[STX])^{n_H})$. Values for IC_{50} and Hill coefficient, n_H , are listed in Table 3. Data points represent mean ± S.E.M. (*n* = 5). Inset, representative time course of block by and washout of 3, 10, and 30 μM STX, where the ~3-min exposures were approximately 10 min apart (vertical and horizontal axes, μA and min, respectively).

TABLE 3

Steady-state and off-rate constants of the block by STX of Na_v1.8 without and with β-subunit coexpressionValues are mean ± S.E.M. (*n* = 5 oocytes).

Na _v 1.8	IC_{50}^a	k_{off}	n_H^a
	$μM$	min^{-1}	
Alone	10.9 ± 1.2	2.87 ± 0.29	1.2 ± 0.2
+ β1	10.9 ± 0.9	2.09 ± 0.18*	1.3 ± 0.1
+ β2	14.0 ± 0.9	2.28 ± 0.15	1.3 ± 0.1
+ β3	11.1 ± 0.4	2.47 ± 0.11	1.1 ± 0.0
+ β4	12.0 ± 0.7	1.77 ± 0.14*	1.1 ± 0.1

^a Data of Fig. 3.* Statistically different from Na_v1.8 expressed alone (*p* = 0.04).

prepared by different laboratories than those used by Chahine's group. Finally, 0.01% DMSO was present in the oocyte bathing solution throughout our experiments (see *Materials and Methods*) to enhance the solubility of MrVIB (Daly et al., 2004; Ekberg et al., 2006); however, for oocytes expressing Na_v1.8 + β1, the activation $V_{1/2}$ value obtained in the presence of DMSO (Table 1) was not significantly different from

that obtained in separate experiments where DMSO was left out (7.9 ± 0.3 mV, mean ± S.E.M., *n* = 5). Likewise, sodium currents in rat DRG neurons are not affected by 0.01% DMSO (Daly et al., 2004).

Effects of β-Subunit Coexpression on the Susceptibility of Na_v1.8 to MrVIB. We (Bulaj et al., 2006) and others (Ekberg et al., 2006) demonstrated previously that sodium currents of oocytes expressing Na_v1.8 were susceptible to block by MrVIB; however, in neither case were the effects of β-subunit coexpression examined. Our present experiments reveal that when any of the four β-subunits was coexpressed a higher-affinity block by MrVIB was observed, with a 5- to 44-fold decrease in K_d depending on the β-subunit; in each case, k_{on} was increased, and k_{off} was decreased slightly (Table 2). Preliminary static-bath experiments that examined the coexpression of a combination of β1- and β2-subunits with Na_v1.8 indicated that the k_{on} increased to a level intermediate between those when each β-subunit alone was coexpressed with Na_v1.8 (data not shown); tests with other combinations of β-subunits remain to be pursued.

We reported previously that MrVIB blocked the TTX-resistant sodium current in rat DRG neurons, probably that of Na_v1.8, with an IC_{50} of approximately 13 nM (Bulaj et al., 2006). This value is closer to the K_d values for Na_v1.8 coexpressed with, than without, β-subunits in oocytes (Table 2); however the block by MrVIB of the neuronal current was more reversible than the currents in oocytes. It remains to be seen what factors, such as those discussed below, are responsible for this difference.

At this point, we do not have a mechanistic explanation for the observed effects of β-subunit coexpression on MrVIB affinity, but there are several alternatives. One obvious hypothesis is that there are binding determinants for MrVIB in both α- and β-subunits. Alternatively, association with β-subunits might allosterically alter the binding sites on the α-subunit for MrVIB. In addition, changes in plasma membrane properties or composition induced by β-subunit expression may be important for affinity, particularly because MrVIB is a rather hydrophobic peptide (Daly et al., 2004; Bulaj et al., 2006). Coexpression of β2 produced the largest change in affinity, and it is notable that expression of β2 significantly increases the area of the oocyte's plasma membrane, which is reflected by larger cell capacitance, through increased size and number of microvilli (Isom et al., 1995).

