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Conotoxins Containing Nonnatural Backbone Spacers: Cladistic-Based Design, Chemical Synthesis, and Improved Analgesic Activity

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SUMMARY

Disulfide-rich neurotoxins from venomous animals continue to provide compounds with therapeutic potential. Minimizing neurotoxins often results in removal of disulfide bridges or critical amino acids. To address this drug-design challenge, we explored the concept of disulfide-rich scaffolds consisting of isostere polymers and peptidic pharmacophores. Flexible spacers, such as amino-3-oxapentanoic or 6-aminohexanoic acids, were used to replace conformationally constrained parts of a three-disulfide-bridged conotoxin, SIIIA. The peptide-polymer hybrids, polytides, were designed based on cladistic identification of nonconserved loci in related peptides. After oxidative folding, the polytides appeared to be better inhibitors of sodium currents in dorsal root ganglia and sciatic nerves in mice. Moreover, the polytides appeared to be significantly more potent and longer-lasting analgesics in the inflammatory pain model in mice, when compared to SIIIA. The resulting polytides provide a promising strategy for transforming disulfide-rich peptides into therapeutics.

INTRODUCTION

Ranging from spider, scorpion, and cone snail venom toxins to plant cyclotides, the molecular diversity of disulfide-rich peptides can be estimated in millions of unique molecules [1–4]. These peptides evolved as neurotoxins to capture prey, for host self-defense, or as neurotrophic and/or neuromodulatory compounds. As a result of millions of years of evolution, the bioactive peptides exhibit high potency and selectivity for their molecular targets, making them attractive as pharmacological tools and therapeutic agents (Figure 1A). Mining these exquisite libraries of disulfide-rich peptides has resulted in such therapeutics as pain-relieving ω-MVIIA, the antihemorrhagic drug aprotinin, and the antidiabetic drug insulin. Many venom peptides are being tested in preclinical and clinical studies as analgesic, anticoagulant, antimicrobial, immunosuppressant, or anticancer therapeutics [5]. One toxin, ziconotide, derived from a cone snail venom was approved by the FDA in 2004 for a treatment of intractable pain [6].

Peptidomimetic approaches have been very effective for short biopolymers, such as opioid peptides or somatostatin [7–9]. However, for multiple-disulfide-bridged peptides, an intrinsic problem of reducing molecular size is a removal of disulfide bridges, often resulting in a loss of the bioactivity [10, 11]. Rational design and peptidomimetic strategies provided compounds with lower potency, as documented in the cases of ω-conotoxin GVIA [12, 13] and ShK toxin [14–16]. One successful example of minimization of a single disulfide-containing peptide by using structure-based and phage-display methods is a 28 aa atrial natriuretic peptide (ANP) [17, 18]. Examples of recent efforts of improving biological stability of conotoxins are the N-to-C-terminal cyclization of MII or MrIA and the replacement of disulfides by diselenocystine bridges [19–21].

To advance engineering technologies for disulfide-rich peptides, we explored a concept of peptide-based pharmacophores grafted into crosslinked, polymer-based scaffolds (Figure 1B). This pseudobiopolymer concept is encapsulated in the term polytide, a neologism describing hybrids of nonpeptidic polymers and peptide-based components. The bioactive conformation of a polytide is stabilized by disulfide bridges (or other crosslinks, such as lactams or thioether bridges). To design polytides, we propose a stepwise replacement of several adjacent amino acid residues with backbone spacers, such as PEG spacers or extended-glycine amino acids. The selection of replacements might be directed by an analysis of nonconserved loci in closely related sequences of peptides (cladistic analysis), as described previously by our

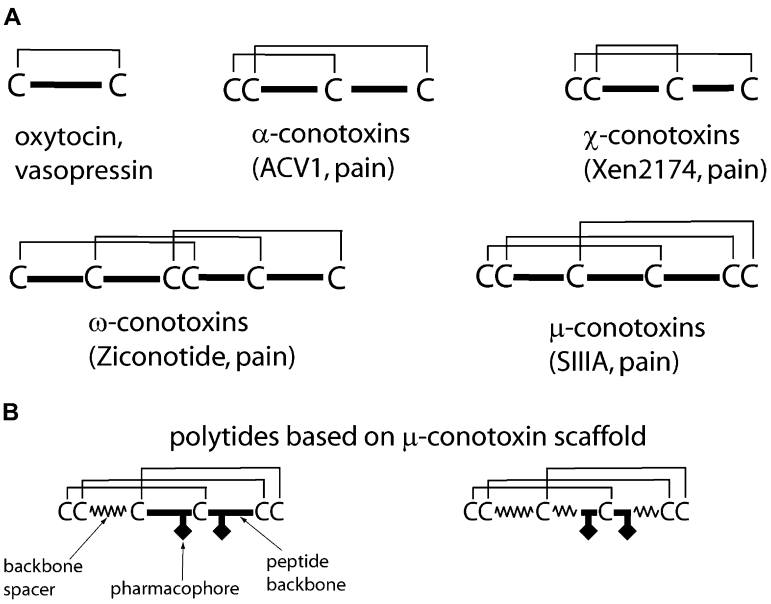


Figure 1. Disulfide-Rich Peptides as Potential Drugs

(A) Diversity of disulfide-rich scaffolds of therapeutically relevant peptides. The names of peptides currently in preclinical and clinical development are shown in parentheses.

