INTRODUCTION
Voltage-gated sodium (Na\textsubscript{v}) channels are the trademark of electro-excitability cells. These transmembrane proteins transiently open in response to membrane depolarizations and thereby provide the Na\textsuperscript{+} current pathway that underlies the initial phase of action potentials. To properly fulfill this crucial physiological role, Na\textsubscript{v} channels are bestowed with three key features: voltage-dependent activation, high selectivity for Na\textsuperscript{+} ions, and spontaneous fast inactivation (Hille, 2001). Na\textsubscript{v} channels are composed of a pore forming ~260-kDa \textalpha-subunit associated with auxiliary \beta-subunits of ~30 kDa. The \textalpha-subunit consists of four homologous, yet non-identical, repeats (DI-IV) connected by intracellular linkers, with each repeat containing six transmembrane segments (S1–6) (Catterall, 2000). The S4 segments in each repeat contain several positively charged Arg or Lys residues in every third position and are identical, repeats (DI-IV) connected by intracellular linkers, with each repeat containing six transmembrane segments (S1–6) (Catterall, 2000). The S4 segments in each repeat contain several positively charged Arg or Lys residues in every third position and are believed to act as voltage sensors, making the channel able to respond to voltage changes across the cell membrane (Stuhmer et al., 1989). Upon membrane depolarization, the positive charges move outward in the electrical field of the membrane, resulting in a conformational change of the protein structure that opens the ion conducting pore (Armstrong, 1981; Kontis et al., 1997). The intracellular loop that connects DIII and IV contains a highly conserved hydrophobic cluster of Ile, Phe, and Met residues, the so-called IFM-motif. This motif is proposed to be the inactivation gate, acting as a hinged lid that closes the ion conducting pore from the cytoplasmic side (West et al., 1992). Numerous studies have indicated that DIV, and more specifically segment IVS4, may play a unique role among the four homologous repeats in coupling activation to inactivation and it has been proposed that movement of this S4 segment facilitates closure of the inactivation gate (Chahine et al., 1994; Chen et al., 1996; Kontis and Goldin, 1997; Sheets et al., 1999). However, the precise molecular mechanism of coupling IVS4 movement to closure of the inactivation gate is still elusive today (Ulbricht, 2005).

Several peptide toxins from the venom of scorpions, sea anemones and spiders have been shown to slow or inhibit the fast inactivation process of Na\textsubscript{v} channels by interacting with overlapping, yet non-identical binding sites, named receptor site 3 (Catterall, 2000). The molecular location of this receptor site is not entirely known but was shown to include several crucial amino acid residues in the extracellular S3–S4 loop in DIV (Rogers et al., 1996; Benzinger et al., 1998). It was proposed that by binding to this loop, site 3 toxins...
Phyla- and subtype-selectivity of a sea anemone toxin

prevent the normal gating movement of the voltage sensor in DIV, thereby hindering the conformational changes associated with fast inactivation (Rogers et al., 1996).

In mammals, nine different genes that encode distinct Na\textsubscript{v} channel subtypes (Na\textsubscript{v}1.1–Na\textsubscript{v}1.9) have been identified until today (Goldin, 1999). These closely related subtypes (49–87% sequence identity among human subtypes) can have very different biophysical properties and are expressed in a tissue-specific manner. Evidently, this differential expression plays an important role in the diversity in electrical properties of excitable tissues and plasticity of nervous tissues. The importance of Na\textsubscript{v} subtype diversity is also reflected in the emerging roles of different Na\textsubscript{v} subtypes play in various channelopathies (for reviews, see Ashcroft, 2006; Catterall et al., 2008; Cannon, 2010). In contrast to the nine mammalian Na\textsubscript{v} channel genes, only one gene encoding Na\textsubscript{v} channels (para) has been identified in insects until today (Loughney et al., 1989). Functional diversity in insect Na\textsubscript{v} channels is very likely to be achieved by alternative splicing and RNA editing of the para transcript, rather than expression of distinct genes (Tan et al., 2002; Song et al., 2004). As a consequence, the insect Na\textsubscript{v} channel orthologs share much more sequence identity (typically 87–98%) than their mammalian counterparts (King et al., 2008). An important feature of animal toxins is that they can discriminate between closely related subtypes with high selectivity. However, for most of these toxins, the subtype-selectivity pattern is unknown. CgNa is a 47-amino acid residue type I toxin isolated from the venom of the Giant Caribbean Sea Anemone Condylactis gigantea. Previous studies showed that CgNa increases the action potential duration by slowing the inactivation of tetrodotoxin-sensitive (TTX-S) sodium currents in rat dorsal root ganglion (DRG) neurons (Standker et al., 2006; Salceda et al., 2007). In this study, we reveal the phyla- and subtype-selectivity of CgNa on Na\textsubscript{v} channels, using cloned Na\textsubscript{v} channel subtypes expressed in Xenopus oocytes.

**MATERIALS AND METHODS**

**TOXIN PURIFICATION**

CgNa was isolated and purified from the Giant Caribbean Sea Anemone Condylactis gigantea as described previously (Standker et al., 2006).