Fig. 4. Depolarizing conditioning (Cond.) pulses accelerate the washout of MrVIB. Oocytes were voltage-clamped, and I_{Na} was evoked by 30-ms test pulses to +20 mV, presented every 20 s, with or without a 300-ms conditioning pulse that preceded each test pulse by 3 s, as described under *Materials and Methods*. **A**, example of results with conditioning pulses to +120 mV. Bars indicate intervals during which MrVIB was present (gray bar) or when conditioning pulses were given (black bar). [MrVIB] were 10, 1, and 1 μM for $\text{Na}_V1.8$, $+\beta1$, and $+\beta2$, respectively. Insets, representative current traces in response to test pulses obtained before peptide addition (a), just before peptide washout (b), and after full recovery from block facilitated by conditioning pulses (c). **B**, time course of recovery from MrVIB block during washout of peptide and its acceleration by conditioning pulses to +120 mV. Each data point represents mean $\pm$ S.E.M. (error bars are both positive- and negative-going for $\text{Na}_V1.8$ expressed alone, positive-going for $+\beta1$, and negative-going for $+\beta2$; $n = 3$ oocytes for $\text{Na}_V1.8$, 9 for $+\beta1$, and 3 for $+\beta2$). Minimal recovery from block is seen until commencement of conditioning pulses (at $t = 10$ min), after which block is relieved with a rate constant, k'_{off} , whose values are presented in the bar graph (see Inset). **C**, semilog plot of k'_{off} versus amplitude of conditioning pulse for oocytes expressing $\text{Na}_V1.8$ with either $\beta1$ or $\beta2$. Values in parentheses indicate numbers of oocytes tested. Data were fit to a single exponential function yielding K values (see *Materials and Methods*) of 0.044 and 0.046 mV^{-1} for $+\beta1$ and $+\beta2$, respectively ($r = 0.99$ for both curves).

We confirmed that $\beta2$ -expression increases the oocyte's electrical capacitance (Table 2).

Lipid composition of, and mechanical forces on, the membrane associated with a channel can influence its gating properties and susceptibility to a gating-modifier toxin, as has been observed with voltage-gated potassium channels in planar lipid bilayers (Schmidt and MacKinnon, 2008). Mechanical forces on the plasma membrane can also affect the kinetics of sodium currents, as was observed with $\text{Na}_V1.4$ exogenously expressed in *X. laevis* oocytes (Shcherbatko et al., 1999). In this regard, it is of interest that the rate of recovery after block by MrVIA of $\text{Na}_V1.2$ expressed in *X. laevis* oocytes was found to be faster from outside-out patches than from whole cells (Terlau et al., 1996). Thus, factors extrinsic to the VGSC complex per se can, at least in principle, affect toxin affinity.

Voltage Dependence of the Depolarization-Induced Acceleration of MrVIB Washout. Heinemann's laboratory observed that MrVIA's block of $\text{Na}_V1.4$ expressed in human embryonic kidney cells was quite reversible, with a k_{off} value of 0.33 min^{-1} (Zorn et al., 2006). The block could be relieved by strong depolarizations, and full recovery from the block by 400 nM MrVIA could be achieved with a single 300-ms pulse to +40 mV (Leipold et al., 2007). In contrast, the k_{off} values for block of $\text{Na}_V1.8$ by MrVIB were 30- to 100-fold smaller (Table 2), and a single 300-ms conditioning pulse, even to +120 mV, only partially "pushed off" the peptide during washout, such that conditioning pulses had to be applied repeatedly during peptide washout to achieve complete recovery from block (Fig. 4).

As described under *Results*, the k'_{off} was disproportionately smaller when the duration of the conditioning pulse was reduced from 300 to 100 ms. This suggests that upon depolarization k'_{off} relaxes rather slowly to its new level, possibly a consequence of a slow conformational change of the

channel. This is reminiscent of slow inactivation (Vijayaragavan et al., 2001).

The slopes of both curves in Fig. 4C are approximately 22 mV per e-fold change in k'_{off} , which suggests an effective transfer of 1.1 elementary charges is involved in gating k'_{off} (see *Materials and Methods*). This is not far from the estimated 1.8 elementary charges reported for the recovery from MrVIA block of $\text{Na}_V1.4$ (Leipold et al., 2007). It should be noted that in the experiments with $\text{Na}_V1.4$ recovery from block was measured in the presence of peptide, whereas in our experiments, the recovery from block was determined in the absence of peptide, i.e., during washout with control solution.

