Role of Hydroxyprolines in the *in Vitro* Oxidative Folding and Biological Activity of Conotoxins†

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ABSTRACT: Hydroxylation of proline residue occurs in specific peptides and proteins derived from plants and animals, but the functional role of this modification has been characterized primarily in collagen. Marine cone snails produce disulfide-rich peptides that have undergone a plethora of posttranslational modifications, including proline hydroxylation. Although *Conus* snails extensively utilize proline hydroxylation, the consequences of this modification remain largely unexplored. In this work, we investigated the function of 4-hydroxyproline (Hyp) in conotoxins from three distinct gene families: μ-, ω-, and α-conotoxins. Analogues of μ-GIIIA, ω-MVIIC, α-GI, and α-ImI were synthesized with either Pro or Hyp, and their *in vitro* oxidative folding and biological activity were characterized. For GIIIA, which naturally contains three Hyp residues, the modifications improved the ability to block $Na_V1.4$ sodium channels but did not affect folding. In contrast, the presence of Hyp in MVIIC had a significant impact on the oxidative folding but not on the biological activity. The folding yields for the MVIIC[Pro7Hyp] analogue were approximately 2-fold higher than for MVIIC under a variety of optimized oxidation conditions. For α-conotoxins ImI and GI, the hydroxylation of the conserved Pro residue improved their folding but impaired their activities against target receptors. Since prolyl-4-hydroxylase and protein disulfide isomerase coexist as a heterotetramer in the ER, we discuss the effects of Hyp on the folding of conotoxins in the context of cis−trans isomerization of Pro and Hyp. Taken together, our data suggest that proline hydroxylation is important for both *in vitro* oxidative folding and the bioactivity of conotoxins.

Hydroxylation of proline, resulting in hydroxyproline (Hyp),[1] is a posttranslational modification best characterized in collagen, a major protein in all vertebrates (*1*, *2*). Hydroxylation of proline in the Pro-Hyp-Gly repeats stabilizes the collagen triple helix, although the mechanism by which the hydroxyl group contributes to protein stability is not fully understood (*3*−*9*). Other animal proteins containing Hyp residues include C1q protein from blood and in invertebrates minicollagens from nematodes and venom toxins from cone snails (*10*−*12*). Hyp residues are used by plants and algae in Hyp-rich glycoproteins that constitute cell walls, such as extensin P1 (*13*). In one extreme case, extracellular matrix (ECM) glycoprotein from Volvox consists of 68% Hyp (*13*). Short signal peptides containing multiple Hyp residues are produced by plants as a defensive system against herbivore attack (*14*−*16*). These 15−20-amino acid Hyp-rich peptides, systemins, can contain up to six Hyp residues and up to 17 carbohydrate moieties (pentoses) (*17*). In the case of muskmelon, *Cucumis melo*, the hydroxyproline-rich glycoprotein seems to be involved in defense mechanisms against fungi (*18*, *19*). Thus, although Hyp is distributed in proteins within the animal and plant kingdoms, only a few specialized systems utilize this posttranslational modification.

Posttranslational modifications occur frequently in the peptide neurotoxins produced by predatory marine cone snails (*11*, *20*). A well-studied modification is γ-carboxyglutamate (Gla). This modification was shown to stabilize the helical secondary structure in members of the conantokin family of venom peptides and to promote calcium-assisted oxidative folding of Cys-rich peptide toxins (*21*−*23*). Among the estimated 50000−100000 conotoxins produced by cone snails, only ∼100 have been isolated and structurally characterized. Many of these contain 4-hydroxyproline (*11*, *20*, *24*−*26*) or other hydroxylated amino acid residues, such as hydroxylysine or hydroxyvaline (*26*−*28*). Figure 1 and Table 1 show selected examples of conotoxins from several families containing Hyp residues. The M-superfamily of conotoxins comprises peptides with multiple Hyp residues. On the other hand, highly conserved Pro residues, such as in the first loop of the majority of α-conotoxins, are not hydroxylated (Table 1), although several α-conotoxins with

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[1] Abbreviations: ACh, acetylcholine; GSH, reduced glutathione; GSSG, oxidized glutathione; Hyp, 4-hydroxyproline; nAChRs, nicotinic acetylcholine receptors; Na_V, voltage-gated sodium channel; P4H, prolyl-4-hydroxylase; PDI, protein disulfide isomerase.

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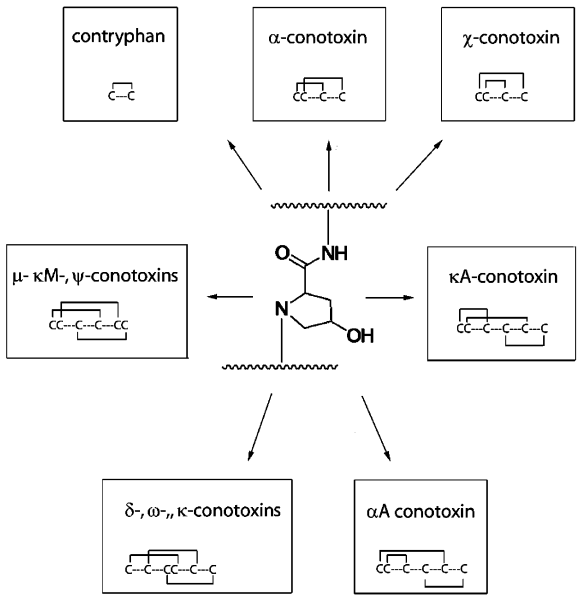

FIGURE 1: Diverse conotoxins belonging to many families contain 4-hydroxyproline. Disulfide bridge connectivity is illustrated beneath each family name.

Hyp in this position have recently been discovered (*26*). The occurrence of Hyp in conotoxins is still difficult to predict with a high level of certainty.

