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Synthetic Ciguatoxins Selectively Activate $Na_v1.8$-derived Chimeric Sodium Channels Expressed in HEK293 Cells*

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The synthetic ciguatoxin CTX3C has been shown to activate tetrodotoxin (TTX)-sensitive sodium channels ($Na_v1.2$, $Na_v1.4$, and $Na_v1.5$) by accelerating activation kinetics and shifting the activation curve toward hyperpolarization (Yamaoka, K., Inoue, M., Miyahara, H., Miyazaki, K., and Hirama, M. (2004) *Br. J. Pharmacol.* 142, 879–889). In this study, we further explored the effects of CTX3C on the TTX-resistant sodium channel $Na_v1.8$. TTX-resistant channels have been shown to be involved in transducing pain and related sensations (Akopian, A. N., Sivilotti, L., and Wood, J. N. (1996) *Nature* 379, 257–262). Thus, we hypothesized that ciguatoxin-induced activation of the $Na_v1.8$ current would account for the neurological symptoms of ciguatera poisoning. We found that 0.1 μM CTX3C preferentially affected the activation process of the $Na_v1.8$ channel compared with those of the $Na_v1.2$ and $Na_v1.4$ channels. Importantly, without stimulation, 0.1 μM CTX3C induced a large leakage current (I_L). The conductance of the I_L calculated relative to the maximum conductance (G_{max}) was 10 times larger than that of $Na_v1.2$ or $Na_v1.4$. To determine the molecular domain of $Na_v1.8$ responsible for conferring higher sensitivity to CTX3C, we made two chimeric constructs from $Na_v1.4$ and $Na_v1.8$. Chimeras containing the N-terminal half of $Na_v1.8$ exhibited a large response similar to wild-type $Na_v1.8$, indicating that the region conferring high sensitivity to ciguatoxin action is located in the D1 or D2 domains.

Ciguatoxin is a lipophilic cyclic polyether with 13 ether rings (Fig. 1) derived from the dinoflagellate *Gambierdiscus toxicus* (1) and is a cause of ciguatera, which is a widespread fish poisoning that presents a variety of neurological symptoms, including hyperalgesia and allodynia. These symptoms might be a consequence of the direct interaction of ciguatoxin with voltage-dependent Na^+ channels expressed in the peripheral nervous system. CTX1B[2] suppressed fast sodium channel inactivation, evoked spontaneous action-potential firing of frog nodal membranes (2), and induced a tetrodotoxin-sensitive (TTX-S) persistent leakage current of rat DRG neurons (3). Furthermore, it has been shown that ciguatoxin competes with brevetoxin binding to site 5 of $Na_v1.2$ channels by radioactive photolabeling techniques (4, 5). However, it is still uncertain how ciguatoxin causes pain or how it modulates sensory inputs by changing the activity of sodium channels expressed in DRG neurons. Multiple sodium channel isoforms are expressed in DRG neurons, including both TTX-S ($Na_v1.1$, $Na_v1.6$, and $Na_v1.7$) and TTX-resistant (TTX-R) ($Na_v1.8$ and $Na_v1.9$) Na^+ channels (6). Among these, $Na_v1.8$ might be of great importance for transmitting pain signals, because it is expressed exclusively by primary afferent neurons (7, 8), and it also carries more than 80% of the current at the rising phase of the action potential of small DRGs (9). In addition, deletion of the $Na_v1.8$ gene in mice produced mutants that were resistant to noxious mechanical stimuli and refractory to the development of inflammatory hyperalgesia (7, 8). Similarly, antisense oligonucleotides directed against $Na_v1.8$ reversed neuropathic pain behavior (10). Acute and selective pharmacological blockade of $Na_v1.8$ sodium channels *in vivo* produces significant antinociception in animal models of neuropathic and inflammatory pain (11). Thus, ciguatoxin might preferentially attack $Na_v1.8$ compared with TTX-S Na^+ channels, explaining the neurological symptoms of ciguatera. We tested the effects of synthetic ciguatoxin CTX3C on heterologously expressed $Na_v1.2$, $Na_v1.4$, and $Na_v1.8$ channels in either HEK293 or ND7-23 cells. To confirm the preferential effects of CTX3C on $Na_v1.8$ channels, we also tested the effects on chimeric channels formed from the N-terminal domains of $Na_v1.8$ and the C-terminal domains of $Na_v1.4$, or vice versa. $Na_v1.8$ expressed in ND7-23 cells had an apparently increased sensitivity to ciguatoxins compared with that of $Na_v1.2$ and $Na_v1.4$ expressed in HEK293 cells. Data from chimeric channels indicated that high sensitiv-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB453238.

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[2] The abbreviations used are: CTX1B, pacific CTX-1; TTX, tetrodotoxin; TTX-S, TTS-sensitive; TTX-R, TTX-resistant; DRG, dorsal root ganglion; SNS, sensory neuron-specific.

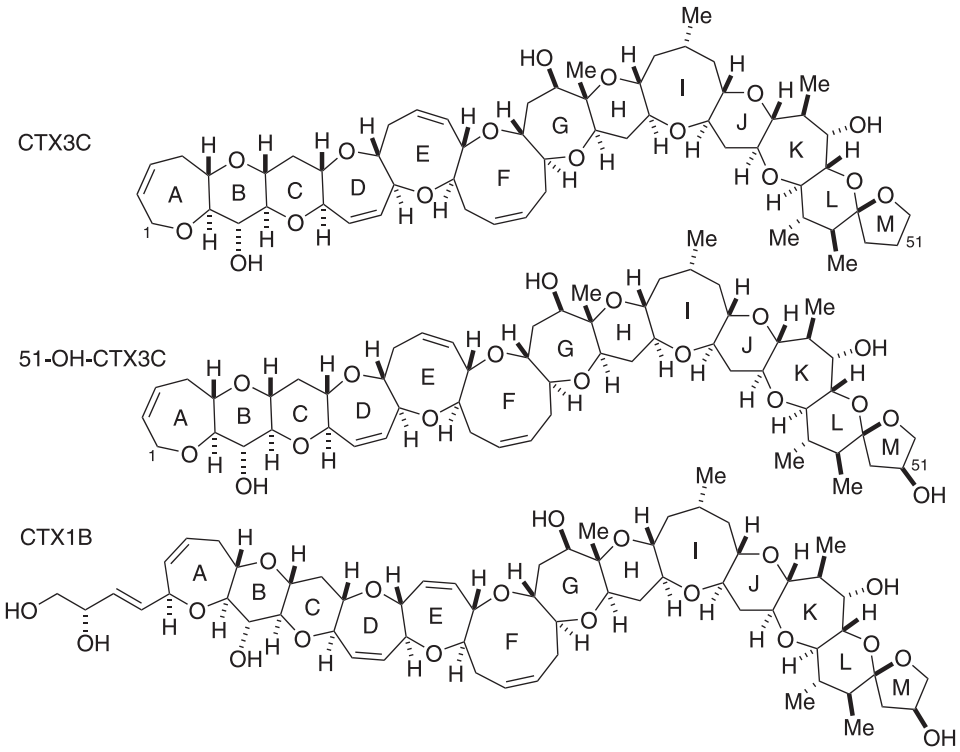

FIGURE 1. **Structures of CTX3C, 51-OH-CTX3C, and CTX1B.**

ity to ciguatoxins of $Na_v1.8$ was derived of the N-terminal half of the $Na_v1.8$ molecule.