**EXPRESSION OF Na\textsubscript{v} CHANNELS**

For expression in *Xenopus laevis* oocytes, the cDNA encoding rNa\textsubscript{v}1,2 and mNa\textsubscript{v}1.6 was subcloned into pLCT1. The rNa\textsubscript{v}1.3, rNa\textsubscript{v}1.4, DmNa\textsubscript{v}1, and tipe cDNA was subcloned into vectors pNa3T, pUI-2, pH19-13-5, and pH19 respectively. For *in vitro* transcription, these plasmids were linearized with NotI. The rNa\textsubscript{v}1.7/pBSTA, rPNl, and h\textsubscript{\beta}/pGEM-HE were linearized with SacII and Nhel respectively. Capped cRNAs were then synthesized from the linearized plasmid using the T7 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The hNa\textsubscript{v}1.5/pSP64T, r\textsubscript{\beta}, pSP64T, and rNa\textsubscript{v}1.8/pSP64T vectors were linearized with XbaI, EcoRI, and XhoI respectively, and transcribed with the SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). Female *X. laevis* frogs were anesthetized by submersion in ice water in the presence of 0.1% 3-aminobenzoic acid ethyl ester (tricaine mselylate). Stage V–VI oocytes were harvested from the ovarian lobes of anesthetized frogs as described previously (Liman et al., 1992). Care and use of *X. laevis* frogs in this study meet with the guidelines of the Catholic University Leuven (K.U. Leuven) and were approved by the ECD (Ethical Commission for Experiments on Animals, Belgian Federal Public Health Service). The oocytes were injected with up to 50 nl of cRNA at a concentration of 1 ng/ml using a microinjector (Drummond, USA). The oocyte incubation solution contained (in mM): NaCl 96, KCl 2, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 2, and HEPES–acid 5 (pH 7.4), supplemented with 50 mg/1 gentamicin sulfate. Whole-cell currents from oocytes were recorded 2–5 days after injection.

**ELECTROPHYSIOLOGICAL STUDY**

Whole-cell currents were recorded in *X. laevis* oocytes using the two-electrode voltage-clamp technique as described by Liman et al. (1992). Experiments were performed at constant temperature 18–24°C using a GeneClamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data acquisition system (Molecular Devices, USA). Data were sampled at a frequency of 20 kHz and low-pass filtered at 2 kHz using a 4-pole low-pass Bessel filter. Leak subtraction was performed using a –PI/4 protocol. The voltage-clamp data recorded in this study were discarded if oocytes exhibited large (>200 nA) or unstable (>10% deviation) leak currents throughout the experiment. To avoid overestimation of a potential toxin-induced shift in the current–voltage relationship as a result of inadequate voltage control when measuring large sodium currents in oocytes, only data obtained from cells with peak currents below 2 μA were considered for analysis. Voltage and current electrodes were filled with 3 M KCl and resistances were kept as low as possible (<1 MΩ). The bath solution contained (in nM): NaCl 96, KCl 2, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 2, and HEPES 5 (pH 7.4).

To examine the electrophysiological effects of CgNa on the cloned Na\textsubscript{v} channels, the following voltage protocols were applied. All protocols were applied from a holding potential of −90 mV and repeated with a start-to-start frequency of 0.2 Hz. (i) Test pulses were elicited by 100-ms depolarizations to the voltage corresponding to maximal activation in control conditions (between −15 and 10 mV, depending on the Na\textsubscript{v} channel subtype). (ii) Current–voltage relationships were determined by 100-ms step depolarizations ranging from −90 to 60 mV with 5-mV increments. The sodium conductance (g\textsubscript{Na}) was calculated according to Ohm’s law (Equation 1): $g_{Na} = \frac{I_{Na}}{(V - V_{rev})}$, where $I_{Na}$ represents the Na\textsuperscript{+} current peak amplitude at a given test potential $V$, and $V_{rev}$ is the reversal potential. The values of $g_{Na}$ were plotted as a function of voltage and fitted using the Boltzmann equation (Equation 2): $g_{Na}/g_{max} = \frac{1 + \exp((V_{rev} - V)/k)}{1 + \exp(-V/\sigma)}$, where $g_{max}$ represents maximal $g_{Na}$, $V_{rev}$ is the voltage corresponding to half-maximal conductance, and $k$ is the slope factor. (iii) To examine the toxin-induced effects on the steady-state inactivation process, a standard two-step voltage protocol was applied. In this protocol, 100-ms conditioning, 5-mV step prepulses ranging from −90 to 60 mV were immediately followed by a 50-ms test pulse to the voltage corresponding to maximal activation in control conditions. Data were normalized to the maximal Na\textsuperscript{+} current amplitude, plotted against prepulse potential, and fitted using the Boltzmann equation (Equation 3): $I_{Na}/I_{max} = \frac{1}{1 + \exp(-((V - V_{rev})/k))} + C$, where $I_{max}$ is the maximal $I_{Na}$, $V_{rev}$ is the voltage corresponding to half-maximal inactivation, $C$ is the test voltage, $k$ is the slope factor, and $C$ is a constant representing a non-inactivating sustained fraction (close
to 0 in control). (iv) The recovery from inactivation was assayed with a double-pulse protocol, where a 100-ms conditioning pulse to the potential corresponding to maximal activation in control was followed by a 50-ms test pulse to the same voltage. Both pulses were interspersed by a repolarization to −90 mV during a gradually increasing time interval (1–40 ms). The \( I_{\text{Na}} \) obtained in the test pulse was normalized to the \( I_{\text{Na}} \) obtained in the conditioning pulse and plotted against the corresponding time interval. To assess the dose–response relationships, data were fitted according to the Hill equation (Equation 4): 
\[
\text{EC}_{50} = \text{toxin} \times (1 + (EC_{50}/[\text{toxin}])^y),
\]
where \( y \) is the amplitude of toxin-induced effect, \( EC_{50} \) is the toxin concentration at half-maximal efficacy, \( [\text{toxin}] \) is the toxin concentration and \( h \) is the Hill coefficient.