The functional role of Hyp residues in conotoxins remains unknown. To investigate the function of Hyp in conotoxins, we selected four conotoxins from three distinct gene families: GIIIA (a μ-conotoxin), MVIIC (an ω-conotoxins), and GI and ImI (both α-conotoxins). These conotoxins were chemically synthesized with either Pro or Hyp, and their *in vitro* oxidative folding and biological activity were compared. Our results indicate that Hyp residues can affect both the folding and bioactivity of conotoxins.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Folding. Synthetic conotoxins were produced using methods identical to those described previously (*29*−*32*). Briefly, the peptides were synthesized on solid support using standard Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] chemistry. The peptides were cleaved from the resin by a 3−4 h treatment with reagent K [trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/water/phenol (90/5/5/2.5/7.5 by volume)]. The peptides were subsequently filtered and precipitated with cold methyl *tert*-butyl ether (MTBE). The linear peptides were purified by reversed-phase HPLC using a semipreparative C18 Vydac column (catalog no. 218TP510, 10 mm × 250 mm, 5 μm particle size) eluted at room temperature at a flow rate of 5 mL/min using a linear gradient from 5 to 30% solution B in 25 min for μ-GIIIA and ω-MVIIC analogues, and a linear gradient from 10 to 60% buffer B was used for α-ImI and α-GI analogues. HPLC solutions were as follows: (A) 0.1% (v/v) TFA in water and (B) 0.1% (v/v) TFA in 60% aqueous acetonitrile (ACN). Absorbance was monitored at 220 nm, and the chromatographic peaks were collected manually.

Oxidative folding of GIIIA or MVIIC and their analogues was carried out in the presence of 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, and either 1 mM GSSH and 1 mM GSH (GIIIA), 1 mM GSSG and 2 mM GSH (20 μM MVIIC), or 5 mM GSSG and 5 mM GSH (1 mM MVIIC). The oxidative folding reactions for 1 mM MVIIC and MVIIC[P7O] were carried out at room temperature or 5 °C with and without 2 M $(NH_4)_2SO_4$ by the method described above. For α-ImI and α-ImI[Hyp6], the folding was performed using conditions identical to those for GIIIA, except at pH 8.7. MVIIC and MVIIC[Hyp7] were folded in presence of 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM reduced and 2 mM oxidized glutathione. Folding of α-GI and α-GI[Hyp5] was performed in 0.1 M Tris-HCl (pH 8.7), 1 mM EDTA, and 0.5 mM reduced and 5 mM oxidized glutathione. After folding was complete, the reaction was quenched by acidification with formic acid (8% final concentration). The folded peptides were purified by semipreparative HPLC using the same gradients mentioned above. Analytical folding reactions were initiated by adding 100 μL of 200 μM reduced linear peptide dissolved in 0.01% TFA to 1 mL of folding buffer. To screen various folding conditions, 20 μM linear peptide was used.

Folding of conotoxins MVIIC and MVIIC[P7O] in the presence of Clear-Ox resin was performed in buffered solutions containing 0.1 M Tris-HCl (pH 7.5) with and without 2 M $(NH_4)_2SO_4$ (*33*). The immobilized Ellman's reagent Clear-Ox was prepared using the method described previously (*34*). The reduced peptides, dissolved in 0.01% TFA, were added to the folding mixture containing 0.1 M Tris-HCl (pH 7.5), Clear-Ox (18 molar equiv over the peptide), and, depending on the specific condition, 2 M $(NH_4)_2SO_4$. The reactions were carried out at room temperature and 5 °C. After 24 h, aliquots were separated from resin by centrifugation and then quenched with formic acid (8% final concentration). The reaction mixtures were analyzed by analytical reversed-phase HPLC.

To produce reference peptides with the native connectivity of disulfide bridges Cys2−Cys8 and Cys3−Cys12 for α-ImI and α-ImI[P6O] and Cys2−Cys7 and Cys3−Cys13 for α-GI and α-GI[P5O], a two-step oxidation with orthogonal Cys protection was employed. The first pair of cysteines was protected with *S*-acetomidomethyl groups, whereas the second pair was protected by *S*-trityl groups. The folding procedure was followed as previously reported (Luo et al., 1999). The identities of the final products were confirmed by mass spectrometry analysis. Positive ion matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained in the linear and reflector modes with a JEOL JMS HX110 double-focusing spectrometer.

NMR Spectroscopy. Peptide samples (20−30 nmol) were dried and resuspended in 250 μL of a 90% H_2O/10% ^2H_2O/1% $C^2H_3COO^2H$ mixture (pH 2.6) (80−120 μM). One-dimensional ^1H NMR spectra were recorded with a Varian Inova 600 NMR spectrometer equipped with a triple-resonance cryogenic probe. Water suppression was achieved with an 11-echo pulse scheme. Spectra were recorded at 20 °C using 4096 complex points and a spectral width of 12 ppm. Proton chemical shifts are referenced to DSS at 0 ppm.

Electrophysiology. (1) nAChR Assay. Recordings were made from *Xenopus* oocytes expressing mouse adult skeletal muscle ($\alpha 1\beta 1\epsilon\delta$) and rat neuronal ($\alpha 7$) nAChR subtypes, in a static bath containing ND-96 as previously described (*35*). Briefly, oocytes were injected with cRNA 1−2 days after being harvested and used for voltage clamp recording 3−8 days after injection. The bathing solution contained

Table 1: Sequences of Selected Conotoxins from Distinct Families Containing either Hyp or Conserved Pro Residues[a]

Hydroxyproline -containing conotoxin		Non-Hydroxyproline containing conotoxins	
μ-conotoxins		**4/3 subfamily of α-conotoxins**	
GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	ImI	GCCSDPRCAWRC*
GIIIB	RDCCTOORKCKDRRCKOMKCCA*	RgIA	GCCSDPRCRYRCR
GIIIC	RDCCTOOKKCKDRRCKOLKCCA*	Reg1c	GCCSDPRCKHQC*
PIIIA	ZRLCCGFOKSCRSRQCKOHRCC*		
κM-conotoxins		**4/7 subfamily of α-conotoxins**	
RIIIK	LOSCCSLNLRLCOVOACKRNOCCT*	MII	GCCSNPVCHLEHSNLC*
ψ-conotoxins		PnIA	GCCSLPPCAANNPDYC*
PIIIE	HOOCCLYGKCRRYOGCSSASCQR*		
4/7 subfamily of α-conotoxin		**3/5 subfamily of α-conotoxins**	
EI	RDOCCYHPTCNMSNPQIC*	MI	GRCCHPACGKNYSC*
δ-conotoxin		SI	ICCNPACGPKYSC*
PVIA	EACYAOGTFCGIKOGLCCSEFCLPGVCFG	CnIA	GRCCHPACGKYYSC*
SVIE	DGCSSGGTFCGIHOGLCCSEFCFLWCITFID	GI	ECCNPACGRHYSC*
αA-conotoxin			
PIVA	GCCGSYONNACHOCSCKDROSYCGQ*		
EIVA	GCCPYONAACHOCGCKVGROOYCDROSGG*		
ω-conotoxin		**ω–conotoxin**	
GVIA	CKSOGSSCSOTSYNCCRSCNOYTKRCY*	MVIIC	CKGKGAPCRKTMYDCCS GSCGRRGKC*
χ-conotoxin			
MrIA	NGVCCGYKLCHOC		
MrIB	VGVCCGYKLCHOC		
Contryphans		**Contryphans**	
contryphan-R	GCOWEPWC*	contryphan-Vn	GDCPWKPWC*
contryphan-Tx	GCOWQPYC*	contryphan-M	NγSγCPWHPWC*
4/3 subfamily of α-conotoxins			
Reg1b	GCCSDORCKHQC*		