EXPERIMENTAL PROCEDURES

All procedures involving animal handling and experimental protocols were approved by the Institutional Animal Care Committee (Hiroshima International University and Hiroshima University) and carried out in accordance with the guidelines issued by the National Institutes of Health.

Construction of Chimeras—The α-subunits of rat skeletal muscle Na^+ channel ($Na_v1.4$) (12) and rat brain type II Na^+ channel ($Na_v1.2$) (13) cDNAs were cloned into mammalian expression vectors of pcDNA3.1(−) (Invitrogen) and pCI-neo (Promega, Madison, WI), respectively. The sensory neuron-specific (SNS) TTX-R Na^+ channel ($Na_v1.8$) cDNA (7) was cloned into pcDNA3.1(−). Chimeras were constructed using the two cDNAs by swapping domains; the N-terminal half-domains (D1D2) of the SNS cDNA were flanked by the C-terminal half-domains (D3D4) of the μ1 cDNA resulting in SS/MM (the first two domains derived from SNS Na^+ channels followed by the last two domains derived from μ1 Na^+ channels), and vice versa resulting in MM/SS, at the junction of the intracellular loop between D2 and D3, corresponding to Pro-918 for μ1 cDNA or Pro-1000 for SNS cDNA. Briefly, the insertion of five bases, GGCCG, at the proline coding codon, C/CC, created the NotI site, GC/GGCCGC, using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Domain swapping was performed at the created NotI sites. Finally, the artificially inserted five bases were deleted using the QuikChange mutagenesis kit. All of the resulting constructs were confirmed with restriction mapping and sequencing using an ABI PRISM ™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Isolation of Rat p11—Total cellular RNA was isolated from myometrial tissue dissected from adult Sprague-Dawley rats following the TRIzol protocol (Invitrogen). mRNA was purified by a FirstTrack mRNA purification kit (Invitrogen). First-strand and second-strand cDNA were synthesized with a Marathon cDNA kit using poly(dT) primer (BD Biosciences). The final cDNA product was used as a myometrium marathon cDNA template for PCR amplification with rat DRG p11-specific forward (5′-ATGCCATCCCAAATGGAGCAT-3′) and reverse primers (5′-CTACTTCTTCTGCTTCATGTGTACTAC-3′). The rat p11 sequence has been deposited with DNA Data Bank of Japan (DDBJ) data libraries under the accession number AB453238.

Transient Transfection and Cell Culture—The wild-type rat $Na_v1.4$ and rat $Na_v1.2$ cDNAs were transiently transfected into HEK293 cells with the S65A bright green fluorescent protein mutant in the vector pCA-GFP (14) to allow the detection of transfected cells. Chimeric mutants of SS/MM and MM/SS were cotransfected into HEK293 cells with the Naβ_1 (15) subunit subcloned into pIRES-hrGFP-1a (Stratagene) to increase the expression levels as well as for the detection of transfected cells. $Na_v1.8$ was poorly expressed in HEK293 cells. Thus, we employed a more suitable heterologous expression system for $Na_v1.8$ Na^+ channels. The wild-type SNS cDNA was transiently transfected into ND7-23 cells purchased from Sigma with p11, subcloned into pIRES-hrGFP-1a. John *et al.* (16) reported that the ND7-23 cell line allowed stable expression of rat $Na_v1.8$ channels with biophysical properties that closely resemble the native TTX-resistant currents in DRG neurons. Also, p11 was reported to promote the translocation of $Na_v1.8$ to the plasma membrane, producing functional channels (17). The transfection of cDNAs into HEK293 cells or ND7-23 cells was performed with Effectine reagent (Qiagen K. K., Tokyo, Japan). Cells were grown to 50% confluence in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 30 units/ml penicillin G (Invitrogen), and 30 μg/ml streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The transfected cells were used for electrophysiological experiments through post-transfection day 3. Transfection-positive cells were identified by epifluorescence microscopy performed using a 100-watt mercury lamp and a standard fluorescein isothiocyanate filter set (Nikon, Tokyo, Japan). Single isolated green fluorescent cells were then studied as described below.

Solutions and Chemicals—The bath solution contained 70 mM NaCl, 67 mM *N*-methyl-D-glucamine, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES (pH 7.4). TTX (0.3