(B) Concept of polytides. Introduction of isostere backbone spacers into conformationally constrained peptides produces polymer-peptide hybrids, polytides. To better illustrate this concept, two μ-conotoxin-based polytides that differ in a number of backbone spacers are shown.

laboratory [22, 23]. Once successful, such "backbone prosthesis" replacements may be combined. The term "protein prosthesis" was first proposed by Raines [24, 25]: we would like to extend this term to peptides as well. In this work, we explored the backbone prosthesis concept by using conotoxins that block sodium channels [26–28]. The resulting conotoxin-polymer hybrids appeared to be at least 10-fold more potent analgesics than the original peptide in the inflammatory pain model in mice after systemic administration. Our data presented here show that employing normally flexible spacers in place of conformationally constrained regions of disulfide-bridged peptides is a valid strategy for producing compounds with a therapeutic potential.

RESULTS

Rational Design of Polytides

The concept of "backbone prosthesis" was inspired by recently described structure-activity relationships of three μ-conotoxins, SmIIIA, SIIIA, and KIIIA, that belong to a single clade of cone snail peptides (Figure 2A) [27]. Despite naturally occurring central truncations, all three conotoxins belonging to a single clade potently blocked Na channels. KIIIA and SIIIA exhibited comparable activity in blocking TTX-R and TTX-S sodium channels in amphibian nerve preparations [27]. Thus, neither the length of the first loop nor the two N-terminal residues seemed to be critical for determining interactions with sodium channels. Since SIIIA contains "nonessential" ZN- at the N terminus, and 2 glycine residues in the first loop, this peptide was selected to test how replacing these residues with backbone spacers may affect folding and bioactivity. Based on the above-described cladistic analysis, two analogs were designed containing two distinct backbone spacers (Figure 2B). In the first analog, PEG-SIIIA, both ZN- and -GG- were replaced with amino-3-oxapentanoic acid. In the second, AHX-SIIIA, 6-aminohexanoic acid was used instead of amino-3-oxapentanoic acid. The structural variation between SIIIA and the prosthesis-containing analogs in the C-terminal region is evident from comparing three model structures shown in Figures 2C and 2D. The region C-terminal to the backbone replacement, however, maintains a similar conformation to the original SIIIA. In particular, the residues critical to activity, K11, W12, and H16 [29], adopt the same spatial arrangement in all three structures.

Chemical Synthesis and Oxidative Folding

Chemical synthesis of PEG-SIIIA and AHX-SIIIA was carried out on solid support by using standard Fmoc protocols. Fmoc-protected amino-3-oxapentanoic acid or 6-aminohexanoic acid were coupled by using identical conditions to those used for other amino acids. Cleaved peptides were purified by using preparative reverse-phase HPLC. Reduced peptides were added to a buffer containing 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM reduced glutathione, and 1 mM oxidized glutathione. Folding was quenched after an appropriate time, and the folding mixtures were separated by analytical C_{18} HPLC. Representative HPLC separations of the folding reactions are shown in Figure 3. Folding equilibria for SIIIA and the hybrids were reached within 10 min. The main folding products accumulated at ~50% for SIIIA, 52% for PEG-SIIIA, and 28% for AHX-SIIIA. The folded hybrids exhibited ^{1}H NMR spectra similar to that of SIIIA (Figure 3). The amide regions of SIIIA and the analogs were well dispersed, with the analogs showing even better dispersion than the parent peptide and lacking minor peaks presumably arising from minor conformers in SIIIA.

Biological Activity

The folded hybrids and SIIIA were tested on TTX-sensitive (TTX_s) sodium currents (I_{Na}) in voltage-clamped mouse dorsal root ganglia (DRG) neurons. Figure 4A illustrates the block of I_{Na} produced by 5 μM of the peptides after ~20 min of exposure. SIIIA only blocked ~20% of the TTX_s I_{Na}, whereas the two hybrids were more active, blocking ~45%–55%. The block by SIIIA and its analogs was only very slowly reversible. Increasing the concentration to 25 μM resulted in ~65% inhibition for SIIIA and ~80% inhibition for the hybrids (Figure 4B). The analogs were then tested in blocking compound action potentials (CAPs) in sciatic nerve preparations in mice. Strikingly, PEG-SIIIA and AHX-SIIIA exhibited faster kinetics in blocking A-CAPs than SIIIA (Figure 4C). When analogs with replacements of either the N-terminal or the first loop -GG- with the PEG spacer were tested, the faster kinetics could be mostly accounted for by the N-terminal PEG moiety. TTX-resistant C-CAPs were not affected by SIIIA or the hybrids (data not shown).