All data were analyzed using Clampfit 8.1 (Molecular Devices, USA), Excel 2003 (Microsoft, USA), and Origin 6.1 (OriginLab, USA) software. Statistical differences were determined using a Student’s t test. A test was considered to be significant when \( p < 0.05 \). All numerical data are presented as the mean ± SE for at least three experiments (\( n \geq 3 \)).

**RESULTS**

CgNa was previously reported to slow the fast inactivation of TTX-S \( Na \) currents in rat DRG neurons (Standker et al., 2006; Salceda et al., 2007). To illustrate the subtype-selectivity pattern behind these actions, we examined the effects of CgNa on a wide range of cloned mammalian \( Na \) channels expressed in *Xenopus* oocytes. The toxin slowed the fast inactivation of specific \( Na \) subtypes, resulting in an increase in \( I_{\text{Na}} \) peak amplitude and an incompletely inactivated or sustained current at the end of the 100-ms test depolarization (Figure 1, left-hand panels). The maximal degree of slowed inactivation was observed with subtypes \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \), where addition of 10 \( \mu M \) CgNa to the bath medium produced a sustained current of 27.1 ± 2.7% \( (n = 7; p < 0.05) \) and 34.7 ± 1.5% \( (n = 6; p < 0.05) \) of \( I_{\text{Na}} \) peak amplitude, respectively. In parallel, their peak \( I_{\text{Na}} \) amplitudes increased by 36.6 ± 6.4% \( (n = 7; p < 0.05) \) and 28.5 ± 4.8% \( (n = 6; p < 0.05) \). In the case of \( hNa_{1.5}/\beta \), the peak \( I_{\text{Na}} \) amplitude was increased by 10.9 ± 3.2% \( (n = 3; p < 0.05) \), while the current was fully inactivated after 100 ms. The slowing of these mammalian \( Na \) subtypes was not associated with a shift in current–voltage relationships nor a change in slope factor or midpoint potential of the activation curves. Neither was the reversal potential of any of the tested \( Na \) subtypes changed, indicating that the ion selectivity of the channels was not altered by the toxin (Figure 1, middle panels). However, the observed effects were accompanied by a small but significant shift in steady-state inactivation of \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \). The midpoint potential of steady-state inactivation \( (V_h) \) was shifted from −27.0 ± 0.2 to −37.0 ± 0.4 mV \( (n = 5; p < 0.05) \) and from −51.3 ± 0.3 to −54.4 ± 0.6 mV \( (n = 3; p < 0.05) \), respectively. Moreover, the steady-state inactivation of \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \) became incomplete in presence of 10 \( \mu M \) CgNa, resulting in the appearance of a non-inactivating component of 28.8 ± 0.5 and 36.7 ± 0.7% (Figure 1, right-hand panels). The other mammalian \( Na \) subtypes examined in this study remained unaffected by CgNa at concentrations up to 10 \( \mu M \).

In contrast to the clear effects, but modest potency, of the toxin on specific mammalian \( Na \) channel subtypes, CgNa affected the inactivation of the insect sodium channel clone DmNa1/1tipE from *Drosophila melanogaster* much more profoundly. This was manifested by a dramatic increase in peak \( I_{\text{Na}} \) up to a maximum of 313.5 ± 19.2% \( (n = 6; p < 0.05) \) of control amplitude and a complete removal of real-time and steady-state inactivation in the presence of 10 \( \mu M \) CgNa (Figure 2B). At the more moderate concentration of 100 nM, the toxin increased the peak \( I_{\text{Na}} \) by 63.8 ± 19.2% \( (n = 4; p < 0.05) \) and induced a sustained current of 101.5 ± 20.3% \( (n = 4; p < 0.05) \) of \( I_{\text{Na}} \) peak amplitude (Figure 2A).