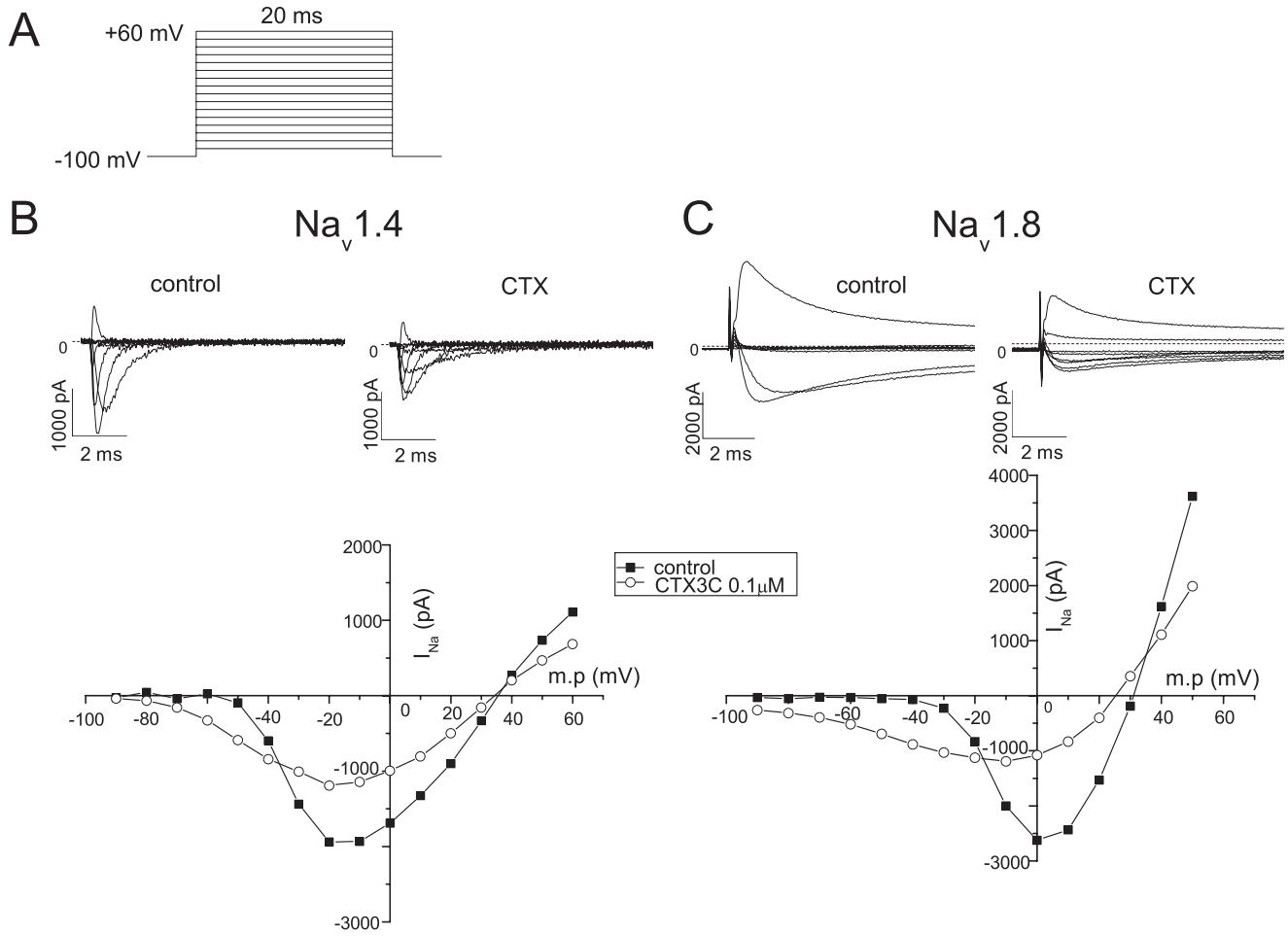

FIGURE 2. **Typical effects of 0.1 μM CTX3C on the current-voltage relationships of $Na_v1.4$ and $Na_v1.8$ Na^+ channels.** *A,* depolarizing test pulses (20 ms) from a holding potential of −100 to 60 mV in 10-mV increments were given every 1 s, before (*left*) and 5 min after (*right*) perfusion with 0.1 μM CTX3C. For clarity, the current records of I_{Na} are displayed only in 20-mV steps. The peak I_{Na} at each voltage step was measured and plotted against the membrane potential (shown at the *bottom* of *B* and *C*) in the absence (■) and presence (○) of 0.1 μM CTX3C. Slower activation and incomplete inactivation of $Na_v1.8$ channels compared with $Na_v1.4$ channels are clearly shown in the current records shown *above* each *I-V* graph. Voltage steps near the resting potential evoked earlier activation in the presence of CTX3C in both channels. Note the suppression of the peak I_{Na} by CTX3C.

μM) was added only when currents from ND7-23 cells were recorded to eliminate I_{Na} other than $Na_v1.8$. The pipette solution contained 70 mM CsF, 60 mM CsCl, 12 mM NaF, 5 mM EGTA, and 5 mM HEPES (pH 7.4). Ciguatoxin congeners, CTX3C, and 51-hydroxy-CTX3C (51-OH-CTX3C) were synthesized *de novo* by the method described previously (18–20). The stock solution of the ciguatoxin congeners (1 mM) in DMSO was diluted with bath solution to a final concentration of 0.1–10 μM. Bath solution was continuously supplied at a rate of 2 ml min^{-1}. Separately, as described previously (21), CTX3C solution was directly infused into the bath chamber with siphon and vacuum tubing in a Y configuration, so that the cells under study were positioned in the center of the stream.

Electrophysiological Recording and Data Analysis—Macroscopic I_{Na} from transfected cells was measured using the whole-cell patch clamp method. Whole-cell patch pipettes with a resistance of 1.5–3 megohms were used to achieve optimum voltage control. Whole-cell currents (filtered at 10 kHz) were recorded using an Axopatch 200B amplifier (Axon Instruments; Foster City, CA), and more than 80% of the series resistance was compensated to minimize voltage errors. Recordings were started 10 min after establishing a whole-cell recording configuration. Whole-cell membrane currents were digitized at a sampling rate of 50–100 kHz with a 12-bit analogue-to-digital converter (DigiData 1321A interface; Axon Instruments), controlled by pClamp software (version 8; Axon Instruments). I_{Na} was determined by subtraction of linear capacitative and leak current with the P/4 protocol (22) in all experiments, except for the case where a relatively large background current was induced with CTX3C or those that were undertaken to measure the voltage dependence of steady-state inactivation. All experiments were conducted at room temperature (22–24 °C). Data are presented as mean ± S.E. along with the number of observations (n), unless otherwise stated. Statistically significant differences between group means were determined by the Student's t test for paired or unpaired observations, as appropriate. The criterion for statistical significance was $p < 0.05$.

RESULTS

Ciguatoxin-induced Background Currents through Na^+ Channels—It has been demonstrated that at least 1 μM CTX3C is necessary to modulate $Na_v1.2$, $Na_v1.4$, and $Na_v1.5$ channels (21) when the holding potential is kept at −140 mV. In this study, the holding potential was set at −100 mV.

Under these conditions, 0.1 μM CTX3C was sufficient to induce activation of $Na_v1.2$ (data not shown) and $Na_v1.4$ channels expressed in HEK293 cells, as demonstrated by a negative shift of threshold potential, a shortening of time to peak, and a suppression of the amplitude of the sodium current I_{Na} (Fig. 2*B*). In the presence of TTX (0.3 μM), we recorded the I_{Na} of $Na_v1.8$ channels expressed in ND7-23 cells (23), the functional expression of which is enhanced with cotransfection of p11 as reported previously (16, 17). $Na_v1.8$ channels were activated and reached maximum currents at more depolarized potentials (from −50 to −30 mV for threshold and from −10 to +10 mV for peak potentials) and were inactivated with a 10 times slower time course than those of $Na_v1.4$ (Fig. 2*C*). The most prominent difference between $Na_v1.4$ and $Na_v1.8$ channels was that background (or leak) currents were induced in the latter without depolarizing pulses at −100 mV (Fig. 3). CTX3C (0.1 μM) induced a large current of more than 400 pA in $Na_v1.8$, but not in $Na_v1.4$ (~6 pA), when no depolarizing pulses were applied from a holding potential of −100 mV, although both cells exhibited comparable maximum peak currents (−2,650 pA *versus* −2,370 pA for $Na_v1.8$ and $Na_v1.4$, respectively) in the absence of CTX3C.