[a] Proline or hydroxyproline amino acid residues are shaded, and asterisks indicate an amidated C-terminus.

Table 2: Structure and Molecular Targets of Conotoxins Studied in This Work[a]

conotoxin	amino acid sequence	Target subtype
μ-GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	$Na_v1.4$
μ-GIIIA[O6P]	RDCCTPOKKCKDRQCKOQRCCA*	
μ-GIIIA[O7P]	RDCCTOPKKCKDRQCKOQRCCA*	
μ-GIIIA[O17P]	RDCCTOOKKCKDRQCKPQRCCA*	
μ-GIIIA[O6,7P]	RDCCTPPKKCKDRQCKOQRCCA*	
μ-GIIIA[O6,7,17P]	RDCCTPPKKCKDRQCKOQPCCA*	
α-ImI	GCCSDPRCAWRC*	α7 nAChR
α-ImI[P6O]	GCCSDORCAWRC*	
α-GI	ECCNPACGRHYSC*	α1β1εδ nAChR
α-GI[P5O]	ECCNOACGRHYSC*	
ω-MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGKC*	P/Q Ca channels
ω-MVIIC[P7O]	CKGKGAOCRKTMYDCCSGSCGRRGKC*	

[a] O is hydroxyproline; asterisks denote amidation.

bovine serum albumin at a concentration of 0.1 mg/mL to minimize nonspecific adsorption of the toxin and atropine at 1 μM to block endogenous muscarinic acetylcholine receptors. The bath was gravity-perfused at a rate of ~3 mL/min, and acetylcholine (ACh)-gated currents were elicited with 1 s pulses of 100 μM ACh applied every minute. The oocyte was allowed to equilibrate with toxin in a static bath for 5 min prior to restoration of the perfusion and ACh pulses. Three oocytes were used for each data point. Dose−response curves were fit to the equation % response = 100/[1 + ([toxin]/IC_{50})^{n_H}], where n_H is the Hill coefficient.

(2) $Na_V1.4$ Assay. The clone for the α-subunit of rat $Na_V1.4$ was a generous gift from A. Goldin (University of California, Irvine). The channel was expressed in *Xenopus* oocytes as described previously (*36*). Whole-cell currents were recorded under two-electrode voltage clamp control using an OC-725C clamp amplifier (Warner Instruments Corp.). The intracellular electrodes were filled with 3 M KCl and had a resistance

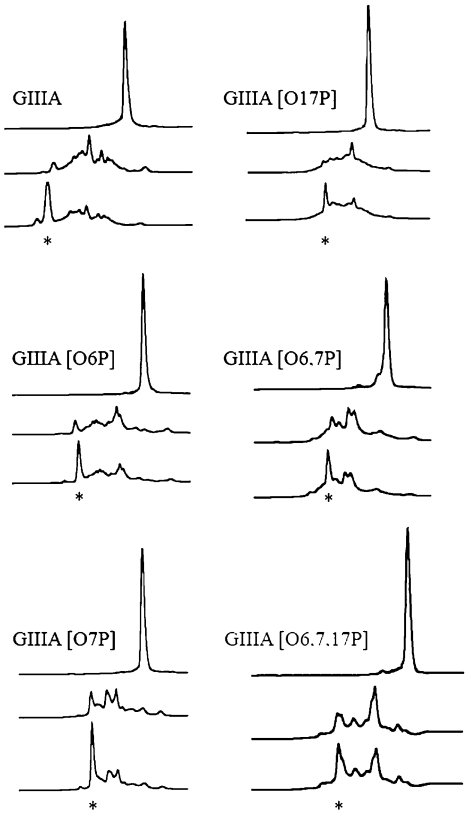

FIGURE 2: Oxidative folding of μ-GIIIA and its analogues in the presence of 1 mM oxidized and 1 mM reduced glutathione and 0.1 M Tris-HCl (pH 7.5). The reaction was quenched at 0, 5, and 120 min. Analytical C18 reversed-phase HPLC was used to separate folding mixtures. An asterisk denotes the folded species that is likely to contain the nativelike disulfide connectivity.

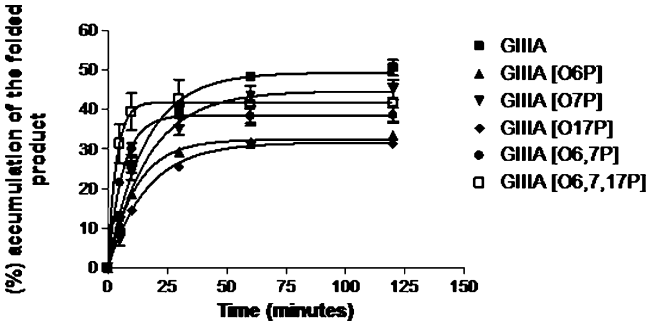

FIGURE 3: Folding kinetics of μ-GIIIA and its analogues. Folding reactions were performed as shown in Figure 2. HPLC peaks were integrated and used to plot a time course of the appearance of properly folded conotoxins. The experimental points were averaged from three independent experiments and fit to a first-order equation.

between 0.5 and 1 MΩ. The membrane potential was held at −100 mV. Currents were low-pass filtered at 2 kHz (−3 dB) and sampled at 10 kHz. The bath solution was ND-96 as described above. Toxin-containing solutions were directly applied to the (static) bath with a Gilson Pipetman. All electrophysiological measurements where performed at room temperature (~22 °C).