The toxin-induced increase in \( I_{\text{Na}} \) was not associated with a shift in current–voltage relationships or reversal potential. However, 100 nM CgNa shifted the voltage dependence of steady-state inactivation \( (V_h) \) from −41.6 ± 0.1 to −43.1 ± 0.3 mV; \( n = 6; p < 0.05 \) and caused the steady-state inactivation to become incomplete (47.8 ± 0.4% non-inactivating component; \( n = 6; p < 0.05 \)). The toxin-induced increase in peak and sustained current amplitude was described by a relatively slow time course, reaching a steady-state after ~12 min (Figure 2C; \( n = 6 \)) and was not reversible by washing the bath medium. In addition, CgNa increased the rate of recovery from inactivation of the insect DmNa1/1tipE channel. The time constant of recovery significantly decreased from 1.89 ± 0.07 ms in control to 1.57 ± 0.11 ms in presence of 100 nM CgNa (Figure 2E; \( n = 7; p < 0.05 \)).

Given the differences in modulation of peak and steady-state current of specific \( Na \) subtypes by CgNa, three different parameters were taken into account to properly quantify the subtype- and phylum-selectivity of the toxin. Therefore, dose–response curves were constructed using: (i) \( I_{\text{Na}} \) peak/\( I_{\text{Na}} \) peak, monitoring the toxin-induced increase in peak current amplitude; (ii) \( I_{\text{Na}} \) 5 ms/\( I_{\text{Na}} \) peak, showing the impairment of inactivation during the fast decay phase of the current; and (iii) \( I_{\text{Na}} \) 100 ms/\( I_{\text{Na}} \) peak, reflecting the fraction of remaining \( I_{\text{Na}} \) at steady-state, which displays the toxin-induced sustained current (Figures 3A–C). All dose–response data were fitted with the Hill equation (Eq. 4) and the resulting \( EC_{50} \) values can be found in Table 1. To facilitate comparison of the selectivity of the toxin for the tested subtypes, the normalized potency (inversed \( EC_{50} \) values with the highest set as 100%) and the normalized efficacy (relative amplitudes with the highest set as 100%) were plotted in bar diagrams (Figures 3D,E). Clearly, the selectivity of CgNa for DmNa1/1tipE is more pronounced in terms of efficacy (i.e., amplitude of the effect at saturating concentrations) than in terms of potency (i.e., the concentration required to produce an effect of a given amplitude). According to the potency, the following rank order was observed for CgNa: DmNa1/1tipE > \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \) \( >> \) \( hNa_{1.5}/\beta \) \( >> \) \( rNa_{1.2}/\beta \), \( rNa_{1.4}/\beta \), \( rNa_{1.7}/\beta \), and \( rNa_{1.8}/\beta \). The \( EC_{50} \) values observed on the insect channel were roughly two-fold lower than those on the most sensitive mammalian channels \( mNa_{1.6}/\beta \) and \( rNa_{1.3}/\beta \) (Table 1).

A similar rank order was observed in terms of efficacy: DmNa1/1tipE \( >> \) \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \) \( >> \) \( hNa_{1.5}/\beta \) \( >> \) \( rNa_{1.2}/\beta \), \( rNa_{1.4}/\beta \), \( rNa_{1.7}/\beta \), and \( rNa_{1.8}/\beta \). In this case, the efficacy of CgNa on the insect channel was roughly 10-fold higher than on \( rNa_{1.3}/\beta \) and \( mNa_{1.6}/\beta \). In general, no great differences were observed between the \( I_{\text{Na}} \) peak, \( I_{\text{Na}} \) 5 ms and \( I_{\text{Na}} \) 100 ms for each channel. The only great exception was seen with \( hNa_{1.5}/\beta \), where the \( I_{\text{Na}} \) 100 ms was not determinable, reflecting the lack of sustained current in this subtype.
FIGURE 1 | Effects of CgNa on cloned mammalian Na\textsubscript{r} channel subtypes Na\textsubscript{r,1.2}, Na\textsubscript{r,1.3}, Na\textsubscript{r,1.4}, Na\textsubscript{r,1.5}, Na\textsubscript{r,1.6}, Na\textsubscript{r,1.7}, and Na\textsubscript{r,1.8}, expressed in *Xenopus* oocytes. The tested mammalian isoforms originate from rat (r), human (h), or mouse (m). Left-hand panels show representative whole-cell current traces in control (black traces) and in presence of 10 μM CgNa (gray traces). Middle panels show normalized current–voltage relationships (n = 3–7) in control (●) and in presence of 10 μM CgNa (○). Right-hand panels show steady-state activation and inactivation curves (n = 3–7) in control (● and ■, respectively) and in presence of 10 μM CgNa (○ and □, respectively), fit with the Boltzmann equation.
FIGURE 2 | Effects of CgNa on the cloned insect Nax channel DmNax1/TipE expressed in Xenopus oocytes. (A,B) Left-hand panels show representative whole-cell current traces in control (black traces) and in presence CgNa (gray traces) at a concentration of 100 nM (A) and 10 µM (B); middle panels show normalized current–voltage relationships (n = 3–6) in control (●) and in presence of CgNa (○) at a concentration of 100 nM (A) and 10 µM (B); right-hand panels display steady-state activation and inactivation curves (n = 3–6) in control (●) and ■, respectively and in presence of CgNa (○ and □, respectively) at a concentration of 100 nM (A) and 10 µM (B). (C) Time course of the increase (n = 6) in peak Ipeak (A, 100 nM; ▲, 10 µM) and sustained Ipeak measured after 100 ms (○, 100 nM; ●, 10 µM). (D) Dose–response curves displaying the concentration dependence of the CgNa-induced increase in Ipeak (●) and sustained Ipeak measured after 100 ms (▲). (E) Recovery from inactivation (n = 7) in control (●) and in the presence of 100 nM CgNa (○).