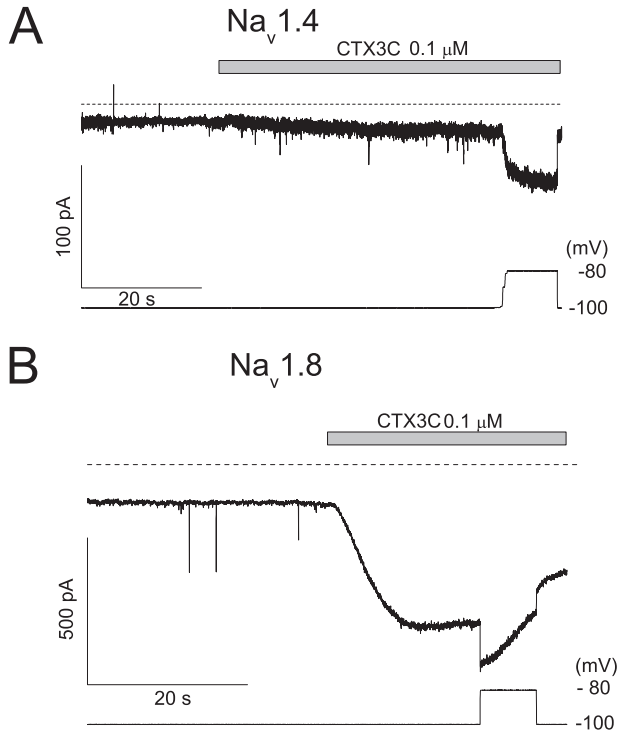

FIGURE 3. **Spontaneous induction of sustained current by CTX3C without depolarized steps.** The membrane was continuously clamped at −100 mV during the perfusion of 0.1 μM CTX3C. This induced a spontaneous leak current of ~6 pA in $Na_v1.4$ (*A*), but a large current in $Na_v1.8$ (*B*). Further depolarization to −80 mV (the *bottom traces* in each panel indicate the membrane potential applied) increased the currents in both $Na_v1.4$ and $Na_v1.8$ channels, but the $Na_v1.8$ channels were already susceptible to inactivation at −80 mV.

Quantification of Ciguatoxin-induced Resting Sodium Conductance—As the number of functionally expressed channels varies from cell to cell, it is not appropriate simply to compare the amplitude of ciguatoxin-induced currents between cells. Maximum conductance in the absence of CTX3C (G_{max}-control) is a good measure of the number of functionally expressed channels in each cell. Thus, we evaluated the potency of CTX3C to induce background conductance (resting sodium conductance) at −100 mV in the presence of 0.1 μM CTX3C relative to G_{max}-control. CTX3C (0.1 μM) induced a resting sodium conductance of 0.84 ± 0.27% (n = 7) relative to the G_{max}-control in $Na_v1.8$, which was significantly larger than that in $Na_v1.4$ (0.08 ± 0.03%, n = 5; $p <$ 0.05; Fig. 4).

Molecular Determinants of $Na_v1.8$ Exhibiting Larger Resting Sodium Conductance in Response to CTX3C—To test whether the effects of CTX3C on $Na_v1.8$ expressed in ND7-23 cells are derived from ND7-23 cell environment or $Na_v1.8$ molecule itself, we made two chimeras from $Na_v1.4$ and $Na_v1.8$ channels that could be successfully expressed in HEK293 cells: MM/SS and SS/MM. The former contained the two N-terminal domains of $Na_v1.4$ followed by the C-terminal two domains of $Na_v1.8$, and vice versa for the latter (Fig. 4*A*). CTX3C (0.1 μM) induced a resting sodium conductance of 0.85 ± 0.12% (n = 6) in SS/MM-expressing cells, relative to the G_{max}-control, which was significantly larger ($p <$ 0.01) than that of the MM/SS-expressing cells (0.05 ± 0.03%; n = 5), but similar to $Na_v1.8$ in ND7-23 cells. We further tested whether the property of SS/MM could be preserved in ND7-23 cells. SS/MM in ND7-23 cells responded to 0.1 μM 51-OH-CTX3C producing a resting sodium conductance of 0.40 ± 0.20% (n = 4) relative to the G_{max}-control, which are comparable with data from SS/MM in HEK293 cells in the presence of 0.1 μM 51-OH-CTX3C, 1.91 ± 1.33%

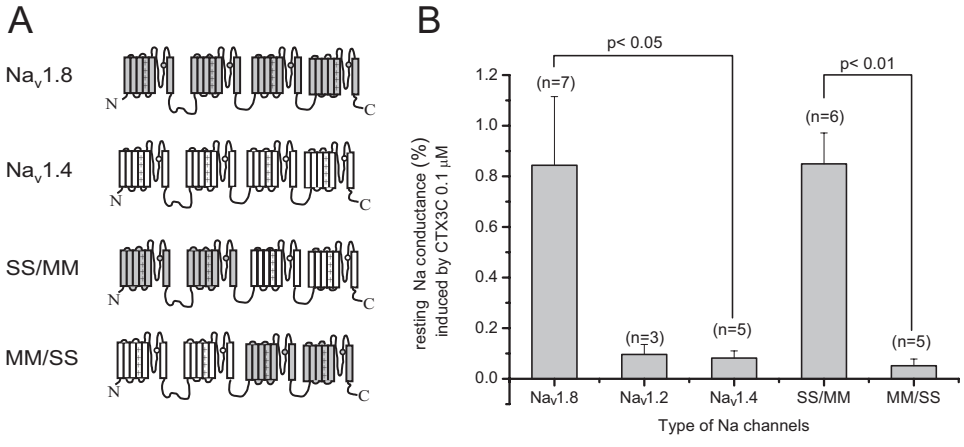

FIGURE 4. **Structure/activity relationships of wild-type and chimeric $Na_v1.8/1.4$ channels.** *A,* schematic presentation of chimeric constructs. *B,* effects of CTX3C on resting Na^+ conductance of three wild-type Na^+ channel subtypes and two chimeras. The ciguatoxin-induced leakage current magnitudes were quantitatively evaluated by dividing the ciguatoxin-induced resting conductance by the maximum conductance (G_{max}) in the absence of CTX3C. The $Na_v1.8$ and SS/MM showed 10 times larger conductance than those of $Na_v1.2$, -1.4, and MM/SS.

