Na\(_\alpha\)\(\beta\) Subunits Modulate the Inhibition of Na\(_\alpha\)1.8 by the Analgesic Gating Modifier \(\mu\)O-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

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ABSTRACT

Voltage-gated sodium channels (VGSCs) consist of a pore-forming \(\alpha\)-subunit and regulatory \(\beta\)-subunits. Several families of neuroactive peptides of Conus snails target VGSCs, including \(\mu\)O-conotoxins and \(\mu\)-conotoxins. Unlike \(\mu\)-conotoxins and the guanidinium alkaloid saxitoxin (STX), which are pore blockers, \(\mu\)O-conotoxins MrVIA and MrVIB inhibit VGSCs by modifying channel gating. \(\mu\)O-MrVIA/B can block Na\(_\alpha\)1.8 (a tetrodotoxin-resistant isoform of VGSCs) and have analgesic properties. The effect of Na\(_\beta\)-subunit coexpression on susceptibility to block by \(\mu\)O-MrVIA/B and STX has, until now, not been reported. Here, we show that \(\beta_1\)-, \(\beta_2\)-, \(\beta_3\)-, and \(\beta_4\)-subunits, when individually coexpressed with Na\(_\alpha\)1.8 in Xenopus laevis oocytes, increased the \(k_{on}\) of the block produced by \(\mu\)O-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 \(\beta\)-subunit coexpression. Thus, coexpression of \(\beta\)-subunits with Na\(_\alpha\)1.8 can strongly influence the affinity of the conopeptide for the channel. This observation is of particular interest because \(\beta\)-subunit expression can be dynamic, e.g., \(\beta_2\)-expression is up-regulated after nerve injury (J Neurosci, 25: 10970–10980, 2005); therefore, the effectiveness of a \(\mu\)O-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\(_\alpha\)1.8 was only marginally, if at all, affected by coexpression of any of the \(\beta\)-subunits. Our results raise the possibility that \(\mu\)O-conotoxins and perhaps other gating modifiers may provide a means to functionally assess the \(\beta\)-subunit composition of VGSC complexes in neurons.

Introduction

The characterization of the effects of the guanidinium alkaloilds 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 that target VGSCs, each via a different mechanism: \(\mu\)-conotoxins, like the action of TTX and STX, block the channel’s pore (Catterall et al., 2007); \(\mu\)O-conotoxins are gating modifiers that block channel activation (Zorn et al., 2006; Heinemann and Leipold, 2007; Leipold et al., 2007); \(\gamma\)-conotoxins promote channel activation (Fiedler et al., 2008); and \(\delta\)-conotoxins inhibit fast inactivation (Leipold et al., 2005; West et al., 2005).

VGSCs are multisubunit complexes, with a single \(\alpha\)-subunit and one or more \(\beta\)-subunits (Catterall et al., 2007). In mammals, there are nine known \(\alpha\)-subunits (Na\(_{\alpha}\)1.1–Na\(_{\alpha}\)1.9), each of which when expressed alone can form a functional channel with distinctive kinetic and pharmacological properties (Catterall et al., 2005). Four Na\(_{\beta}\)-subunits (\(\beta_1\)–\(\beta_4\)) have been identified thus far, and coexpression of \(\beta\)-subunits with \(\alpha\)-subunits can have several functional consequences, including elevated sodium currents, faster inactivation kinetics, and alterations in voltage-sensitivity profiles; furthermore, \(\beta\)-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\(_{\alpha}\)1.8 and Na\(_{\alpha}\)1.9, 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.,...
1998; Tate et al., 1998), unlike all other Na\textsubscript{\textit{v}}s, except Na\textsubscript{\textit{v}}1.5, which is modestly resistant to TTX (Satin et al., 1992). Mutant mice in which the genes for Na\textsubscript{\textit{v}}1.8 or Na\textsubscript{\textit{v}}1.9 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 Na\textsubscript{\textit{v}}1.8 and Na\textsubscript{\textit{v}}1.9, 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 \textit{\mu}-conotoxins are one of the few ligands known to block Na\textsubscript{\textit{v}}1.8 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., 2006), although its precise target in this regard remains to be established.

As already mentioned, \textit{\mu}-conotoxins are gating modifiers that block channel activation. Other gating modifiers, such as scorpion toxins, can interact with \textit{\beta}-subunits; in fact, a scorpion toxin played a role in the initial discovery of \textit{\beta}-subunits (Hartshorne and Catterall, 1981). Thus, we were motivated to examine whether coexpression of \textit{\beta}-subunits with Na\textsubscript{\textit{v}}1.8 influenced the block by MrVIB. We demonstrate here, using cloned channels exogenously expressed in \textit{Xenopus laevis} oocytes, that the block produced by MrVIB is markedly affected by which \textit{\beta}-subunit is coexpressed with Na\textsubscript{\textit{v}}1.8.