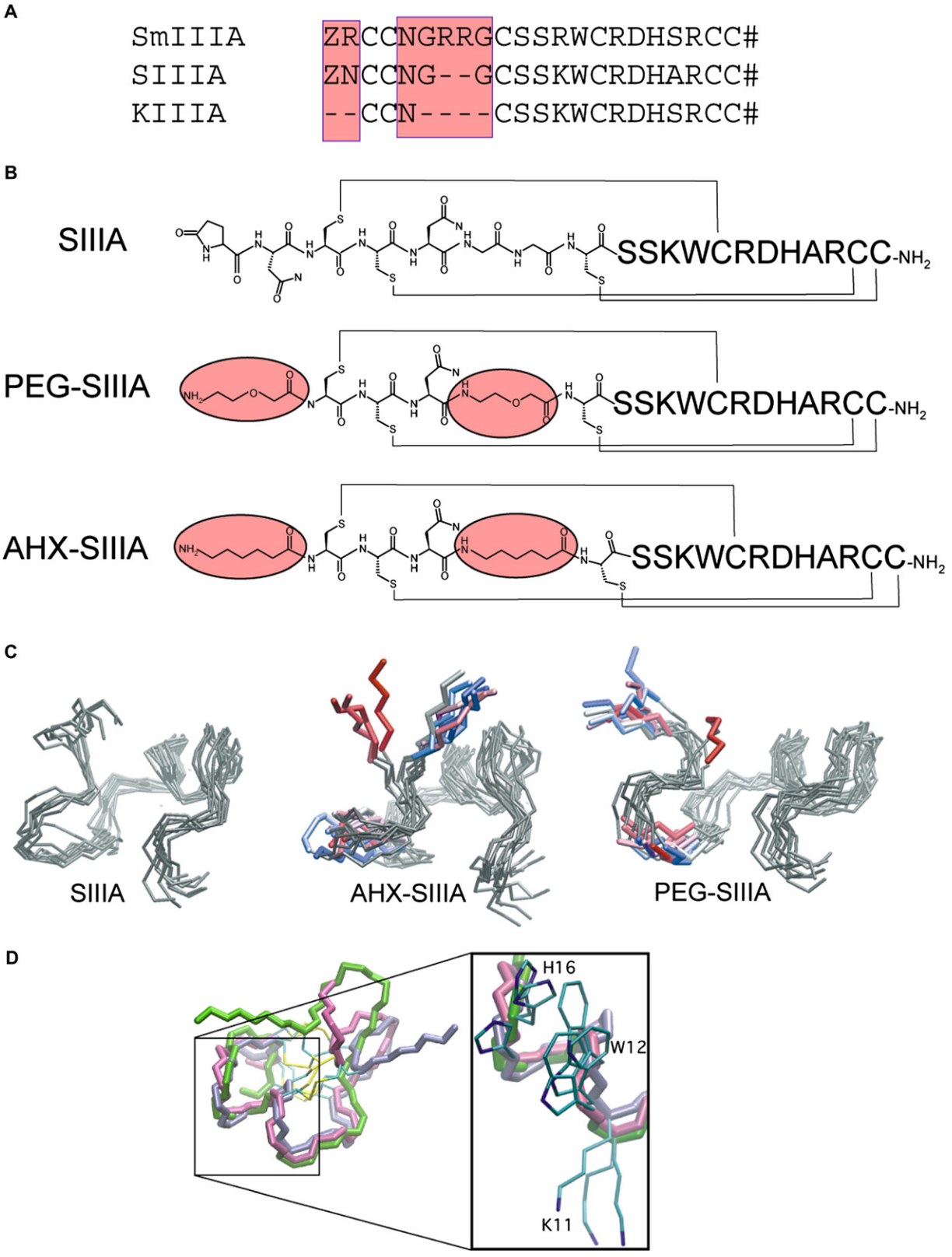
To evaluate analgesic activity, the analogs were tested in the inflammatory pain assay in mice (formalin test). Peptides were injected intraperitoneally (i.p.) prior to injection of formalin. As shown in Figure 5A, all three compounds were analgesic at doses of 10 nmol per animal (corresponding to ~0.7 mg/kg), although both hybrids were more active in the Phase II response than SIIIA. Since PEG-SIIIA acted more quickly than SIIIA in blocking A-CAPs, a time course of the nociceptive response was determined (Figure 5B). At doses of 10 nmol per animal, PEG-SIIIA exhibited more pronounced and longer-lasting analgesic activity in the inflammatory phases. We also observed a significantly faster response for PEG-SIIIA in the acute response phase, when formalin was injected 30 s or 5 min after the i.p. injection of the compounds. The analgesic effects were dose dependent, with an estimated ED_{50} of 12 nmol per mouse (0.9 mg/kg) for SIIIA and 0.7 nmol per mouse (0.05 mg/kg) for PEG-SIIIA. Thus, the PEG-spacer-containing polytide was significantly more potent compared to the original conotoxin, SIIIA.