Bioassay. Swiss Webster mice (18 days old) were treated intracranially with either ω-MVIIIC or ω-MVIIIC[P7O]. Mice were treated with 20 μL of different doses of lyophilized peptides dissolved in a normal saline solution in control experiments and placed in a cage for observation.

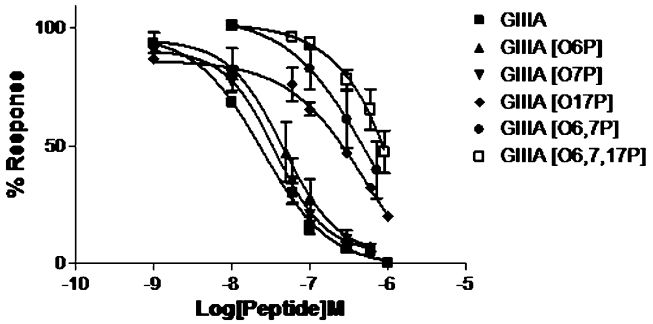

FIGURE 4: Activity of μ-GIIIA and its analogues in blocking $Na_V1.4$ expressed in *Xenopus* oocytes. Dose−response curves for all μ-GIIIA analogues were obtained by measuring the peak sodium current in voltage-clamped oocytes as described in Experimental Procedures. Each data point is the average of the response obtained from three oocytes.

Table 3: Folding Properties and Activity of GIIIA Analogues Containing Hyp → Pro Substitutions

analogue	k (min^{-1})	steady state (% accumulation of total fold species)	activity,[a] IC_{50} (nM)
μ-GIIIA	0.06	49.3	21.8 (18.4−25.7)
μ-GIIIA[O6P]	0.08	32.4	43.7 (30.1−63.4)
μ-GIIIA[O7P]	0.05	44.9	31.2 (23.9−40.7)
μ-GIIIA[O17P]	0.06	31.6	215.0 (156.3−295.7)
μ-GIIIA[O6,7P]	0.15	38.5	137.7 (109−173.1)
μ-GIIIA[O6,7,17P]	0.27	41.9	870.3 (674−1123)

[a] Against $Na_V1.4$. Parentheses indicate the 95% confidence interval.

RESULTS

To characterize the functional role of Hyp residues in conotoxins, representative members from three distinct gene families were selected: μ-, α-, and ω-conotoxins. Structures of the conotoxins studied are summarized in Table 2. μ-GIIIA contains three Hyp residues, at positions 6, 7, and 17. These Hyp residues are found in GIIIA, GIIIB, and GIIIC, whereas only two Hyp residues are found in PIIIA. We synthesized μ-GIIIA analogues with single Hyp/Pro replacements, as well as with double and triple replacements. In the case of MVIIC, the original peptide was synthesized without Hyp (*37*), but other members of this family contain Hyp (Table 1). Interestingly, there is a possibility that the venom-derived natural MVIIC may have contained Hyp, and the synthesized version, which was based on the sequence of a cDNA clone, was simply synthesized in the unmodified form (B. M. Olivera, unpublished observations). This aspect of MVIIC remains to be elucidated. In α-conotoxins ImI and GI, the Pro residue in the first loop is highly conserved (Table 1), making these peptides ideal candidates as the "negative control". Noteworthy is the fact that Hyp residues were found in α-conotoxins from *Conus regius* (*26*) or *Conus victoriae* (*38*, *39*), but a vast majority of venom-derived α-conotoxins have a Pro residue in the first loop. To examine why Pro, rather than Hyp, in this position was evolutionarily selected, we synthesized both conotoxins with Pro or Hyp (Table 2). For each analogue, the folding kinetics and thermodynamics were studied and bioactivity was assessed.

μ-Conotoxins. Oxidative folding of GIIIA and the Pro-containing analogues was carried out in the presence of