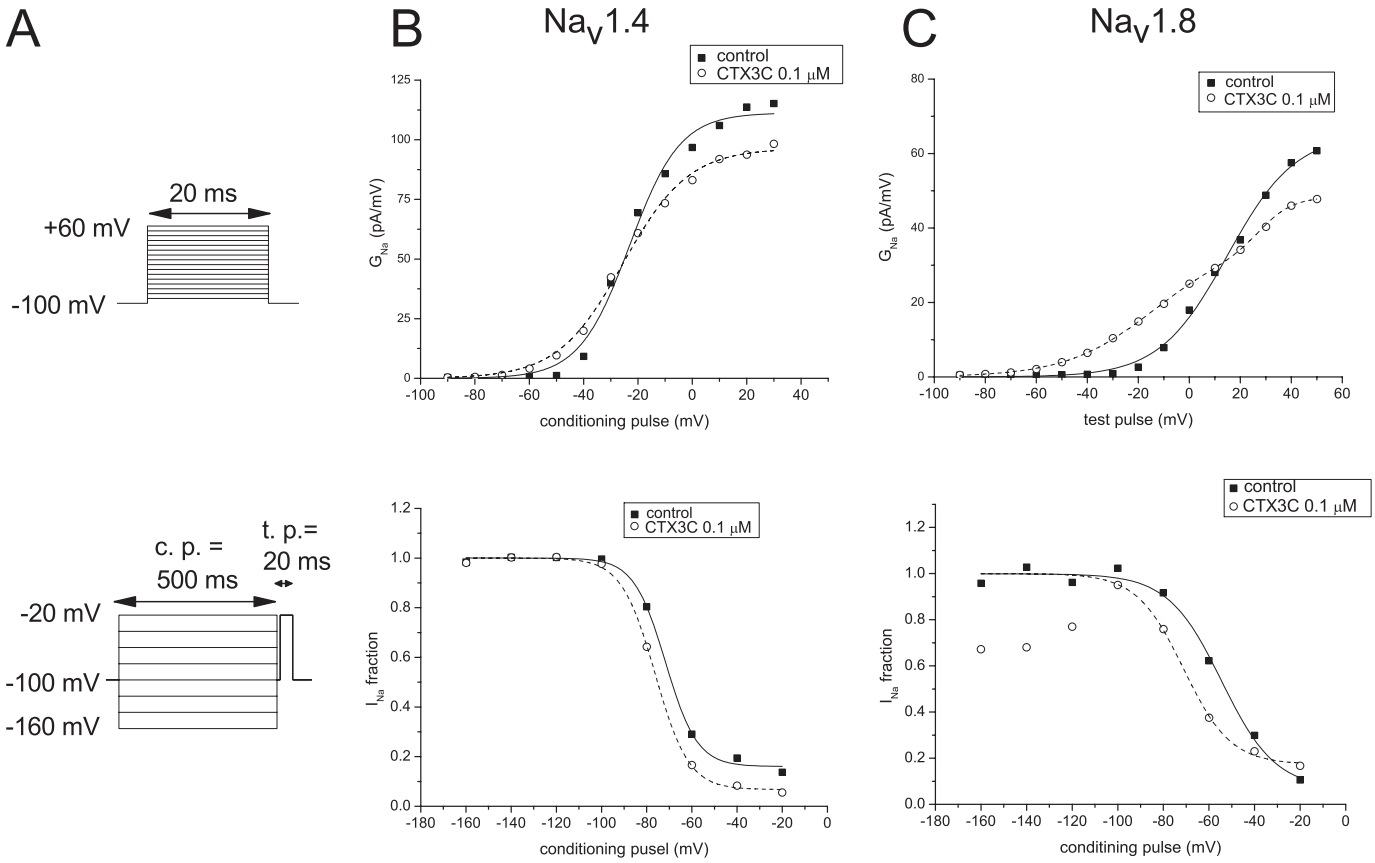

FIGURE 5. **Representative plots of activation and steady-state inactivation curves for $Na_v1.4$ and $Na_v1.8$ channels.** *A*, pulse protocols for activation curves (*top*) and inactivation curves (*bottom*). *B* and *C*, *symbols* indicate data in the absence (*solid lines* and *closed squares*) and presence (*dotted lines* and *open circles*) of 0.1 μM CTX3C. Boltzmann functions (*smooth lines*) were fitted to each set of activation curves

$$\left(\frac{1}{1+\exp\left(V-\frac{V_{act1/2}}{k_{act}}\right)}\right)$$

or inactivation curves

$$\left(\frac{1}{1+\exp\left(V-\frac{V_{inact1/2}}{k_{inact}}\right)}+c\right)$$

In the case of ciguatoxin-modified $Na_v1.8$ channels, activation curves were fitted by a sum of two Boltzmann distributions

$$\left(\frac{1}{1+\exp\left(V-\frac{V1_{act1/2}}{k1_{act}}\right)}+\frac{1}{1+\exp\left(V-\frac{V2_{act1/2}}{k2_{act}}\right)}\right)$$

During standard double-pulse protocols for the steady-state inactivation measurement, giving conditioning pulses of −120 to −100 mV, increased the availability of Na^+ channels in the presence of CTX3C before the transition to inactivation, especially in $Na_v1.8$, making a peak in the curve at around −100 mV. In this case, a fit was obtained by not fitting data points showing potentials more negative than −100 mV. The parameters obtained by the fitting procedure for all of the constructs are shown in Table 1 for the activation curve and in Table 2 for the steady-state inactivation curve. The activation curves were calculated from the peak I_{Na} value obtained at each membrane potential divided by the driving force. The steady-state inactivation curves were obtained by a double-pulse protocol. The I_{Na} evoked with a test pulse to −20 mV was preconditioned by a 500-ms clamp step to a variable membrane potential (−160 to −20 mV in 20-mV increments) with a gap of 0.25 ms, and normalized by the unconditioned current elicited with a test pulse from the holding potential (−100 mV).

($n = 3$). We used 51-OH-CTX3C here, because of a lack of availability of CTX3C. 51-OH-CTX3C had a quantitatively similar effect on resting sodium conductance of SS/MM in HEK293 cells to that of CTX3C (see Fig. 4*B*).