TTX-resistant currents in rat DRG neurons and Na\textsubscript{\textit{v}}1.8 are considerably more sensitive to STX than to TTX (Roy and Narahashi, 1992; Sivilotti et al., 1997); thus, we compared the inhibition of Na\textsubscript{\textit{v}}1.8 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 \textit{\beta}-subunit coexpression.

**Materials and Methods**

**Clones.** The clone for rat Na\textsubscript{\textit{v}}1.8, provided by Prof. John Wood (University College, London, UK) in pRK7, was linearized with HpaI. Clones for rat \textit{\beta}1 and \textit{\beta}2 (Na\textsubscript{\textit{v}}1.1 and Na\textsubscript{\textit{v}}1.2), provided by Prof. Alan Goldin (University of California, Irvine, CA), in pBlSTa and pLCT1, respectively, were linearized with NotI. Clones for rat \textit{\beta}3 and \textit{\beta}4 (Na\textsubscript{\textit{v}}1.3 and Na\textsubscript{\textit{v}}1.4), in pcDNA3.1zeo(+) and pcDNA3.1zeo(-), provided by Prof. Lori Ison (University of Michigan, Ann Arbor, MI), were linearized with XbaI and BamHI, respectively. cRNA for Na\textsubscript{\textit{v}}1.8 was prepared using SP6 RNA polymerase. cRNAs for Na\textsubscript{\textit{v}}1.8–4 were constructed using T7 RNA polymerase (mMessage mMachine kit; Ambion, Austin, TX). A poly(A) tail was subsequently added to the cRNAs for Na\textsubscript{\textit{v}}1.3 and Na\textsubscript{\textit{v}}1.4 subunits [poly(A) tailing kit; Ambion].

**Injection of \textit{X. laevis} Oocytes.** Oocytes were prepared essentially as described previously (Fiedler et al., 2008). Each oocyte was injected with 69 nl of Na\textsubscript{\textit{v}}1.8 cRNA without or with \textit{\beta}-subunit cRNA (35 ng of each). Oocytes were incubated at 16°C for 6 to 11 days in a solution of ND96 (+ 196 mM NaCl, 2 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{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-\textmu l well in a wafer of Sylgard (Dow Corning, Midland, MI). Intracellular electrodes contained 3 M KCl (<0.3 M\Omega resistances). Sodium currents (I\textsubscript{\textit{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 $g_{\text{Na}} = I_{\text{Na}}/(V_{\text{step}} - V_{\text{rest}})$, where $g_{\text{Na}}$ is the conductance, $I_{\text{Na}}$ is the peak current amplitude in response to the potential step, $V_{\text{step}}$ is the test potential, and $V_{\text{rest}}$ 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 $V_{1/2}$ is the normalized $g_{\text{Na}}$, or $I_{\text{Na}}$. $V_{\text{step}}$ 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 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 $k_{\text{off}}$, and its voltage dependence was determined by plotting $k_{\text{off}}$ as a function of the amplitude of the conditioning pulse; the resulting curve was fit to a single-exponential function, $k_{\text{off}}(V) = \exp(kT \times V_{\text{cond}})$, where $V_{\text{cond}}$ 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 $k_{\text{off}}$ by assuming that $K = (Z_q \times q_s)/k_{\text{B}} \times T$, where $Z_q$ is the gating charge, $q_s$ is the elementary charge (i.e., that of a proton), $k_{\text{B}}$ is Boltzmann’s constant, and $T$ is absolute temperature (cf. Leipoldt et al., 2007; see also Hille, 2001). The factor $q_s/k_{\text{B}} \times T$ has a value close to 1/25 mV\textsuperscript{2} for room temperature, so $Z_q \approx 25$ mV\textsuperscript{2}/K.

It might be noted that the strong depolarizing pulses we used induced a potentiation of the I\textsubscript{\textit{Na}}, of oocytes expressing Na\textsubscript{\textit{v}}1.8. This “run-up” of I\textsubscript{\textit{Na}}, was frequently seen regardless of whether and which \textit{\beta}-subunit was coexpressed. No differences have been discerned in the biophysical or pharmacological properties of the I\textsubscript{\textit{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\textsubscript{\textit{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 l/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 l/min for 48 s to rapidly replace the fluid in the well, then at a rate of 30 \mu l/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 l/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 homemade 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 ± S.E.M., with n values representing the number of oocytes tested.