DISCUSSION
Because of their prominent role in electro-excitability, Nax channels have become one of the foremost important targets of venomous animals. Most of the presently known Nax channel toxins isolated from sea anemones modulate channel function by slowing or inhibiting the channel’s inactivation process and are able to discriminate between closely related Nax subtypes. Yet, for most known sea anemone toxins, the exact pattern of Nax subtypes they target is either unknown, or at best incomplete. In this work, we present the elaborate study of a sea anemone toxin on a broad range of mammalian Nax subtypes (Nax1.2–Nax1.9), and report its phyla-selectivity using the insect DmNax1 channel from D. melanogaster.

This study demonstrates that CgNa causes a slowing of the fast inactivation of the neuronal Nax channel subtypes rNax1.3/β1 and mNax1.6/β1 and, to a lesser extent, of the cardiac isofrm hNax1.5/β1 (Figure 1). In contrast, CgNa failed to affect the other mammalian Nax channel subtypes rNax1.2/β1, rNax1.4/β1, rNax1.7/β1, and rNax1.8/β1, at concentrations up to 10 µM. Because of current difficulties in the expression of the rNax1.1 clone in oocytes, this subtype was not included in the electrophysiological assay. The other mammalian Nax subtype that was not examined in this study, Nax1.9, fails to express in standard heterologous systems. However, because Nax1.9 channels are endogenously expressed in rat DRG neurons (Dib-Hajj et al., 1998) and CgNa was previously shown to be inactive in experiments with tetrodotoxin-resistant (TTX-R) Nax channels in rat DRG neurons (Salceda et al., 2007), we can exclude the possibility of CgNa affecting Nax1.9. The presently described Nax subtype-selectivity pattern accords well with previous studies which showed the activity of CgNa on TTX-S Nax channels in DRG neurons (Standker et al., 2006; Salceda et al., 2007).
FIGURE 3 | Dose-response relationships of the effects of CgNa on the insect and mammalian Na_\text{v} channels. Dose-response curves are constructed using the following three parameters to quantify the effects induced by CgNa: increase in peak \( I_{\text{Na}} \) (A), increase in \( I_{\text{Na}} \) 5 ms (B), and increase in \( I_{\text{Na}} \) 100 ms (C). Left-hand graphs show phyla-selectivity between insect and mammalian Na_\text{v} channels, while right-hand graphs zoom in to show the subtype-selectivity among the mammalian Na_\text{v} subtypes. (D,E) Bar diagram illustrating the differences in potency and efficacy of CgNa on the variety of assayed insect and mammalian Na_\text{v} channels.
Table 1 | Summary of the effects of CgNa on Na channels.

| Control | CgNa | CgNa | CgNa |
|---------|------|------|------|
| \(V_m\) (mV) | \(-10.6 \pm 0.2\) | \(-22.2 \pm 0.6\) | \(-38.8 \pm 0.1\) |
| \(\Delta V_m\) (mV) | \(-20.2 \pm 0.1\) | \(-20.2 \pm 0.1\) | \(-61.3 \pm 0.3\) |
| \(\tau\) (ms) | \(125 \pm 2.3\) | \(2.8 \pm 0.3\) | \(11.0 \pm 0.9\) |
| \(\Delta \tau\) (%) | \(11.0 \pm 0.2\) | \(11.0 \pm 0.2\) | \(3.2 \pm 0.8\) |
| Peak | \(213.5 \pm 19.2\) | \(36.6 \pm 4.6\) | \(1.1 \pm 0.3\) |
| \(100\) ms | \(271 \pm 2.7\) | \(11.1 \pm 0.3\) | \(34.7 \pm 1.5\) |
| EC\(_{50}\) (nM) | \(1874 \pm 34.9\) | \(661.7 \pm 1676\) | \(467.0 \pm 59.5\) |

- \(V_m\): midpoint potential of activation; \(V_r\): midpoint potential of steady-state inactivation; \(\tau\): time constant of fast inactivation; \(\Delta V_m\): relative increase in sodium current amplitude; N.D.: not determined because of a toxin-induced removal of voltage dependency; N.D.: not determined because of a lack of toxin-induced effect on \(V_m\); 100 ms in the tested concentration range. To facilitate comparison, all data (except control and EC\(_{50}\)) refer to a toxin concentration of 10 nM. EC\(_{50}\) values were obtained from fit of the dose–response data (see Figure 3) according to the Hill equation (Eq. 4).