Changes in Sodium Channel Kinetics Induced by CTX3C—Induction of resting sodium conductance by CTX3C might be an extreme form of leftward shift of the activation curve that can be observed using the conductance-voltage relationship (Fig. 5). The voltage dependences of activation of $Na_v1.4$, $Na_v1.8$, and the related chimera (SS/MM and MM/SS) channels in the absence of CTX3C were adequately described by a single Boltzmann distribution; however, the sum of two Boltzmann distributions was required for ciguatoxin-modified $Na_v1.8$ (Fig. 5*B*) and SS/MM channels. The voltages required for half-maximal activation ($V_{act1/2}$; midpoint) and k_{act} (slope factor) for each construct are shown in Table 1. The $V_{act1/2}$ for $Na_v1.8$ and

TABLE 1

Ciguatoxin-induced changes in activation properties measured with conductance-voltage relationship

		$V_{act1/2}$ (mV)			% Reduction in G_{max}
	n	*Control*	*CTX3C*		
$Na_v1.2$	3	–20.3 ±1.8	–28.7± 5.5		21.6 ± 6.7
$Na_v1.4$	5	–25.9 ± 3.8 *	–28.6 ± 4.5		21.4 ± 8.0
$Na_v1.8$	6	4.9 ± 2.0	15.8 ± 5.8	–22.7 ± 10.8	18.2 ± 12.3
			40.9 ± 11.9 %		
SS/MM	3	–0.1 ± 3.0	8.3 ± 6.1	–28.0 ± 3.5	16.3 ± 17.5
			30.0 ± 20.8 %		
MM/SS	4	–23.5 ± 3.9	–31.9 ± 6.5		37.2 ± 8.0

		k_{act} (mV)		
	n	*Control*		*CTX3C*
$Na_v1.2$	3	–9.7 ± 0.2 *	–13.2 ± 0.5	
$Na_v1.4$	5	–9.1 ± 0.7 *	–12.5 ± 1.4	
$Na_v1.8$	6	–11.1 ± 0.88	–7.0 ± 1.0	–18.3 ± 2.8
SS/MM	3	–11.2 ± 1.6	–4.4 ± 2.0	–17.8 ± 1.4
MM/SS	4	–9.3 ± 0.4 *	–11.5 ± 0.8	

* $p < 0.05$, statistical comparisons were performed using the paired two-tailed Student's *t* test.

TABLE 2

Ciguatoxin-induced changes in inactivation properties measured with steady-state inactivation curves

		$V_{inact1/2}$ (mV)	
	n	*Control*	*CTX3C*
$Na_v1.2$	3	–67.8 ± 1.0 *	–76.8 ± 2.6
$Na_v1.4$	5	–73.8 ± 2.0 *	–82.1 ± 2.7
$Na_v1.8$	5	–63.4 ± 3.7 *	–71.5 ± 2.3
SS/MM	3	–62.8 ± 1.5	–64.2 ± 1.1
MM/SS	4	–58.2 ± 2.5 **	–70.7 ± 3.9

		k_{inact} (mV)	
	n	*Control*	*CTX3C*
$Na_v1.2$	3	6.0 ± 0.4	6.3 ± 0.7
$Na_v1.4$	5	7.4 ± 0.3	8.0 ± 0.4
$Na_v1.8$	5	10.3 ± 1.4	9.8 ± 1.1
SS/MM	3	8.6 ± 2.2	5.4 ± 0.7
MM/SS	4	7.9 ± 1.4	10.4 ± 1.1

* $p < 0.05$.
** $p < 0.01$; statistical comparisons were performed using the paired two-tailed Student's *t* test.

SS/MM in control cells was more depolarized by ~20–30 mV than those for $Na_v1.4$ and MM/SS, respectively, indicating that the high threshold nature of $Na_v1.8$ is derived from the N-terminal domains of the molecule (D1/D2). Ciguatoxin application induced a leftward shift of the $V_{act1/2}$ of $Na_v1.4$ to a limited extent (~3 mV), but more substantially in MM/SS, although the slopes of the two constructs were not notably changed (Table 1). A significant effect on the $V_{act1/2}$ was seen in both $Na_v1.8$ and SS/MM; an extra component of the activation curve (the low threshold component) was created in a more hyperpolarized range. The $V_{act1/2}$ values for the high threshold component for $Na_v1.8$ and SS/MM, respectively, were depolarized by 11 and 8 mV compared with that of the single component in each respective control (Table 1). In addition, the slope factors of the ciguatoxin-created low threshold components of $Na_v1.8$-expressing and SS/MM-expressing cells were remarkably large (−18.3 ± 2.8 and −17.8 ± 1.4 mV, respectively). The creation of this $Na_v1.8$-selective component may be related to the induction of resting sodium conductance.

Depolarizing square pulses to −10 mV from a holding potential of −100 mV in a voltage clamp condition activated the channels and let their currents reach peak values within 0.44 ± 0.05 ms ($n = 3$) in $Na_v1.2$-expressing cells and 0.43 ± 0.04 ms ($n = 5$) in $Na_v1.4$-expressing cells; however, this was much slower in $Na_v1.8$-expressing cells as the time to peak for $Na_v1.8$ was 1.51 ± 0.28 ms ($n = 6$). The values of the time to peaks of the related constructs, SS/MM and MM/SS, fell between these limits (1.01 ± 0.05 ms, $n = 3$, for SS/MM and 0.76 ± 0.16 ms, $n = 4$, for MM/SS). CTX3C (0.1 μM) accelerated the activation process of the Na^+ channels by shortening the time to peaks by 26.0 ± 8.2% ($n = 3$) in $Na_v1.2$ and by 11.3 ± 3.3% ($n = 5$) in $Na_v1.4$, but this effect was significantly larger ($p < 0.05$) in $Na_v1.8$ (by 31.9 ± 6.9%, $n = 6$). Again, the related constructs, SS/MM and MM/SS, showed values between those of $Na_v1.4$ and $Na_v1.8$.

The suppressive effect of CTX3C on the Na^+ channels can be seen in the changes in maximum conductance (G_{max}) estimated by fitting conductance-voltage relationships using the Boltzmann equation (Fig. 5). When the activation curve was made of the sum of two components, the G_{max} was treated as the sum of each amplitude ($G_{max} = G_{max1} + G_{max2}$). CTX3C (0.1 μM) equally reduced the G_{max} of $Na_v1.2$, $Na_v1.4$, $Na_v1.8$, and SS/MM by around 18–22%, except in MM/SS-transfected cells, where it was reduced by 37% (Table 1).