Results

Functional Consequences of Coexpression of Each of Four Na<sub>v</sub>-β-Subunits with Na<sub>v</sub>1.8. Sodium currents ($I_{Na}$) of Na<sub>v</sub>1.8 expressed in X. laevis oocytes either alone or coexpressed with β-subunits are illustrated in Fig. 1. Coexpression with β-subunits significantly affected the time course of fast inactivation as well as the voltage sensitivities of activation and inactivation of Na<sub>v</sub>1.8 (Table 1).

Coexpression of Each of the Four β-Subunits with Na<sub>v</sub>1.8 Increased the Rate of Block by MrVIB. The effects of MrVIB on Na<sub>v</sub>1.8 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, $k_{off}$. 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<sup>−1</sup>, for Na<sub>v</sub>1.8 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 $K_p$ values (Table 2). IC<sub>50</sub> values, however, were not obtained because with toxin concentrations near the expected nanomolar IC<sub>50</sub> 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<sub>v</sub>1.8 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<sub>50</sub> and $k_{off}$ values were not obtained because with toxin concentrations near the expected nanomolar IC<sub>50</sub> values, the times to reach steady-state block can be predicted to take a few hours, given the results in Fig. 2B and Table 2, which were outside the time window of our experiments.

**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

<table>
<thead>
<tr>
<th>$Na_v$1.8</th>
<th>Activation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inactivation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inactivation&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ mV</td>
<td>$k$</td>
<td>$n$</td>
</tr>
<tr>
<td>Alone</td>
<td>13 ± 0.2</td>
<td>8 ± 0.1</td>
<td>44</td>
</tr>
<tr>
<td>+ β1</td>
<td>6.5 ± 0.1*</td>
<td>7.2 ± 0.1*</td>
<td>47</td>
</tr>
<tr>
<td>+ β2</td>
<td>0.9 ± 0.1*</td>
<td>6.4 ± 0.1*</td>
<td>47</td>
</tr>
<tr>
<td>+ β3</td>
<td>3.4 ± 0.1*</td>
<td>7.3 ± 0.1*</td>
<td>18</td>
</tr>
<tr>
<td>+ β4</td>
<td>9.9 ± 0.1*</td>
<td>8.1 ± 0.1</td>
<td>35</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from Fig. 1B, left.
<sup>b</sup> Data from Fig. 1B, right.
<sup>c</sup> Statistically different from $Na_v$1.8 expressed alone (p < 0.007).

*Subunits with $Na_v$1.8.

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**TABLE 1**

Activation and inactivation parameters and time constants of fast inactivation of $Na_v$1.8 without and with β-subunit coexpression.

Values represent mean ± S.E.M., with n values as tabulated.
indicating that to readily observe possible effects of
terparts in Fig. 2B (i.e., those obtained with a holding poten-
point represent values of
interaction of MrVIB with the channel, the block by MrVIB of
interaction of MrVIB with the channel, larger perturbations
were obtained for use in B. Inset, recovery from block after toxin washout
of recovery followed an exponential function characterized by
of recovery from block, and the recovered traces seemed essentially identical to those of control (Fig. 4A insets). The time course
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
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 Na$_v$1.8 + β1 and Na$_v$1.8 + β2 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).

Discussion

Effects of β-Subunit Coexpression on the Biophysical Parameters of Na$_v$1.8. In our previous report concerning MrVIB and Na$_v$1.8, the latter was coexpressed in oocytes with β1 (Bulaj et al., 2006). In the present experiments, we examined five conditions: Na$_v$1.8 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 β1 coexpression are qualitatively consistent with those reported by Chahine’s group (Vijayaragavan et al., 2001); however, the changes induced by β2 or β3 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 Na$_v$1.8 and β-subunit clones we used were
TABLE 2
Effects of β-subunit coexpression on block of NaV1.8 by MrVIB and electrical capacitance of oocytes
Values are mean ± S.E.M., with n values as tabulated.

<table>
<thead>
<tr>
<th>NaV1.8</th>
<th>k_ona</th>
<th>k_offb</th>
<th>K_d c</th>
<th>n</th>
<th>Cell Capacitance c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min⁻¹µM⁻¹</td>
<td>min⁻¹</td>
<td>nM</td>
<td></td>
<td>µF</td>
</tr>
<tr>
<td>Alone</td>
<td>0.25 ± 0.01</td>
<td>0.0110 ± 0.0009</td>
<td>44 ± 4.6</td>
<td>22</td>
<td>0.27 ± 0.00</td>
</tr>
<tr>
<td>+ β1</td>
<td>0.75 ± 0.22 **</td>
<td>0.0064 ± 0.0011 *</td>
<td>8.5 ± 2.9</td>
<td>16</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>+ β2</td>
<td>7.93 ± 0.21 **</td>
<td>0.0076 ± 0.0007 *</td>
<td>1.0 ± 0.2</td>
<td>22</td>
<td>0.89 ± 0.04 **</td>
</tr>
<tr>
<td>+ β3</td>
<td>0.57 ± 0.03 **</td>
<td>0.0037 ± 0.0009 *</td>
<td>6.5 ± 1.8</td>
<td>5</td>
<td>0.30 ± 0.01 *</td>
</tr>
<tr>
<td>+ β4</td>
<td>1.74 ± 0.03 **</td>
<td>0.0062 ± 0.0024 *</td>
<td>3.5 ± 1.2</td>
<td>4</td>
<td>0.26 ± 0.01</td>
</tr>
</tbody>
</table>

Values of k_on were calculated from slopes of linear regression curves illustrated in Fig 2B.