Although MrVIB was discovered several years ago (Fainzilber et al., 1995; McIntosh et al., 1995), its synthesis has been difficult (caused in large part by its hydrophobic nature) and was accomplished only relatively recently (Bulaj et al., 2006). Studies with synthetic mutants of MrVIB are expected to help identify the important elements of the peptide in blocking the α -subunit as well as the modulating influence of β -subunits.

Lack of Effect of Coexpression of β -Subunits on the Susceptibility of $\text{Na}_V1.8$ to Saxitoxin. STX blocked $\text{Na}_V1.8$, regardless of β -subunit coexpression, with an IC_{50} approximately 10 μM (Fig. 3 and Table 3), which is within a factor of 2 of STX's IC_{50} for block of TTX-resistant currents in rat DRG neurons (Roy and Narahashi, 1992; Leffler et al., 2005). These results suggest that β -subunit coexpression does not affect the susceptibility of $\text{Na}_V1.8$ toward a pore blocker, like STX. To our knowledge, this is the first report to document the relative insensitivity of the block of any $\text{Na}_V\alpha$ -subunit isoform by STX to coexpression with any of the four β -subunits.

The discovery that β -subunits are important for the interaction of MrVIB with a VGSC complex may be relevant to the

development of MrVIB and related conopeptides, as well as other gating-modifier toxins, for diagnostic and therapeutic applications. The observation that the coexpression of the β2-subunit has the largest effect is particularly interesting because this β-subunit has been reported to be up-regulated in rat and mouse models of neuropathic pain (Pertin et al., 2005). MrVIB has been shown to be a potential analgesic based on its effects in studies using chronic pain rat models (Ekberg et al., 2006), and it is possible that the affinity of MrVIB for Na_v1.8 may have been enhanced by the coexpression of β2-subunits.

The present experiments used the *X. laevis* oocyte expression system, principally because of the relative ease with which the functional activities of Na_v1.8 with the various β-subunits could be stably recorded over a period of up to 1 h. However, it is known that a given VGSC can have a different phenotype when expressed in oocytes versus transfected mammalian cells (Baroudi et al., 2000). Our results with the oocyte system provide a strong incentive to extend the studies with MrVIB to transfected mammalian cell lines, if not DRG neurons themselves, in future experiments. In particular, it would be interesting to see whether the susceptibility to MrVIB of sodium currents mediated by Na_v1.8 is altered in DRG neurons of rats subjected to nerve injury, and, if so, whether β-subunit expression is involved. Such experiments may serve to test the possibility that μO-conotoxins and perhaps other gating modifiers can be used to functionally probe the β-subunit composition of VGSC complexes in neurons.

Acknowledgments

We thank Prof. John N. Wood for providing the Na_v1.8 clone; Prof. Alan A. Goldin for providing the Na_vβ1 and β2 clones; and Prof. Lori L. Isom for providing the Na_vβ3 and β4 clones.

Authorship Contributions

Participated in research design: Wilson, Zhang, Olivera, Bulaj, and Yoshikami.

Conducted experiments: Wilson.

Contributed new reagents or analytic tools: Azam and Bulaj.

Performed data analysis: Wilson.

Wrote or contributed to the writing of the manuscript: Wilson, Zhang, Azam, Olivera, Bulaj, and Yoshikami.