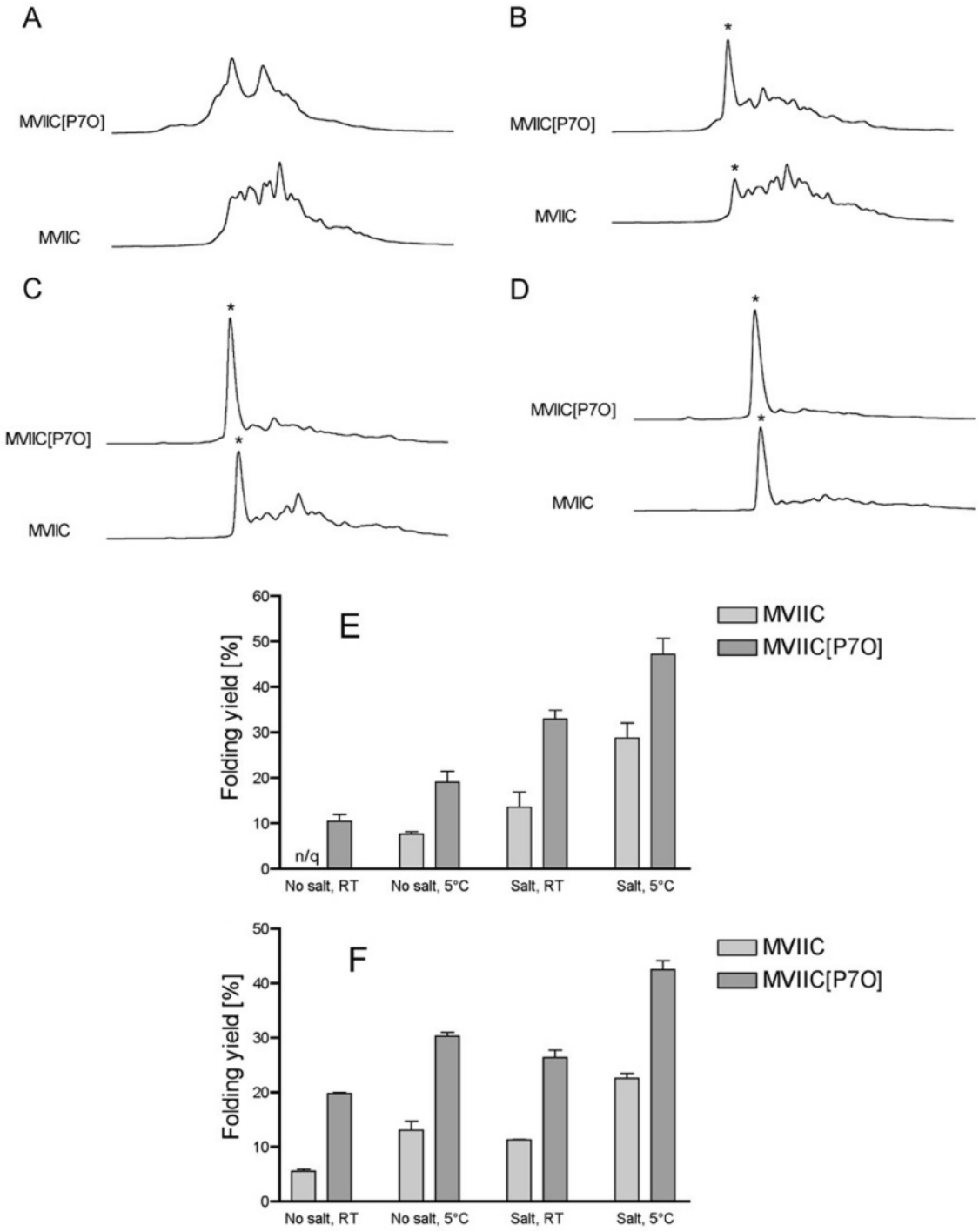

FIGURE 5: Effects of various folding conditions on the accumulation of MVIIC and MVIIC[P7O]. The folding reactions were carried out in the presence of 5 mM oxidized and 5 mM reduced glutathione (pH 7.5), with and without salt, at room temperature and 5 °C. After 24 h, the reactions were quenched with formic acid and the mixtures analyzed on the analytical C18 HPLC column. (A) Folding of MVIIC and MVIIC[P7O] without salt, at room temperature. (B) Folding of MVIIC and MVIIC[P7O] without salt, at 5 °C. (C) Folding with salt, at room temperature. (D) Folding with salt, at 5 °C. (E and F) Bar graphs summarizing folding of MVIIC and MVIIC[P7O] in the presence of glutathione (E) or Clear-Ox (F).

reduced and oxidized glutathione, as described previously (*31*, *32*). Representative HPLC fractionation of folding mixtures quenched after 0, 5, and 120 min for μ-GIIIA analogues is shown in Figure 2. The multiple folded species were detectable at early times for all analogues. The steady-state distribution was established after 1 h. To determine whether the main folded species were fully oxidized, HPLC peaks from 120 min folding samples were collected and analyzed by MALDI and one-dimensional proton NMR. All molecular masses of the peaks agreed with the calculated masses. For each μ-GIIIA analogue, one-dimensional NMR analysis was performed. The amide and aromatic proton resonance positions and intensities were qualitatively similar to those of native μ-GIIIA (Figure S1 of the Supporting Information), suggesting the μ-GIIIA analogues are similarly folded species. A single set of dispersed resonances is also consistent with a single and highly populated conformer in solution. Folding rates and equilibria are summarized in Table 3. The folding yields decreased from approximately 50% for GIIIA to 30% (for O6P and O17P analogues).

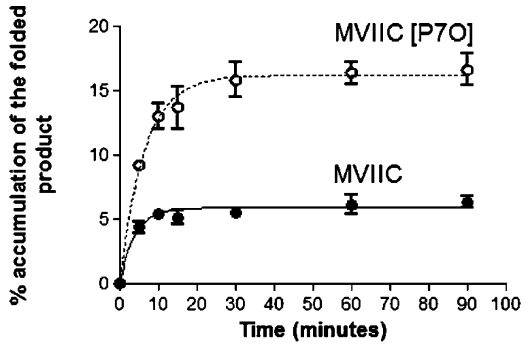

FIGURE 6: Comparison of folding kinetics of MVIIC and MVIIC-[P7O]. Both peptides were folded in the presence of 1 mM reduced and 2 mM oxidized glutathione (pH 7.3). The aliquots from the folding reactions were quenched by acidification and fractionated by reversed-phase HPLC. Quantification of the folding species was performed by HPLC. The fit yielded apparent rates (k_{app}) of 0.26 and 0.15 min^{-1} for formation of the native MVIIC and MVIIC-[P7O], respectively. The calculated levels of steady-state accumulation of MVIIC and MVIIC[P7O] were 6 and 16%, respectively.

Interestingly, the GIIIA analogue lacking all three Hyp residues folded significantly faster than the Hyp-containing peptides (Figure 4).

Analogues of μ-GIIIA were further tested for their ability to block the rat $Na_V1.4$ sodium channel subtype expressed in oocytes. The dose−response curves are shown in Figure 4, and the IC_{50} values are summarized in Table 3. The μ-GIIIA[O6P] and μ-GIIIA[O7P] analogues were found to have similar affinities for $Na_V1.4$ compared to native μ-GIIIA (43.7, 31.2, and 21.8 nM, respectively). However, μ-GIIIA-[017P], μ-GIIIA[O6,7P], and, in particular, μ-GIIIA[O6,7,-17P] exhibited lower potencies than μ-GIIIA.

ω-Conotoxins. Since MVIIC is a commonly used pharmacological tool for the study of structure and function of calcium channels (*40*, *41*), ω-MVIIC was first synthesized with proline at position 7 (*37*). Despite the possibility that venom-derived MVIIC may contain Hyp rather than Pro, the effects of having a 4-hydroxyproline residue at this position have never been evaluated. MVIIC exhibits very poor folding properties; the judicious combination of high ionic strength and low temperature were established as being most optimal for folding (*42*). Here, we applied an identical strategy, as described by Kubo et al. (*42*), to evaluate effects of salt and temperature on the oxidative folding of Pro- and Hyp-containing MVIIC analogues. The results from these experiments are summarized in Figure 5. Both analogues folded very poorly under low-ionic strength, ambient-temperature conditions, but the folding yields significantly improved when the temperature was lowered to 5 °C or the ionic strength was increased to 2 M $(NH_4)_2SO_4$. Remarkably, the folding of the MVIIC[P7O] analogue was improved to a greater extent than that of MVIIC under any of the folding conditions studied. Since improvements in the oxidative folding of MVIIC or MVIIC[P7O] are of potential commercial value, we explored how the immobilized folding reagent, Clear-Ox (*34*), may affect folding of both analogues. As shown in Figure 5E, the folding yields for MVIIC[P7O] were again significantly higher compared to those for MVIIC.