Rat DRG neurons express two types of Na\(_2\) channels: TTX-S (carried mainly by Na\(_{1.1}, \text{Na}_{1.3}, \text{Na}_{1.6}, \text{and} \text{Na}_{1.7}\)) and TTX-R (Na\(_{1.8}\) and Na\(_{1.9}\)) (Roy and Narahashi, 1992; Black et al., 1996; Dib-Hajj et al., 1998).

Beside these clear effects, but modest potency, observed on mammalian Na\(_2\) channels, CgNa exhibits a marked phyla-selectivity in its actions. When tested at the same concentration as on the mammalian Na\(_2\) channels (10 \(\mu\)M), CgNa drastically increases the peak current and causes complete removal of fast inactivation and steady-state inactivation of the insect DmNa\(_{1.1}\)/tipE channel (Figure 2B). At more moderate concentrations (100 nM), CgNa induces a sustained steady-state current and non-inactivating fraction of the steady-state inactivation of DmNa\(_{1.1}\)/tipE. Similar to the mammalian channels, the activation of the insect channel remains unaltered by the toxin (Figure 2A). In addition, CgNa produces a significant increase in the repriming kinetics of the insect channel when returned to the resting state following activation (Figure 2E). Similar to previous reports in DRG neurons, these effects are not reversible by washing the bath medium (Salceda et al., 2007).

The apparent selectivity of CgNa for the insect DmNa\(_{1.1}\)/tipE channel over mammalian Na\(_2\) channels is more pronounced in terms of efficacy (~10-fold higher amplitude) than in terms of potency (only ~2-fold lower EC\(_{50}\)) (see Figures 3D,E). Although CgNa exhibits a selectivity for DmNa\(_{1.1}\)/tipE, it is not specific for insect Na\(_2\) channels. While a few insect-selective sea anemone toxins are known, no truly insect-specific sea anemone toxin has been reported to this date. NvI from Nematostella vectensis was demonstrated to exert a high selectivity for insect channels (Moran et al., 2008). Although no dose–response data are available to quantify the reported selectivity and the tested mammalian channels in this study were limited to Na\(_{1.2}, \text{Na}_{1.4}, \text{and} \text{Na}_{1.5}\), NvI was shown to affect DmNa\(_{1.1}\)/tipE profoundly (~100% increase in peak current) at 1 \(\mu\)M while only small effects (~20% increase in peak current) were observed on the mammalian Na\(_2\) subtypes at 25 \(\mu\)M. Similarly, Av3 from Atemasia viridis (originally known as ATX III) showed selectivity for the insect channel over Na\(_{1.2}, \text{Na}_{1.4}, \text{Na}_{1.5}, \text{and} \text{Na}_{1.6}\) (Moran et al., 2007). The toxins BgII and BgIII from Bunodosoma granulifera were also shown to target insect Na\(_2\) channels with a remarkably high selectivity (Bosmans et al., 2002). The EC\(_{50}\) values of BgII and BgIII on the most sensitive mammalian Na\(_2\) channels were ~50-fold and ~5-fold higher than those described on the insect DmNa\(_{1.1}\)/tipE channel, respectively. Interestingly, this difference in selectivity was ascribed to a single N16D mutation, the sole difference in their amino acid sequences. This mutation seems to cause a great decrease in potency of BgIII for the DmNa\(_{1.1}\)/tipE channel, yet it slightly increased the efficacy of the toxin on the insect channel. This suggests that the introduction of a negatively charged residue at this position might be unfavorable for binding to the receptor site, but at the same time beneficial for the activity of the toxin. The only other type I sea anemone toxins reported until today that also exhibit this N16D substitution are CgTx II and III from Bunodosoma cangi­catt (Zaharenko et al., 2008; Wanke et al., 2009). Although these toxins were not assayed on insect Na\(_2\) channels, this substitution did not seem to affect the activity of the toxins on mammalian Na\(_2\) channels to the same extent as in the case of BgIII (Salceda et al., 2002). In CgNa, position 16 is occupied by the highly conserved Asn residue, but the role of a putative “unfavorable negative charge” might be taken over by neighboring acidic side chains, as the solution structure of CgNa revealed a significantly higher percentage of exposed negatively charged residues than is typical for type I sea anemone toxins (Salceda et al., 2007). The role of such a N16D substitution in the efficacy and potency of CgNa on insect channels certainly deserves further attention in future mutagenesis studies.