References

- Akopian AN, Sivilotti L, and Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* **379**:257–262.
- Akopian AN, Souslova V, England S, Okuse K, Ogata N, Ure J, Smith A, Kerr BJ, McMahon SB, Boyce S, et al. (1999) The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. *Nat Neurosci* **2**:541–548.
- Al-Sabi A, McArthur J, Ostroumov V, and French RJ (2006) Marine toxins that target voltage-gated sodium channels. *Marine Drugs* **4**:157–192.
- Baroudi G, Carbonneau E, Pouliot V, and Chahine M (2000) SCN5A mutation (T1620M) causing Brugada syndrome exhibits different phenotypes when expressed in *Xenopus* oocytes and mammalian cells. *FEBS Lett* **467**:12–16.
- Billen B, Bosmans F, and Tytgat J (2008) Animal peptides targeting voltage-activated sodium channels. *Curr Pharm Des* **14**:2492–2502.
- Bulaj G, Zhang MM, Green BR, Fiedler B, Lyster RT, Wei S, Nielsen JS, Low SJ, Klein BD, Wagstaff JD, et al. (2006) Synthetic μO-conotoxin MrVIB blocks TTX-resistant sodium channel Na_v1.8 and has a long-lasting analgesic activity. *Biochemistry* **45**:7404–7414.
- Catterall WA, Cestèle S, Yarov-Yarovsky V, Yu FH, Konoki K, and Scheuer T (2007) Voltage-gated ion channels and gating modifier toxins. *Toxicon* **49**:124–141.
- Catterall WA, Goldin AL, and Waxman SG (2005) International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev* **57**:397–409.
- Cummins TR, Sheets PL, and Waxman SG (2007) The roles of sodium channels in nociception: implications for mechanisms of pain. *Pain* **131**:243–257.
- Daly NL, Ekberg JA, Thomas L, Adams DJ, Lewis RJ, and Craik DJ (2004) Structures of μO-conotoxins from *Conus marmoreus*. Inhibitors of tetrodotoxin (TTX)-sensitive and TTX-resistant sodium channels in mammalian sensory neurons. *J Biol Chem* **279**:25774–25782.
- Dib-Hajj SD, Tyrell L, Black JA, and Waxman SG (1998) Na_v, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. *Proc Natl Acad Sci USA* **95**:8963–8968.
- Ekberg J, Jayamanne A, Vaughan CW, Aslan S, Thomas L, Mould J, Drinkwater R, Baker MD, Abrahamson B, Wood JN, et al. (2006) μO-conotoxin MrVIB selectively blocks Na_v1.8 sensory neuron specific sodium channels and chronic pain behavior without motor deficits. *Proc Natl Acad Sci USA* **103**:17030–17035.
- Fainzilber M, van der Schors R, Lodder JC, Li KW, Geraerts WP, and Kits KS (1995) New sodium channel-blocking conotoxins also affect calcium currents in *Lymnaea* neurons. *Biochemistry* **34**:5364–5371.
- Fiedler B, Zhang MM, Buczek O, Azam L, Bulaj G, Norton RS, Olivera BM, and Yoshikami D (2008) Specificity, affinity and efficacy of iota-conotoxin RXIA, an agonist of voltage-gated sodium channels Na(V)1.2, 1.6 and 1.7. *Biochem Pharmacol* **75**:2334–2344.
- Hartshorne RP and Catterall WA (1981) Purification of the saxitoxin receptor of the sodium channel from rat brain. *Proc Natl Acad Sci USA* **78**:4620–4624.
- Heinemann SH and Leipold E (2007) Conotoxins of the O-superfamily affecting voltage-gated sodium channels. *Cell Mol Life Sci* **64**:1329–1340.
- Hille B (2001) *Ion Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA.
- Isom LL, Ragsdale DS, De Jongh KS, Westenbroek RE, Reber BF, Scheuer T, and Catterall WA (1995) Structure and function of the β2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* **83**:433–442.
- Leffler A, Herzog RI, Dib-Hajj SD, Waxman SG, and Cummins TR (2005) Pharmacological properties of neuronal TTX-resistant sodium channels and the role of a critical serine pore residue. *Pflugers Arch* **451**:454–463.
- Leipold E, DeBie H, Zorn S, Borges A, Olivera BM, Terlau H, and Heinemann SH (2007) μO conotoxins inhibit Na_v channels by interfering with their voltage sensors in domain-2. *Channels (Austin)* **1**:253–262.
- Leipold E, Hansel A, Olivera BM, Terlau H, and Heinemann SH (2005) Molecular interaction of δ-conotoxins with voltage-gated sodium channels. *FEBS Lett* **579**:3881–3884.