Comparison of folding kinetics for MVIIC and MVIIC-[P7O] was performed under conditions described previously (*42*, *43*). The overall distributions of the folding products for both MVIIC and the P7O form are similar to that described previously (*43*). As shown in Figure 6, the folding rates for the Hyp-containing analogue were approximately 2 times faster, whereas the calculated yields were 3-fold higher. Biological activity for ω-MVIIIC and ω-MVIIIC-[P7O] was tested in a mouse bioassay following *icv*

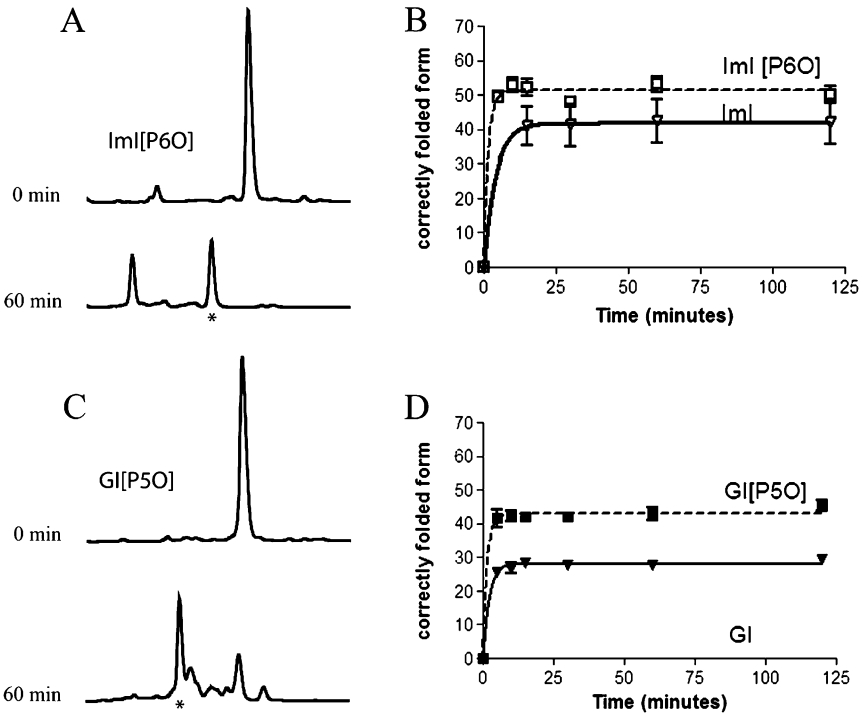

FIGURE 7: HPLC chromatograms and folding kinetics for α-conotoxins ImI and GI and theirs analogues. (A) Direct oxidative folding of α-ImI in the presence of 0.1 M-Tris-HCl (pH 8.7) and 1 mM oxidized and 2 mM reduced glutathione. (B) The correctly folded form was determined by HPLC as shown in panel A. The filled and empty squares depict data for native and analogue forms, respectively. The plotted values are an average from three experiments. (C) The folding condition for α-GI was identical to that for α-ImI except at 0.5 mM reduced and 5 mM oxidized glutathione. (D) The curves were generated from the relative area of the peak labeled with an asterisk in panel C. Asterisks denote correctly folded forms.

Table 4: Folding Properties of α-Conotoxin Analogues Containing Pro or Hyp Residues

analogue	k (min^{-1})	steady state (% accumulation of total fold species)
α-ImI	0.24	41.9
α-ImI[P6O]	0.68	51.3
α-GI	0.47	28.1
α-GI[P5O]	0.69	43.0

administration of each peptide. Both the proline and the hydroxylated proline peptides caused the striking "shaker" syndrome in mice (*44*, *45*); symptoms could be detected right after injections at doses of 100 pmol/g of body weight, and after 40 to 50 min, these injections caused death. When the doses were increased 3-fold, the same shaking syndrome was elicited but the mice died in 30 min in both cases.

α-*Conotoxins*. Analogues for α-ImI and α-GI conotoxins containing a Hyp residue in the second loop were synthesized and tested on oocytes expressing neuronal α7 and skeletal muscle nAChRs, respectively. The oxidative folding of GI and ImI conotoxins was performed as previously described (*30*, *46*). To verify the correctly folded species for the Hyp-containing analogues, the orthogonal protection of the Cys residues was applied; the Cys connectivity Cys2−Cys8 and Cys3−Cys12 for α-ImI[P6O] and Cys2−Cys7 and Cys3−Cys13 for α-GI[P5O] were achieved using a standard two-step oxidation protocol. The first pair of Cys residues was oxidized with $K_3[Fe(CN)_6]$, and the second pair was oxidized with iodine. Subsequently, the oxidative folding reactions with peptides containing all four unprotected Cys residues were carried out. The HPLC separation of the folding reaction mixture and the time course of appearance of the correctly folded species are shown in Figure 7. The apparent rate constants were found to be similar for α-GI and α-GI-[P5O] unlike those for α-ImI and α-ImI[P6O] (Table 4). In both cases, the Hyp-containing analogues folded more efficiently than the native peptides.

As illustrated in Figure 8 and Table 5, α-ImI was found to exhibit an IC_{50} value (300 nM) similar to that described previously (*47*, *48*). However, a 50-fold higher concentration of α-ImI[P6O] elicited a <5% inhibition. In the case of α-GI and α-GI[P5O], surprisingly similar potencies were found for the adult muscle nAChR, although replacing the proline residue in α-GI with hydroxyproline resulted in a more rapid dissociation from the adult muscle nAChRs (Figure 9).