Because sea anemone toxins and scorpion α-toxins were shown to induce similar electrophysiological effects on Na\(_2\) channels, they were examined in binding competition assays, which concluded that these toxins share a common binding site on Na\(_2\) channels (Catterall and Beress, 1978). In fact, these toxins share an overlapping but non-identical binding site, later referred to as receptor site 3 (Catterall, 1979, 1980). Several mutagenesis studies indicated that the extracellular loop between S3 and S4 of DIV represents a substantial part of this receptor site (Thomsen and Catterall, 1989; Rogers et al., 1996; Benzinger et al., 1998; Sheets et al., 1999; Ulbricht, 2005). Moreover, a recent study using chimera channels indicated that scorpion and spider toxins that slow the inactivation of Na\(_2\) channels target the voltage sensor paddle motif (S3b–S4) of DIV exclusively; additional interactions with paddle motifs from other domains will alter channel activation (Bosmans et al., 2008). Upon binding to the extracellular IVS3–S4 loop, site 3 toxins are thought to prevent normal voltage sensor movement in DIV, thereby affecting the coupling of activation and inactivation, and the conformational changes associated with fast inactivation of...
the channel (Rogers et al., 1996). Although CgNa has not been examined in competitive binding studies, it is not unreasonable to surmise, in the light of its typical electrophysiological behavior, that this toxin slows inactivation by interacting with receptor site 3 of the Na\textsubscript{\textalpha} channel.

Several extensive mutagenesis studies identified individual amino acid residues in IVS3–S4 as important determinants for binding of site 3 toxins (see Figure 4, IVS3, residues in bold). More specifically, the most extracellularly located negatively charged residue in the IVS3 segment was pointed out as a “hot spot” residue (Rogers et al., 1996; Benzinger et al., 1998). Interestingly, this negative charge is well conserved among mammalian and insect sodium channels, only being absent in rN\textalpha{\textalpha}, 1.8 and rN\textalpha, 1.9. Neutralization or reversal of this charge in hN\textalpha{\textalpha}, 1.5 (D1612R or D1612N) caused a strong decrease in affinity for sea anemone toxin ApB (Benzinger et al., 1998). In contrast, when the corresponding Glu\textsuperscript{1613} residue in rN\textalpha, 1.2 was mutated into a charge-conserving Asp, the binding affinity of sea anemone toxin ATX II increased significantly (Rogers et al., 1996). Markedly, CgNa appears to have a preference for the Na\textsubscript{\textalpha} subtypes that contain an Asp residue in this position over subtypes that possess a Glu residue (see Figure 4, gray background). Only rN\textalpha, 1.4 deviates from this trend, as it contains an Asp residue, but is not targeted by CgNa. On the other hand, substitution of Glu\textsuperscript{1616} (shown in bold in Figure 4, IVS3–S4) into a Gln residue resulted in a strong decrease in affinity of ATX II for the rN\textalpha, 1.2 subtype (Rogers et al., 1996). Similarly, the Gln residue in this position in rN\textalpha, 1.4 might therefore account for the low affinity of CgNa for this Na\textsubscript{\textalpha} subtype. This E-Q substitution is also present in the CgNa-sensitive hN\textalpha{\textalpha}, 1.5, where it might be responsible for the lower affinity of CgNa compared to rN\textalpha, 1.3 and mN\textalpha, 1.6. Further comparison of the CgNa-insensitive rN\textalpha, 1.4 with hN\textalpha{\textalpha}, 1.5

![Sequence alignment of IVS3–S4 from insect and mammalian Na\textsubscript{\textalpha} subtypes. Amino acid alignment of the transmembrane segments S3 and S4 from the homologous repeat DIV connected by the extracellular loop. The two sequences on top are from insect channels (MdNav1 and DmNav1; Md, house fly; Dm, fruit fly), the other sequences are from mammalian channel subtypes (Na\textsubscript{\textalpha}, 1–9; r, rat; m, mouse; h, human). The Na\textsubscript{\textalpha} channel subtypes affected by CgNa are shown on a gray background, residues that differ to those in the sequence of Na\textsubscript{\textalpha}, 1–3 are colored red. Amino acid residues discussed in the text are printed in bold. Positions of the outermost N-terminal residues in the aligned sequence of each subtype are: MdNav1, 1688 (Uniprot accession number Q94615); DmNav1, 1680 (P35500); rN\textalpha, 1.1, 1002 (P04774); rN\textalpha, 1.2, 1592 (P04775); rN\textalpha, 1.3, 1538 (P08104); hN\textalpha, 1.1, 1407 (Q15390); hN\textalpha, 1.5, 1589 (Q14524); mN\textalpha, 1.6, 1581 (Q9YVT5); rN\textalpha, 1.7, 1574 (Q14524); rN\textalpha, 1.8, 1495 (Q14524); rN\textalpha, 1.9, 1497 (O89427).]
draws the attention to the V-F substitution in the IVS3–S4 loop in hNa_{1.5}. The otherwise highly similar loop regions of rNa_{1.4} and hNa_{1.5} suggests that this residue might also be important for the subtype-selectivity of CgNa. Mutation of this Val residue in rNa_{1.2} into Ala reduced affinity of ATX II (Rogers et al., 1996), but substitution into Phe seems to be beneficial for CgNa binding in Na_{1.5}. Interestingly, CgNa slows the inactivation of hNa_{1.5} without inducing a persistent steady-state current, as observed with rNa_{1.3} and mNa_{1.6} (see Figure 1 and Table 1). However, a sustained Na^{+} current component has long been recognized to be present in the plateau phase of the action potential of cardiomyocytes and Purkinje fibers (Zaza et al., 2008) and this sustained current was shown to be increased by site 3 toxins such as ATX II (Oliveira et al., 2004). It remains to be clarified why the steady-state inactivation of hNa_{1.5} is not affected by CgNa. One possible explanation could be that this is the only human clone examined in this study. On the one hand, the mammalian orthologs (i.e., encoded by homologous genes in different species) hNa_{1.5} and rNa_{1.5} are extremely well conserved and their IVS3–S4 sequences are completely identical. On the other hand, there are several pathogenic mutations whose effect depends on the clone used, even if the amino acid sequence is very well conserved.