DISCUSSION

To our knowledge, this work describes the first example of successful conotoxin engineering in which the resulting analogs exhibited both improved in vitro and in vivo pharmacological properties. Replacing peptidic parts of SIIIA with backbone spacers produced peptide-polymer hybrids with reduced molecular size (19 aa instead of 22 aa), and yet with a significantly more potent and longer-lasting analgesic activity. To our knowledge, we describe here for the first time that μ-conotoxins, such as SIIIA, are analgesic after systemic administration; this activity is likely due to blocking of peripheral sodium channels. These data complement a growing list of diverse analgesic conotoxins (reviewed in [30]). Mechanistic aspects of why the PEG-SIIIA hybrid was a more potent analgesic than SIIIA are being investigated with more structure-activity relationship and pharmacological studies. However, independent of mechanisms by which backbone prosthesis improved the pharmacological profile of SIIIA, this work provides the proof-of-concept data for the hypothesis that engineering conotoxins by using backbone spacers may be a useful strategy by which to develop therapeutics. Indeed, the very high potency of PEG-SIIIA (50 μg/kg, i.p.) justifies additional efforts in exploring this, or related analogs, as an analgesic for treatment of neuropathic pain.

Although aminohexanoic acid is commonly used in peptides, to the best of our knowledge, this is the first example of successful introduction of Ahx into a conformationally constrained peptide. Previously, aminohexanoic acid was proven useful in engineering centrally truncated analogs of neuropeptide Y [31, 32]. Similarly, introduction of Ahx into GLP-1 improved its hypoglycemic duration of action [33]. In another study, 8-amino-3,6-dioxaoctanoic acid was used in backbone-substituted analogs of the decapeptide antibiotic tyrocidine A [34]. Finally, PEGylation of peptides and proteins is a commonly used strategy for improving their therapeutic properties [35]. In contrast to the previous work, we propose to use diverse flexible spacers as isosteric replacements of conformationally constrained parts of disulfide-rich peptides. As illustrated in Figure 6, such spacers may be of different length or chemical nature. In fact, there is a growing number of commercially available spacers compatible with the solid-phase peptide synthesis using Fmoc chemistry: we hope that this work might spur even more interest in developing diverse spacers. We would like to speculate here that replacing nonessential amino acid residues with different spacers may provide a means to more rational modifications of bioavailability of the resulting polytides. Another advantage of this approach is that such isostere replacements are less likely to compromise the biological activity, because the bioactive conformation is stabilized by crosslinks such as the disulfide bridges. Formation of the native disulfide crosslinks in polytides might be achieved by either direct oxidative folding, as shown here, or by orthogonal protection of cysteine residues [36, 37]. Interestingly, the SIIIA-based polytides maintained a high tendency for the efficient oxidative folding, further emphasizing an important role of the cysteine patterns in determining folding mechanisms. This is very encouraging for the synthetic accessibility of polytides derived from different conotoxins or other disulfide-rich peptides.

Extending the concept of polytides, the backbone spacers may be considered for the N-to-C-terminal cyclization, producing cyclotide-polymer hybrids with additional backbone spacer crosslinks [3]. Similarly, many peptide inhibitors of proteinases, antimicrobial peptides, insect toxins, or snake toxins may be amenable to backbone prosthesis, making them more attractive drug candidates compared with peptidomimetics or small molecules. Since solid-phase synthesis methods have become more and more applicable to larger polypeptides, backbone spacers may be used in protein engineering