The steady-state voltage dependence of fast inactivation was evaluated by applying a 500-ms conditioning prepulse to various potentials from a holding potential of −100 mV, followed by a brief repriming interval (500 μs) and finally a test pulse to −20 mV. The relationship between the test I_{Na} and membrane potential in the presence and absence of CTX3C was fitted by the Boltzmann equation with two parameters, $V_{inact1/2}$ (half-inactivation voltage) and slope factor (k_{inact}). The values of $V_{inact1/2}$ in the controls indicated a depolarized voltage-dependent relationship in the steady-state inactivation of $Na_v1.8$, SS/MM, and MM/SS compared with that of $Na_v1.4$ (Table 2). In contrast to activation-curve studies, ciguatoxin did not create an extra component of steady-state inac-

tivation. The slope factors were relatively constant. However, the voltage dependence was partly affected by 0.1 μM CTX3C, as the $V_{inact1/2}$ values were significantly hyperpolarized in $Na_v1.2$, $Na_v1.4$, and $Na_v1.8$ (Table 2). The $V_{inact1/2}$ was less affected in SS/MM, whereas a large shift of $V_{inact1/2}$ was observed in MM/SS (Table 2).

Effects on Rate of Recovery from Slow Inactivation—It has been demonstrated that TTX-R sodium channels show faster repriming kinetics from inactivation than TTX-S sodium channels, and CTX1B further shortened their recovery time, with consequent increases in excitability, such as an easy transition to repetitive firing (3). We did similar experiments with a standard two-pulse protocol and a variable interpulse interval (5, 10, 20, 40, 128, 256, 512, 1,000, and 2,000 ms). A conditioning prepulse to 0 mV was used to inactivate sodium channels, after which a 4-ms depolarizing test pulse to 0 mV was applied. A 500-ms prepulse duration was used to fully inactivate the sodium channels. Typical recovery time courses from inactivation for $Na_v1.4$ and $Na_v1.8$ are shown in Fig. 6. In the control, the $Na_v1.8$ channels recovered with two time constants of τ_1 = 15.1 ± 3.5 ms and τ_2 = 670 ± 238 ms (n = 4), and the relative amplitude between the two components, $A\tau_2/A\tau_1$, was 0.27 ± 0.1; the corresponding values for $Na_v1.4$ were τ_1 = 32.4 ± 15.6 ms, τ_2 = 580 ± 113 ms, and $A\tau_2/A\tau_1$ = 1.15 ± 0.24 (n = 5). CTX3C (0.1 μM) slowed the time constants to τ_1 = 18.4 ± 4.0 ms, τ_2 = 17,500 ± 14,200 ms, and $A\tau_2/A\tau_1$ = 0.23 ± 0.03 for $Na_v1.8$ (n = 4), and τ_1 = 21.0 ± 9.2 ms, τ_2 = 706 ± 252 ms, and $A\tau_2/A\tau_1$ = 1.48 ± 0.34 for $Na_v1.4$ (n = 5), respectively (Table 3). From these data, it is difficult to determine which channel has the faster recovery kinetics. For clarity, the available, noninactivated fraction of channels at the 5-ms (R5) and 128-ms (R128) interval are indicated in Table 3. $Na_v1.8$ recovered by 52 ± 4% for 5 ms and 93 ± 0.2% for 128 ms in the control, but this was slowed by CTX3C to 43 ± 7 and 87 ± 13% (n = 3), respectively. $Na_v1.4$ recovered more slowly than $Na_v1.8$ and to a more limited extent (by 78 ± 1.9% in the control and 58 ± 8.3% in the presence of CTX3C during 128 ms). These results do not support the contribution of change in repriming kinetics by CTX3C to the hyperexcitability of DRG neurons, in contrast with the findings of Strachan *et al.* (3).

DISCUSSION

Typical and persistent signs of ciguatera induced by ciguatoxin are neurological symptoms such as hyperalgesia or allodynia. Recent studies on TTX-R Na^+ channels by deletion of the gene encoding $Na_v1.8$ (7, 8, 24–26), by ablating $Na_v1.8$-mRNA by antisense oligodeoxynucleotides (10, 27–30), or by evaluating changes in the expression levels of $Na_v1.8$ after nerve injury (10, 27, 29) have revealed the role of $Na_v1.8$ channels in pain-sensing pathways. Thus, it is reasonable to suggest that ciguatoxin might have profound effects on $Na_v1.8$ channels in particular. Yet ciguatoxin has not been documented to exhibit a clear preference for Na^+ channel subtypes. In our previous study, we found no significant difference in the effects of CTX3C on $Na_v1.2$, $Na_v1.4$, and $Na_v1.5$ channels (21). By contrast, Strachan *et al.* (3) have reported differential actions of CTX1B (P-CTX-1) between TTX-S and TTX-R Na^+ channels of DRG neurons. CTX1B induced a leakage cur-

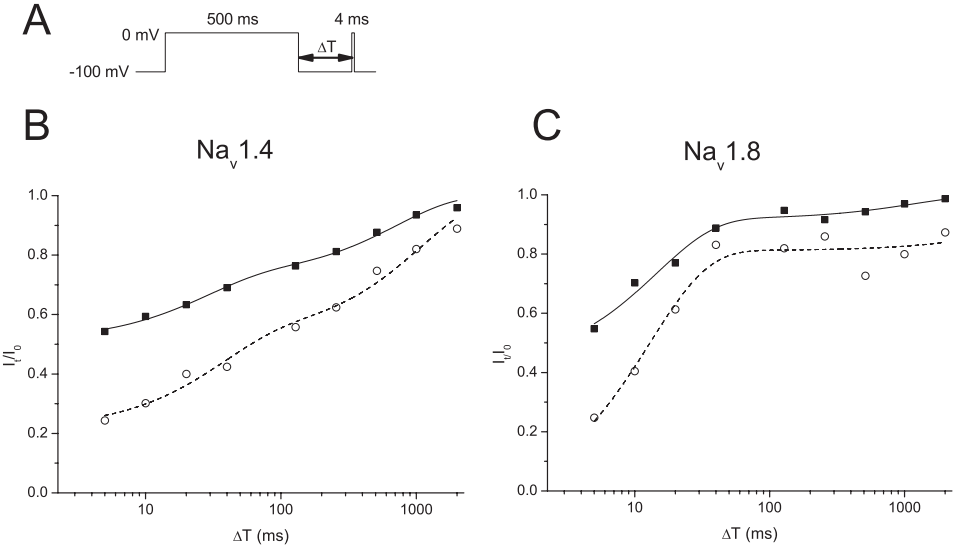

FIGURE 6. **Slowed and suppressed recovery from inactivation by CTX3C.** *A,* pulse protocol with variable intervals (Δ*T*) between the conditioning pulse and the test pulse. Representative recovery data from $Na_v1.4$ (*B*) and $Na_v1.8$ (*C*) are shown. The duration of the conditioning depolarization was set to 500 ms. The recovered I_{Na} values evoked by the test pulse (I_t) relative to those without the conditioning pulse (I_0) were plotted against the interpulse intervals in milliseconds (Δ*t* in the axis). The recovery time courses were fitted by the sum of two exponentials as follows: $(I_t/I_0) = A_1\tau_1 \cdot \exp(-(t/\tau_1) + A_2\tau_2 \cdot \exp(-(t/\tau_2) + C$. See Table 3 for the fitted parameters. $Na_v1.8$ recovered slightly faster than $Na_v1.4$. However, the slower time constants for $Na_v1.8$ tended to be larger than that of $Na_v1.4$.