Membrane capacitances of oocytes were determined as described under Materials and Methods.

* Statistically different from NaV1.8 expressed alone (p < 0.05).
** Statistically different from NaV1.8 expressed alone (p < 0.0001).

prepared by different laboratories than those used by Cha- 
hine’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 NaV1.8 + 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 NaV1.8 to MrVIB. We (Bulaj et al., 2006) and others (Ekberg et al., 2006) demonstrated previously that sodium currents of oocytes expressing NaV1.8 were suscepti-
ble to block by MrVIB; however, in neither case were the effects of β-subunit coexpression examined. Our present ex-
periments 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-subs-
unit; 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 NaV1.8 indicated that the k_on increased to a 
level intermediate between those when each β-subunit alone 
was coexpressed with NaV1.8 (data not shown); tests with 
other combinations of β-subunits remain to be pursued.

We reported previously that MrVIB blocked the TTX-resis-
tant sodium current in rat DRG neurons, probably of 
NaV1.8, with an IC_{50} of approximately 13 nM (Bulaj et al., 2006). This value is closer to the K_d values for NaV1.8 coex-
pressed 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 respon-
sible 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 hy-
pothesis 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 mem-
brane properties or composition induced by β-subunit expres-
sion 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 mem-
brane, which is reflected by larger cell capacitance, through 
increased size and number of microvilli (Isom et al., 1995).
We confirmed that β2-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 NaV1.4 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 NaV1.2 expressed in X. laevis oocytes was found to be faster than from outside-out patches (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 NaV1.4 expressed in human embryonic kidney cells was quite reversible, with a \( k_{\text{off}} \) 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 \( k_{\text{off}} \) values for block of NaV1.8 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 \( k_{\text{off}} \) was disproportionately smaller when the duration of the conditioning pulse was reduced from 300 to 100 ms. This suggests that upon depolarization \( k_{\text{off}} \) 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 \( k_{\text{off}} \), which suggests an effective transfer of 1.1 elementary charges is involved in gating \( k_{\text{off}} \) (see Materials and Methods). This is not far from the estimated 1.8 elementary charges reported for the recovery from MrVIA block of NaV1.4 (Leipold et al., 2007). It should be noted that in the experiments with NaV1.4 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 NaV1.8 to Saxitoxin. STX blocked NaV1.8, 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 NaV1.8 toward a pore-forming peptide, like STX. To our knowledge, this is the first report to document the relative insensitivity of the block of any NaV1α-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

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**Fig. 4.** Depolarizing conditioning (Cond.) pulses accelerate the washout of MrVIB. Oocytes were voltage-clamped, and \( I_{\text{Na}} \) 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 NaV1.8, +β1, and +β2, 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 ± S.E.M. Error bars are both positive- and negative-going for NaV1.8 expressed alone, positive-going for +β1, and negative-going for +β2; \( n = 3 \) oocytes for NaV1.8, 9 for +β1, and 3 for +β2. Minimal recovery from block is seen until commencement of conditioning pulses (at \( t = 10 \) min), after which block is relieved with a rate constant, \( k_{\text{off}} \), whose values are presented in the bar graph (see Inset). C, semilog plot of \( k_{\text{off}} \) versus amplitude of conditioning pulse for oocytes expressing NaV1.8 with either β1 or β2. Values in parentheses indicate numbers of oocytes tested. Data were fit to a single exponential function yielding \( \Delta K \) values (see Materials and Methods) of 0.044 and 0.046 mV\(^{-1}\) for +β1 and +β2, respectively (\( \sigma = 0.99 \) for both curves).
development of MrVIB and related conotoxins, as well as other gating-modifier toxins, for diagnostic and therapeutic applications. The observation that the coexpression of the β-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\textsubscript{\textalpha}1.8 may have been enhanced by the coexpression of β-subunits.

The present experiments used the X. laevis oocyte expression system, principally because of the relative ease with which the functional activities of Na\textsubscript{\textalpha}1.8 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\textsubscript{\textalpha}1.8 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 μ-conotoxins and perhaps other gating modifiers can be used to functionally probe the β-subunit composition of VGSC complexes in neurons.

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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.

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