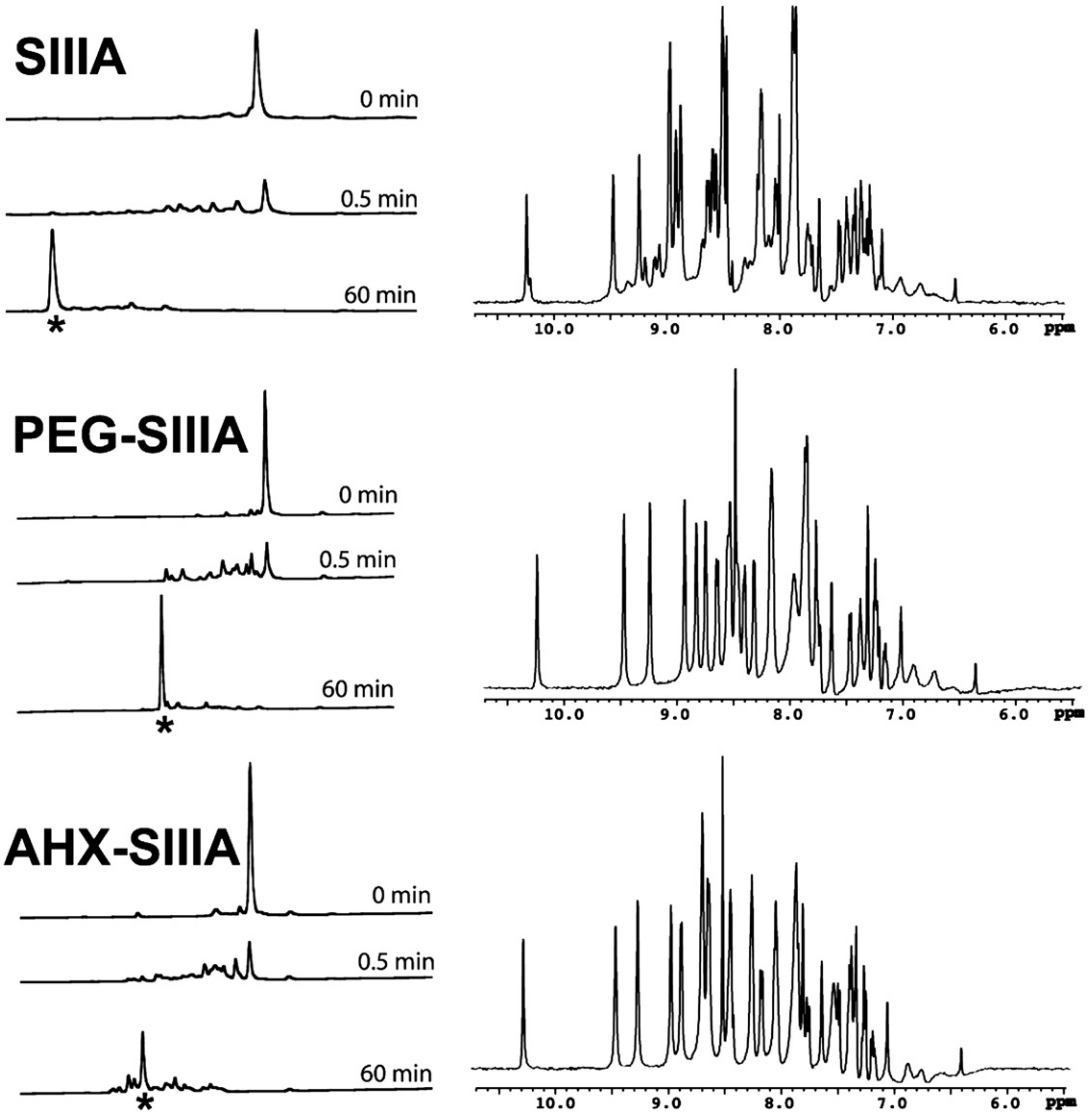


Figure 3. Oxidative Folding of SIIIA and the Peptide-Polymer Hybrids

Folding reactions were carried out in the presence of 0.1 M Tris/HCl (pH 7.5), 1 mM GSSG, and 1 mM GSH at ambient temperature (23°C–25°C). Reactions were quenched by acidification, followed by analytical HPLC analysis. The asterisk denotes the folded species characterized by NMR and used in functional assays. ^{1}H NMR spectra of the folded forms of SIIIA, PEG-SIIIA, and AHX-SIIIA. All spectra were recorded at 600 MHz in $H_2O/^2H_2O/C^2H_3COO^2H$ (50%/50%/1%) at pH 2.6 and 5°C.

with either disulfide-rich scaffolds [38] or mini-protein motifs [39]. It is obvious that more studies will be needed to validate the broader applicability of polytides, but our present work indicates that such an approach is an efficient strategy for transforming disulfide-rich peptides into drugs.

Figure 2. Design of the μ-Conotoxin SIIIA and the Polytides

(A) Cladistic analysis of SmIIIA, SIIIA, and KIIIA. The key structural differences are emphasized by shaded areas.

(B) Structures of SIIIA and two hybrid analogs containing backbone spacers: amino-3-oxapentanoic acid (PEG spacer), yielding PEG-SIIIA; and 6-aminohexanoic acid, yielding AHX-SIIIA.

(C) Model structures of SIIIA, PEG-SIIIA, and AHX-SIIIA. A backbone of eight representative conformations is shown for each analog. The amide backbone is gray; the backbone spacers are shown in red, white, and blue.

(D) Superimposition of the backbone atoms of the final structures of SIIIA (dusty-pink), PEG-SIIIA (steel-blue), and AHX-SIIIA (olive-green) from the MD calculations. The backbone atoms from S9–C20, encompassing the pseudo helical segment, adopt a similar geometry. There are large structural differences between the three peptides in the N-terminal residues prior to S9, including the backbone replacement groups in the prosthetic derivatives. A closer zoom at the 11–16 region shows that residues K11, W12, and H16 in the three peptides adopt similar positions in the superimposition. The atoms of the side chains are colored turquoise (carbon) and blue (nitrogen).