TABLE 3
Ciguatoxin-induced changes in recovery time course from slow inactivation

	n	R_5	R_{128}	τ_1	τ_2	$A\tau_2/A\tau_1$
				ms	*ms*	
Control						
$Na_v1.2$	3	0.60 ± 0.07	0.84 ± 0.05	30.5 ± 27	475 ± 171	0.79 ± 0.26
$Na_v1.4$	5	0.55 ± 0.02	0.78 ± 0.02	32.4 ± 15.6	580 ± 113	1.15 ± 0.24
$Na_v1.8$	4	0.52 ± 0.04	0.93 ± 0.02	15.3 ± 3.5	670 ± 238	0.27 ± 0.1
CTX3C						
$Na_v1.2$	3	0.26 ± 0.08	0.59 ± 0.13	30.6 ± 16.0	1970 ± 397	1.23 ± 0.5
$Na_v1.4$	5	0.40 ± 0.11	0.58 ± 0.09	21.0 ± 9.2	706 ± 252	1.48 ± 0.34
$Na_v1.8$	4	0.43 ± 0.07	0.87 ± 0.02	18.7 ± 4.0	17,500 ± 14,200	0.23 ± 0.03

rent for TTX-S, although it hastened the repriming kinetics (recovery from inactivation) of TTX-R Na^+ channels. These data suggest a greater contribution of TTX-S currents to increased neuronal excitability. In this study, our results were to the contrary. First, CTX3C (0.1 μM) shifted the activation curves to the more hyperpolarized direction or created extra low threshold components with a larger k_{act} (less steep) in $Na_v1.8$. Second, CTX3C exhibited an ability to induce 10 times larger resting sodium conductance in the $Na_v1.8$-expressing cells than in the others ($Na_v1.2$, $Na_v1.4$, and MM/SS). Third, CTX3C slowed the repriming kinetics of both $Na_v1.4$ and $Na_v1.8$ channels. The first two properties of ciguatoxin modulation listed above are in good accordance with the neurological symptoms of ciguatera, assuming that $Na_v1.8$ is the main regulator of the pain-sensing pathway in DRG neurons.

In this study, three subgroups of wild-type sodium channel α-subunits were transiently expressed in HEK293 cells for $Na_v1.2$ and $Na_v1.4$ and in ND7-23 cells for $Na_v1.8$ (these currents could not be recorded in HEK293 cells possibly because of a lack of some cofactors). These environmental differences in ND7-23 cells might therefore favor ciguatoxin actions, resulting in larger effects on $Na_v1.8$. However, this was not the case in our study. We were able to express chimeric channels of $Na_v1.4$ and $Na_v1.8$ (SS/MM and MM/SS) in HEK293 cells. The SS/MM chimera mimicked the wild-type $Na_v1.8$ high sensitivity to CTX3C by showing a similar large resting sodium conductance. In addition, the ciguatoxin-sensitive chimera, SS/MM expressed in ND7-23 cells exhibited a large resting sodium conductance comparable with that in HEK293 cells, in the presence of 0.1 μM 51-OH-CTX3C. These findings suggested that the ND7-23 environment of the cell was not a major factor in inducing higher sensitivity to CTX3C; in other words, it indicated a critical role of the N-terminal domains of $Na_v1.8$ for preferential CTX3C binding.

Our data, in contrast to those reported by Strachan *et al.* (3), suggest that the primary means of ciguatoxin modulation of pain sensing is mediated by TTX-R and not TTX-S Na^+ channels. The TTX-S currents recorded by Strachan *et al.* (3)could be mainly from $Na_v1.7$, which has been reported to be expressed abundantly throughout the peripheral nervous system (31). $Na_v1.7$ might behave differently from $Na_v1.2$ or $Na_v1.4$ against ciguatoxin, as knock-out mice with nociceptor-specific gene deletion of $Na_v1.7$ exhibited reduced sensitivity to noxious mechanical stimuli and inflammation-induced hyperalgesia (32). Also, mutations in $Na_v1.7$ channels have been found to be the cause of human erythermalgia, characterized by intermittent burning pain with redness and heat in the extremities (33). Examining the effects of ciguatoxin on $Na_v1.7$ in comparison with $Na_v1.8$ would resolve this question. In severely intoxicated cases of ciguatera, autonomic dysfunction might appear as bradycardia or hypotension. These symptoms imply that $Na_v1.7$ predominantly expressed in the autonomic nervous system might be affected, but they need a higher dose of ciguatoxin than $Na_v1.8$. Another possibility to explain the discrepancy between our results and those reported by Strachan *et al.* (3) is that chemically modified states of Na^+ channels from freshly isolated DRG might differ from our heterologously expressed Na^+ channels. For example, in diabetic states, $Na_v1.8$ currents were augmented by serine/threonine phosphorylation (15). It is well known that chemically mediated inflammation leads to alteration in both TTX-S (34, 35) and TTX-R Na^+ channels (8, 36–38). A GTP-induced TTX-R Na^+ current has been reported (39), implying that differences in experimental conditions, including the internal solutions in patch pipettes, might affect the results.

Finally, differences in the ciguatoxin structure of CTX3C and CTX1B might affect their potency. CTX1B has almost the same ether ring skeleton as that of CTX3C (a small difference found at E-ring; CTX1B has 7-membered E-ring instead of 8-membered one for CTX3C, see Fig. 1) with the M-ring hydroxylated at the 51st position, and has a dihydroxylated extra-alkyl chain attached at position 1 (Fig. 1). We have observed that hydroxylation of the M-ring of CTX3C, resulting in 51-hydroxy-CTX3C, has no significant effect on its potency (data not shown). Hydroxy residues near the A-ring could be significant for ciguatoxin binding activity. This will be systematically tested in the near future.

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