Overall, the observations discussed above accord quite well with the previous studies that highlight the importance of individual amino acid residues in the IVS3–S4 loop, supporting the importance of this channel region in interaction of CgNa with Na_{1x} channels.

An interesting observation is that, though their IVS3–S4 sequences are completely identical, the rNa_{1.3} subtype is clearly modulated by CgNa while rNa_{1.2} remains unaffected. Although this does not rule out the presence of substantial contacts in IVS3–S4, it indicates that other critical residues outside this loop are responsible for the ability of CgNa to discriminate so thoroughly between these two subtypes. This finding strongly supports previous suggestions that sea anemone toxins interact with a discontinuous receptor site which may include channel regions outside the IVS3–S4 loop that contribute to the heterogeneity in Na_{1x} subtype selectivity (Rogers et al., 1996; Oliveira et al., 2004; Moran et al., 2009). Unfortunately, the presently available data do not give a quantitative idea concerning the extent of involvement of the IVS3–S4 loop in receptor site 3. Further structure–function studies analyzing the sensitivity of chimera or mutant channels will be necessary to explore the complete molecular identity of this receptor site. In the light of the observed preference of CgNa for insect Na_{1x} channels, the substitution of the outermost C-terminally aromatic Phe residue in the IVS3–S4 loop into a Leu residue definitely deserves further attention in these mutagenesis studies.

Another intriguing observation is that the TTX-R peripheral nervous system subtypes rNa_{1.8} (and rNa_{1.9}) are in general quite resistant to site 3 toxins from sea anemones (Bosmans et al., 2002; Salceda et al., 2002, 2006), scorpions (Saab et al., 2002; Maertens et al., 2006), and spiders (Nicholson et al., 2004; Yamaji et al., 2009). This could, at least in part, be due to low conservation of important amino acid residues and longer size of the IVS3–S4 loop (see Figure 4). More specifically, in the IVS3–S4 loop of Na_{1.8}, a motif of four consecutive amino acid residues (Ser, Leu, Glu, and Asn or “SLEN”) was found to play a role in the resistance of this channel against venom from the scorpion Lestes quinquestriatus (Saab et al., 2002). Transfer of this SLEN-motif from the venom-resistant Na_{1.8} subtype to the analogous position in the venom-sensitive Na_{1.4} subtype, rendered Na_{1.4} resistant to the scorpion venom. This suggests an important contribution of either the length, or the specific residues of the SLEN-motif in toxin resistance of Na_{1.8}. Unfortunately, these experiments were not carried out in the context of sea anemone toxins. The search for potent and selective ligands for the neuronal TTX-R subtypes is of particular interest, as these channels play an important role in neuropathic and inflammatory pain (Black et al., 2004; Dib-Hajj et al., 2010). A better understanding of the molecular basis of the resistance of Na_{1.8} and Na_{1.9} to CgNa could therefore significantly contribute in the development of novel pharmacological agents for the treatment of pain. Although toxins that slow Na_{1x} inactivation cause an increased Na^{+} influx, their actions result in a depolarization of the cell. This renders a fraction of the channels unavailable for a new activation by driving them into steady-state inactivation. In this way, the impairment of channel inactivation can ultimately lead to a decrease in Na_{1x} signaling. A good example of a toxin that exerts strong analgesic and induces persistent currents in Na_{1} channels is Batrachotoxin from the poison Dart frog (Bosmans et al., 2004).

In summary, our data highlight that specific insect and mammalian Na_{1x} channel subtypes can be pharmacologically distinguished by their sensitivity to CgNa, as has only been partially described for other sea anemone toxins. Together with the previously determined three-dimensional structure (Salceda et al., 2007) and the presently described subtype-selectivity, future mutagenesis studies could give us more structural information on the interaction between CgNa and specific Na_{1x} channels. Because CgNa interacts with extracellular regions of the channel and most channel regions known to contribute to inactivation are located within membrane or at the intracellular side, this structural information might yield a better insight into the coupling of activation and inactivation in Na_{1x} channels.

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Billen et al.

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Frontiers in Pharmacology | Pharmacology of Ion Channel and Channelopathies

November 2010 | Volume 1 | Article 133 | 10
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