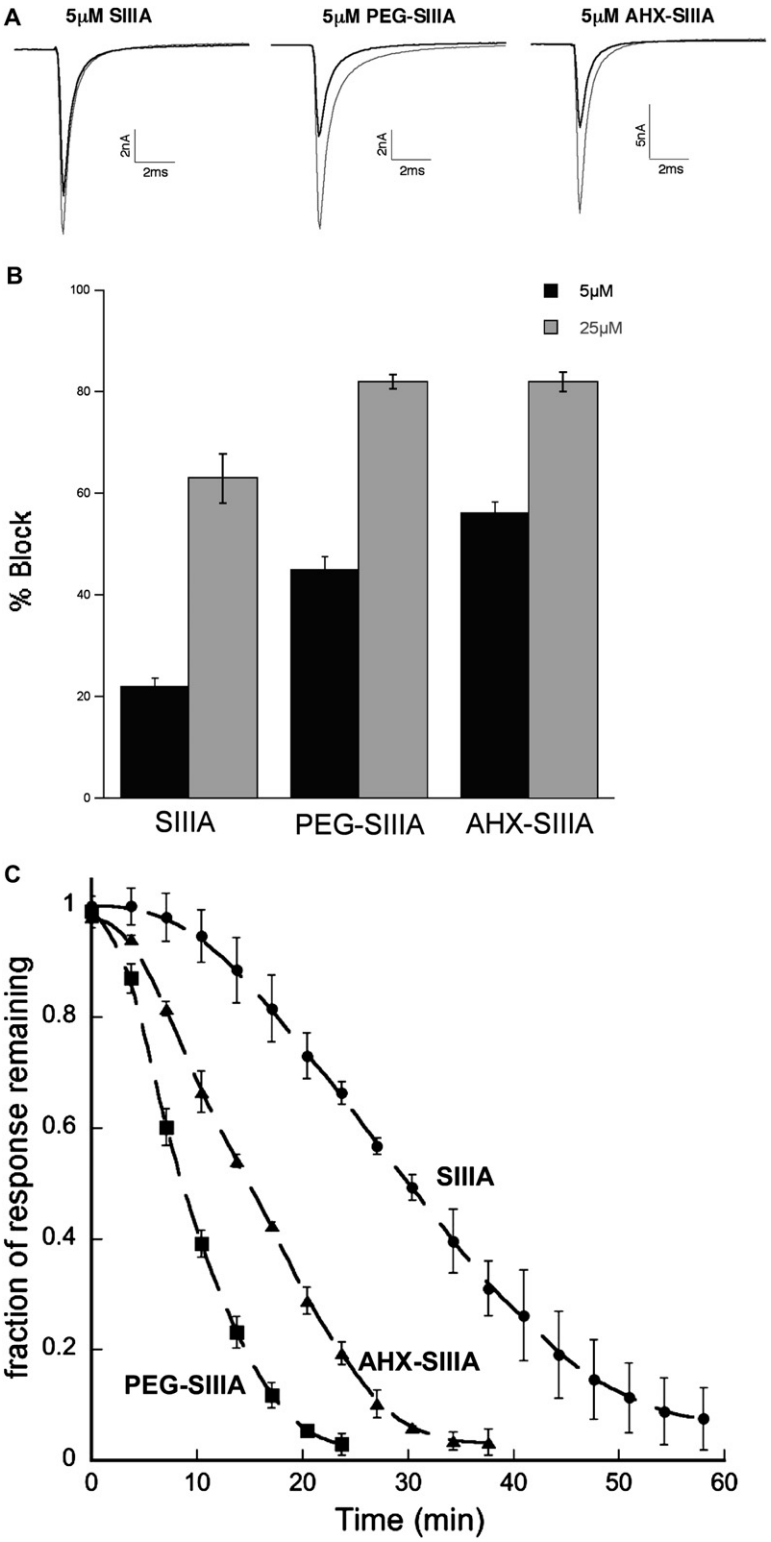


Figure 4. SIIIA and the Hybrids Block Sodium Currents in Mouse Preparations of DRG Neurons and in the Sciatic Nerve

(A) Representative TTX-sensitive sodium currents in DRG neurons recorded in the absence of (control traces, gray) or after an ~20 min exposure to 5 μM SIIIA or analogs (black traces). Each trace represents the average of five responses. DRG neurons were dissociated and whole-cell voltage clamped as described in Experimental Procedures.

(B) Percent block of the peak of the TTX-sensitive sodium current after approximately 20 min of exposure to either 5 μM (black bars) or 25 μM (gray bars) peptide (mean ± SEM, n = 3–4).