- McIntosh JM, Hasson A, Spira ME, Gray WR, Li W, Marsh M, Hillyard DR, and Olivera BM (1995) A new family of conotoxins that blocks voltage-gated sodium channels. *J Biol Chem* **270**:16796–16802.
- Momin A and Wood JN (2008) Sensory neuron voltage-gated sodium channels as analgesic drug targets. *Curr Opin Neurobiol* **18**:383–388.
- Narahashi T (2008) Tetrodotoxin: a brief history. *Proc Jpn Acad Ser B Phys Biol Sci* **84**:147–154.
- Patino GA and Isom LL (2010) Electrophysiology and beyond: multiple roles of Na⁺ channel β-subunits in development and disease. *Neurosci Lett* **486**:53–59.
- Pertin M, Ji RR, Berta T, Powell AJ, Karchewski L, Tate SN, Isom LL, Woolf CJ, Gilliland N, Spahn DR, et al. (2005) Up-regulation of the voltage-gated sodium channel β2 subunit in neuropathic pain models: characterization of expression in injured and non-injured primary sensory neurons. *J Neurosci* **25**:10970–10980.
- Priest BT, Murphy BA, Lindia JA, Diaz C, Abbadie C, Ritter AM, Liberator P, Iyer LM, Kash SF, Kohler MG, et al. (2005) Contribution of the tetrodotoxin-resistant voltage-gated sodium channel Na_v1.9 to sensory transmission and nociceptive behavior. *Proc Natl Acad Sci USA* **102**:9382–9387.
- Roy ML and Narahashi T (1992) Differential properties of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons. *J Neurosci* **12**:2104–2111.
- Satin J, Kyle JW, Chen M, Bell P, Cribbs LL, Fozzard HA, and Rogart RB (1992) A mutant of TTX-resistant cardiac sodium channels with TTX-sensitive properties. *Science* **256**:1202–1205.
- Schmidt D and MacKinnon R (2008) Voltage-dependent K⁺ channel gating and voltage sensor toxin sensitivity depend on the mechanical state of the lipid membrane. *Proc Natl Acad Sci USA* **105**:19276–19281.
- Shcherbatko A, Ono F, Mandel G, and Brehm P (1999) Voltage-dependent sodium channel function is regulated through membrane mechanics. *Biophys J* **77**:1945–1959.
- Sivilotti L, Okuse K, Akopian AN, Moss S, and Wood JN (1997) A single serine residue confers tetrodotoxin insensitivity on the rat sensory-neuron-specific sodium channel SNS. *FEBS Lett* **409**:49–52.
- Tate S, Benn S, Hick C, Trezise D, John V, Mannion RJ, Costigan M, Plumptre C, Grose D, Gladwell Z, et al. (1998) Two sodium channels contribute to the TTX-R sodium current in primary sensory neurons. *Nat Neurosci* **1**:653–655.
- Terlau H and Olivera BM (2004) *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev* **84**:41–68.
- Terlau H, Stocker M, Shon KJ, McIntosh JM, and Olivera BM (1996) MicroO-conotoxin MrVIA inhibits mammalian sodium channels, but not through site I. *J Neurophysiol* **76**:1423–1429.
- Vijayaragavan K, O'Leary ME, and Chahine M (2001) Gating properties of Na(v)1.7 and Na(v)1.8 peripheral nerve sodium channels. *J Neurosci* **21**:7909–7918.
- Vijayaragavan K, Powell AJ, Kinghorn IJ, and Chahine M (2004) Role of auxiliary β1-, β2-, and β3-subunits and their interaction with Na(v)1.8 voltage-gated sodium channel. *Biochem Biophys Res Commun* **319**:531–540.
- West PJ, Bulaj G, and Yoshikami D (2005) Effects of δ-conotoxins PVIA and SVIE on sodium channels in the amphibian sympathetic nervous system. *J Neurophysiol* **94**:3916–3924.
- Zhu S, Gao B, Deng M, Yuan Y, Luo L, Peigneur S, Xiao Y, Liang S, and Tytgat J (2010) Drosotoxin, a selective inhibitor of tetrodotoxin-resistant sodium channels. *Biochem Pharmacol* **80**:1296–1302.
- Zorn S, Leipold E, Hansel A, Bulaj G, Olivera BM, Terlau H, and Heinemann SH (2006) The μO-conotoxin MrVIA inhibits voltage-gated sodium channels by associating with domain-3. *FEBS Lett* **580**:1360–1364.

Address correspondence to: Doju Yoshikami, Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112. E-mail: yoshikami@bioscience.utah.edu