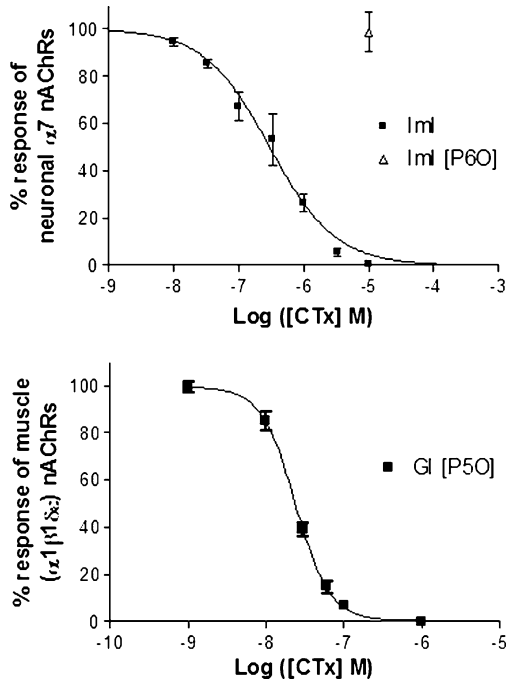

FIGURE 8: Inhibition of human α7 and mouse α1β1$\delta\epsilon$ nAChRs by (top) α-ImI (■) and α-ImI[P6O] (△) and (bottom) α-GI[P5O]. The receptors were expressed in *Xenopus* oocytes that were voltage-clamped as described in Experimental Procedures. The graphs show the peptide concentration dependence of inhibition of peak ACh-gated currents. The error bars are ±the standard error of the mean for at least three repetitions. Corresponding IC_{50} values are given in Table 5.

Table 5: Activity of α-Conotoxin Analogues Containing Pro or Hyp Residues

	IC_{50} (95% confidence interval)			
nAChR subtype	α-ImI	α-ImI[P6O]	α-GI	α-GI[P5O]
α7	300 nM (213−421 nM)	>10 M	not determined	
α$\beta\epsilon\delta$				23.9 nM (21.1−27 nM)
α1β1$\delta\gamma$			20 nM	

DISCUSSION

This work systematically examined, for the first time, the role of Hyp modifications in conotoxins. Relatively little is known about the role of Hyp in proteins and peptides, with the exception of collagen. Conotoxins derived from cone snail venoms are extensively modified with Hyp residues (*11*, *20*): Hyp residues were discovered in conotoxins from at least 10 families, ranging from one- to four-disulfide peptides. Hyp residues can be uniformly distributed throughout the conotoxin sequence (*49*, *50*), as in κM-RIIIK (Table 1). Hyp residues often coexist with other posttranslationally modified amino acids such as 6-bromotryptophan (*Conus* peptides: tx5a, bromocontryphans) or glycoamino acids (κA-SIVA) (*51*−*54*). Hyp can coexist with Pro residues, as shown for α-conotoxins κA-SIVA and r11a (also known as ι-RXIA) (*52*, *55*−*57*). Furthermore, α-conotoxins were recently shown to be underhydroxylated (variably hydroxylated); two identical peptides, RgIB and RgIC, containing either Hyp or Pro were isolated from the venom of *C. regius* (*26*). Despite the unprecedented abundance of Hyp residues in conotoxins, the role of this posttranslational modification in these peptides has not previously been systematically studied.

There are prior studies which suggest that Hyp may affect the biological activity of some conotoxins (Table 6). The results presented in this work indicate that Hyp can be important both for the *in vitro* oxidative folding and for the activity of conotoxins; with regard to the latter, we speculate that cone snails "employ" hydroxylation to improve bioactivity. Our data for μ-conotoxin GIIIA suggest that hydroxylation of Pro contributes to the bioactivity, rather than folding, of this peptide. All substitutions in μ-GIIIA showed only small changes in the accumulation of the folded species, with an increase of the apparent rate of 2.5- and 4.5-fold for μ-GIIIA[O67P] and μ-GIIIA[O6,7,17P], respectively. Analogues of μ-GIIIA with more than one substitution showed a substantial loss of activity in inhibiting $Na_V1.4$ sodium channels. The triple replacement of Hyp with Pro resulted

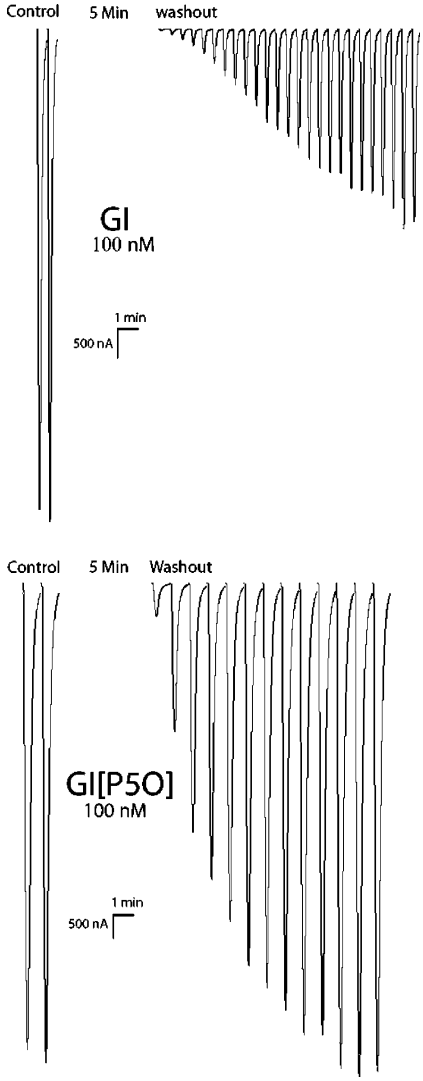

FIGURE 9: Time course of inhibition of mouse $\alpha 1\beta 1\delta\epsilon$ nAChRs by α-GI and its [P5O] analogue. Oocytes expressing the receptor were voltage clamped, and pulses of ACh were applied as described in Experimental Procedures. In each graph, the first two responses are controls, and the 5 min hiatus in responses reflects the time during exposure to peptide in a static bath, after which perfusion and ACh pulses were recommenced to determine the time course of recovery from toxin block. α-GI (100 nM) blocked the ACh-gated current completely and washed out very slowly (top). α-GI-[P5O] (100 nM) blocked the ACh-gated current nearly completely and washed out rapidly (bottom).

in 40-fold decrease in IC_{50}, the highest change in activity compared to the native peptide. This is consistent with the 62-fold increase in K_D seen with the triple replacement reported previously (*58*). Likewise, there was significant loss of activity in the case of μ-GIIIA[O17P], and this result is supported by a previous study in which Hyp17 in μ-GIIIA was found to be important for binding (*59*, *60*). As illustrated in Table 6, in two more Hyp-containing conotoxins, the hydroxylation of Pro was shown to be important for bioactivity: TxIX (*61*) and contryphan Am975 (*62*). Our current data combined with those previously described support the general notion that Hyp residues are important in determining interactions of conotoxins with ion channels and receptors.