(C) Block of A-compound action potentials (TTX-sensitive) in sciatic nerve preparation from mouse by 1 μM peptide concentrations.

SIGNIFICANCE

There is a continuous need to develop drugs derived from peptide-based natural products, such as neurotoxins, antimicrobial peptides, plant peptides, and others. Traditional structure-based peptidomimetic approaches are less applicable for these disulfide-rich peptides, since reducing their molecular size is

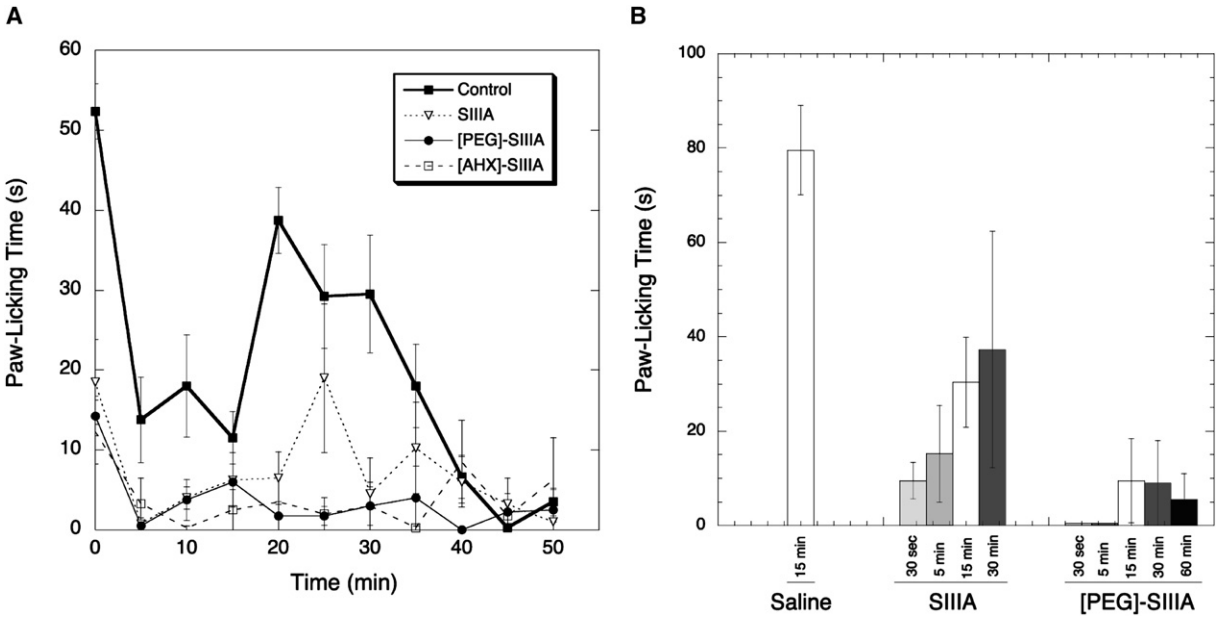


Figure 5. Analgesic Activity of SIIIA and the PEG-SIIIA Hybrid in the Inflammatory Pain Assay in Mouse

(A) Effect of saline (solid square), SIIIA (open triangle), PEG-SIIIA (solid circle), and AHX-SIIIA (open square) on formalin-induced paw licking during Phases I and II. Treatments were administered intraperitoneally 15 min prior to formalin injection.

(B) Effects of 10 nmol SIIIA or the PEG-SIIIA hybrid on paw licking during Phase II (the combined licking time during 15–30 min) at different times before injection of formalin into a paw. Data are expressed as means ± SEM for four animals.

often associated with a removal of stabilizing cross-links or the removal of critical amino acid residues, leading to a loss of bioactivity. This work explored the concept and feasibility of polymer-peptide hybrids, in which conformationally constrained, but non-essential, amino acid residues are replaced with isostere polymeric spacers. The key advantage to this minimization strategy is that the resulting polytides maintain the overall size of the molecule and thus don't compromise the spatial orientation of key functional residues. Replacing parts of the peptidic backbone with various nonpeptidic spacers provides a means of improving bioavailability by reducing susceptibility to proteolysis, and reducing hydrogen bond donors/acceptors (affecting logP values). Although our work provides proof-of-concept data for the use of conotoxin SIIIA, we hope that this approach will be explored in many other disulfide-rich peptides that have a therapeutic potential.

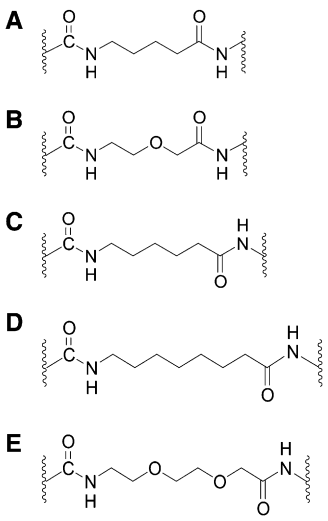


Figure 6. Examples of Isostere Spacers Incorporated into a Peptide Backbone

(A–E) Various F-moc-protected spacers are commercially available. (A) 5-aminopentanoic acid. (B) 5-amino-3-oxapentanoic acid (PEG spacer). (C) 6-aminohexanoic acid. (D) 8-aminooctanoic acid. (E) 8-amino-3,6-dioxaoctanoic acid (PEG spacer). Please note that most spacers shown here have a number of backbone atoms equivalent to either two or three amino acid residues.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

Peptides were synthesized on amide MBHA resin by using Fmoc (N-[9-fluorenyl]methoxycarbonyl) chemistry. For coupling of backbone spacers, the following derivatives were used: Fmoc-aminohexanoic acid and Fmoc-3-oxapentanoic acid (Fmoc-O1Pen from IRIS Biotech). The linear forms were purified by reversed-phase HPLC with a semi-preparative C_{18} Vydac column (218TP510) in a linear gradient of acetonitrile (in 0.1% TFA). Oxidative folding was performed by resuspending the linear peptides in 0.01% TFA in water and injecting this solution into a buffered solution (0.1 M Tris-HCl [pH 7.5]) containing 1 mM EDTA, 1 mM reduced glutathione, and 1 mM oxidized glutathione. The final peptide concentration was 20 μM. Folding reactions were monitored by analytical reversed-phase HPLC separations with a C_{18} Vydac column (218TP54) in a linear gradient of acetonitrile. The identity of the peptides was confirmed by mass spectrometry.

NMR Spectroscopy

Peptide samples (20 nmol) were dried in $H_2O/^2H_2O/C^2H_3COO^2H$ and resuspended in 250 μl 90% H_2O/10% ^2H_2O/1% $C^2H_3COO^2H$ (pH 2.6). One-dimensional ^1H NMR spectra were recorded with a Varian Inova 600 NMR spectrometer. Water suppression was achieved with an 11-echo scheme. Spectra were recorded at 5°C with 4096 complex points and a spectral width of 12 ppm; chemical shifts are referenced to DSS at 0 ppm.

Molecular Modeling

An initial model of SIIIA was created with the MODELER (6v2) program [40] by using the solution structure of SmIIIA [29] (PDB code: 1Q2J, representative structure 13) as a template and by following the procedure described earlier [27]. Backbone prostheses were built into the initial model of SIIIA by using InsightII (Accelrys, 2000). All structures were subjected to molecular dynamics (MD) simulation by using the GROMACS (v3.3.1) package of programs [41] and by applying a similar approach as had been used to model SIIIA previously [27]. All simulations consisted of an initial minimization of water molecules, followed by 100 ps of MD with the peptide fixed. After positional restraints MD, the restraints on the peptide were removed, and MD continued for an additional 10 ns.

Electrophysiology

DRGs were dissected from adult mice of either sex and dissociated as described previously [28]. Briefly, dissociated neurons were perfused with extracellular solution containing (in mM) 35 NaCl, 105 N-methyl-D-glucammonium-Cl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 TEA, and 0.1 $CdCl_2$ (pH 7.3). Recording pipettes contained (in mM) 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES (pH 7.3). To record TTX-sensitive currents, large DRG neurons (diameter > 35 μm) were selected. Conotoxins were dissolved in extracellular solution and were applied to the neurons under study by bath exchange; the bath remained static throughout the toxin exposure. The membrane potential was held at −80 mV, and the sodium channels were activated by a 50 ms test pulse to −20 mV, applied every 30 s. Each test pulse was immediately preceded by a 50 ms, −120 mV conditioning pulse to remove channel inactivation. A MultiClamp 700A amplifier (Axon Instruments, Union City, California) was used, and current signals were low-pass filtered at 3 kHz, digitized at a sampling frequency of 10 kHz, and leak-subtracted by following a P/6 protocol by using in-house software written in LabVIEW (National Instruments, Austin, Texas). All experiments were done at room temperature. Compound action potentials were recorded as described in [30].

Inflammatory Pain Assay

A formalin-induced nociception test in mice was performed as described elsewhere [42]. Swiss Webster mice (25–30 g) were placed in individual open glass cylinders. A total of 15 min prior to intraplantar injection of 10 μl 4.4% formalin into one hind paw, mice were intraperitoneally (i.p.) injected with the tested peptides or saline solution (control). Mice (two at a time) were observed for 50 min, and paw-licking time was determined every 5 min in 2 min intervals. The acute (first) phase of the nociceptive response was quantified within the first 5 min. The inflammatory (second) phase was quantified from 15 to 30 min. For each dose, at least four animals were used. All animal procedures were carried out according to the approved protocol of the IUCAC at the University of Utah.

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