Results of experiments with Hyp-containing α-conotoxins showed that the hydroxylation of Pro located in the first loop (between Cys II and Cys III) may improve their *in vitro* oxidative folding and the stability of the native conformation. However, despite folding advantages of Hyp over Pro, *Conus* snails utilize Pro in α-conotoxins. The explanation of this conservation might be that in both α-conotoxins studied, the Pro → Hyp replacement had a significant negative impact on their activity: α-ImI[P6O] was found to be inactive against α7 nAChRs, and α-GI[P5O] dissociated much faster from the $\alpha 1\beta 1\delta\epsilon$ nAChRs. Similar observations of effects of hydroxylation of the conserved Pro were previously described for another α-conotoxin, PnIB (*63*). Thus, from an evolutionary perspective, the advantages and disadvantages of Pro versus Hyp seem to be carefully "considered" in each family of conotoxins. Equally importantly, one must take into account the fact that *Conus* snails evolved their venom components to target ion channels and receptors of fish and worms, rather than those of mammals. Whether hydroxylation of Pro has comparable effects on those molecular targets requires further investigation.

Hydroxylation of Pro improved the *in vitro* folding yield of ω-MVIIC approximately 2-fold. This finding is remarkable since this conotoxin is well-known to produce low folding yields (*42*, *43*). Thus, the role of Hyp in MVIIC seems to be more structural than functional (the activities of both Hyp- or Pro-containing peptides were comparable). MVIIC most likely contains two conformations in which Pro7 has both cis and trans configurations; the existence of two conformers may explain slower folding and poor yields for this peptide (*64*, *65*). Thus, we speculate here that in the MVIIC[P7O] analogue, Hyp7 has only trans conformation. The steric effects between Ala6 and Hyp7 probably do not play an important role in MVIIC[P7O] because Ala is too small to introduce a steric block. It is more likely that an electronic effect of the hydroxyl group (discussed later) stabilizes the trans conformation, thus affecting the rates and yields of MVIIC folding. A result similar to that of MVIIC was found for ω-GVIA, where substitutions of two Hyp residues at positions 10 and 21 by proline did not change activity but appear to be more structural (*66*−*69*).

Differences in the cis−trans isomerization of Pro or Hyp residues may at least in part account for the contribution of

Table 6: Summary of Studies on Conotoxins in Which a Role of Hyp Was Investigated

peptide	sequence[a]	role of hydroxyproline	ref
ϵ-TxIX	γCCγDGWCCT@AA**O**	important in Ca channel binding	*61*
contryphan Am975	GC**O**$^{+}$WDPWC*	inhibits HVA Ca channels	*62*
ω-GVIA	CKSOGSSCSOTSYNCCRSCN**O**YTKRCY*	no effect on potency, except for the right atrium	*68*
ω-GIIIA	RDCCTOOKKCKDRQCK**O**QRCCA*	contribution to the strength of binding for skeletal muscle sodium channels	*59*

[a] Shaded residues are involved in activity. Abbreviations: O, hydroxyproline; W, bromotryptophan; γ, γ-carboxyglutamic acid; *, amidated C-terminus; @, glycosylation; +, D-amino acid.

Hyp to the *in vitro* oxidative folding of conotoxins. (This could be quantified if one could access the Cγ and Cβ shifts of the proline and/or NOE intensities.) The Pro cis−trans isomerization rates and equilibria greatly depend on local interactions with neighboring residues and on the overall conformation of polypeptides (*65*, *70*−*73*). The inductive effects of 4-hydroxylation of proline were shown to stabilize the trans isomer around the peptidic bond (*74*, *75*). Indeed, all four Hyp residues in RIIIK were found in the trans conformation (*50*). In conotoxin μ-GIIIA, Hyp6 and Hyp17 were defined as the trans conformation and Hyp7 was defined as the cis conformation, but the cis conformation in Hyp7 was not verified by NMR data (*76*). NMR studies on PIIIA revealed two conformations: the major conformer (75%) had Hyp8 in the trans configuration, whereas the minor one had Hyp8 in the cis conformation. However, both conformers had Hyp18 in the trans configuration. The structural consequences of cis−trans isomerization of Hyp residue were also observed in contryphan-R (*77*).

The relationship between proline hydroxylation and the *in vitro* oxidative folding might be biologically significant, since prolyl-4-hydroxylase (P4H) and protein disulfide isomerase (PDI) coexist as heterotetramers in the ER (*78*, *79*). Thus, it is conceivable that proline hydroxylation and formation of disulfide bridges in vivo may occur almost concurrently. However, despite cooperative interactions between P4H and PDI that have been identified to date (*80*), to the best of our knowledge, no studies have shown that P4H can catalyze the oxidative folding of polypeptides. This work may also have broader implications with respect to a role of Hyp in polypeptides. Our observation that proline hydroxylation in conotoxins plays a role in molecular recognition is reminiscent of proline hydroxylation that occurs in hypoxia-inducible factors (HIF) that regulate cellular responses to changes in oxygen (*81*, *82*). Hydroxylation of critical proline residues is a key for molecular recognition of HIF by the von Hippel−Lindau tumor suppressor, leading to polyubiquitination and proteosomal degradation (*83*, *84*). In summary, our studies emphasize the importance of proline hydroxylation in the structure and function of proteins and peptides.

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SUPPORTING INFORMATION AVAILABLE

Proton NMR spectra of μ-GIIIA and the analogues containing Hyp → Pro replacements (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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