κ-Conotoxin PVIIA Is a Peptide Inhibiting the Shaker K⁺ Channel

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κ-Conotoxin PVIIA (κ-PVIIA), a 27-amino acid toxin from Conus purpurascens venom that inhibits the Shaker potassium channel, was chemically synthesized in a biologically active form. The disulfide connectivity of the peptide was determined. κ-Conotoxin PVIIA has the following structure.

CRIONQKCFLHDDCSCRKCNRFKCV

STRUCTURE I

This is the first Conus peptide known to target K⁺ channels. Although the Shaker K⁺ channel is sensitive to κ-PVIIA, the rat brain Kv1.1 subtype is resistant. Chimeras between Shaker and the Kv1.1 K⁺ channels were constructed and expressed in Xenopus oocytes. Only channels containing the putative pore-forming region between the fifth and sixth transmembrane domains of Shaker retained toxin sensitivity, indicating that the toxin target site is in this region of the channel. Evidence is presented that κ-PVIIA interacts with the external tetraethyl-ammonium binding site on the Shaker channel.

Although both κ-PVIIA and charybdotoxin inhibit the Shaker channel, they must interact differently. The F425G Shaker mutation increases charybdotoxin affinity by 3 orders of magnitude but abolishes κ-PVIIA sensitivity.

The precursor sequence of κ-PVIIA was deduced from a cDNA clone, revealing a prepropeptide comprising 72 amino acids. The N-terminal region of the κ-PVIIA prepropeptide exhibits striking homology to the ω-, μO-, and δ-conotoxins. Thus, at least four pharmacologically distinct superfamilies of Conus peptides belong to the same “O” superfamily, with the ω- and κ-conotoxins forming one branch, and the δ- and μO-conotoxins forming a second major branch.

The venoms of the 500 species of predatory marine snails belonging to the genus Conus have proven to be a rich source of biologically active peptides that target voltage-gated calcium and sodium channels, as well as the acetylcholine receptor, and have been characterized. However, the peptides that target potassium channels in Conus venoms are relatively unexplored; recently, we described an initial characterization of κ-PVIIA, a peptide from the fish-hunting snail Conus purpurascens, which inhibited the Shaker K⁺ channel but had no effects on any voltage-gated Ca²⁺ or Na⁺ channel tested (1). The sequence of κ-PVIIA bears little apparent homology to the dendrotoxins or to scorpion and spider toxins, which block the Shaker K⁺ channels.

The Shaker potassium channel is one of the most intensively investigated neuronal signaling macromolecules. Several polypeptide toxins, notably charybdotoxin and the agitoxins (2), have been used to probe this channel; this approach has made the outer vestibule of the Shaker potassium channel the most well mapped region of any ion channel complex. A novel toxin based on a different structural framework from existing ligands could potentially interact with unmapped extracellular regions of the ion channel complex.

In this report, we describe the successful chemical synthesis of κ-PVIIA and determination of the disulfide connectivity of the biologically active peptide. We have also determined the predicted precursor sequence for the peptide, derived from the nucleic acid sequence of a cDNA clone encoding the peptide. These data confirm the amino acid sequence assignment for κ-conotoxin PVIIA reported previously (1). Finally, we have investigated interactions between the toxin and chimeric or mutagenized analogs of the Shaker K⁺ channel.

EXPERIMENTAL PROCEDURES

Native Peptide Purification—κ-Conotoxin PVIIA was purified from milked venom obtained as described by Hopkins et al. (3) from specimens of C. purpurascens collected from the Gulf of California. Milked venom was diluted to 10 ml with a solution of 0.1% trifluoroacetic acid, 99.9% H₂O and quickly injected onto a C₁₈ Vydac preparative column (22.0 × 250 mm, 10-μm particle size, 300-A pore size). Elution of peptides was carried out at a flow rate of 20 ml/min, using a gradient of buffer A containing 0.1% trifluoroacetic acid, 99.9% H₂O and buffer B containing 0.085% trifluoroacetic acid, 9.915% H₂O, 90% CH₃CN as described previously (1). Each peak from the run was collected and stored at 70 °C as stock solutions, and further purification was carried out from these stock fractions. A C₁₇ Vydac analytical column (4.6 × 250 mm, 5-μm particle size) was used for the secondary purification with flow rate of 1 ml/min and B buffer containing 0.085% trifluoroacetic acid, 39.915% H₂O, 60% CH₃CN.

Partial Reduction and Amino Acid Sequence Analysis—The stepwise reduction and differential alkylation of κ-PVIIA to determine disulfide connectivity was carried out as described previously (3–5). The alkylated peptides were purified by HPLC, and sequencing was performed

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1 The abbreviations used are: κ-PVIIA, κ-conotoxin PVIIA; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; TEA, tetraethyl-ammonium.
with Edman chemistry on an Applied Biosystems model 477A Protein Sequencer on the Protein/DNA Core Facility at the University of Utah; we are grateful to Dr. Robert Schackmann for the sequence analysis. Mass spectra were measured with a JEOL JMS-HX110 double-focusing spectrometer fitted with a Cs+ gun.

Solid-phase Peptide Synthesis—The protected peptide resin was built using standard Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry as described by Shon et al. (4) and with two of the cysteines (position 8 and 20) protected by S-acetamidomethyl and the other cysteines with S-trityl. Other side chains were protected as follows: pentamethylenediamine (Arg), t-butyli (Hyp, Acp, and Ser), trityl (Asn, His), and t-butyloxycarbonyl (Lys). Initially, a two-step oxidation protocol was used to generate three fully disulfide-bonded peptide isomers as detailed by Monje et al. (6); only one isomer was biologically active. This resulted in a very poor yield and led us to try conditions in which the linear peptide without acethylamidomethyl-protected Cys was diluted in 0.1% trifluoroacetic acid to less than 50 μl in the presence of 1 mM reduced and 0.5 mM oxidized glutathione (pH adjusted to 7.5). The equilibrium shift induced by the presence of excess reduced glutathione oxidized the peptide to a number of fully oxidized isomers. The major product, which was >90% of this mixture, proved identical to the native peptide (see "Results") and provided a far better yield than the initial two-step oxidation protocol. Five to six hours at the ambient temperature is usually enough to complete the folding reaction using these conditions.

Identification and Sequencing of C. purpurascens cDNA Clones—5 μl of venous duct RNA from C. purpurascens (5) was annealed to 3 pmol of a pol(Y) oligonucleotide, and cDNA was synthesized by avian myeloblastosis virus reverse transcriptase (5 units; Promega, according to the manufacturer's suggested protocol). The resulting cDNA was used as a template for PCR reaction in 10 μl sealed capillary tubes using an Idaho Technology air thermocycler. Each reaction contained 50 ng of template cDNA, 5 pmol each of oligonucleotides corresponding to the 5'- and 3'-untranslated regions sequence of ω-conotoxin prepeptides, 5 nmol of each of the four dNPs, and 0.5 units of Taq polymerase (Boehringer Mannheim) in a buffer consisting of 50 mM Tris, pH 8.3, 250 μM/ml bovine serum albumin, and 2 mM MgCl₂. The PCR consisted of 40 cycles (94 °C pulse, 54 °C pulse, and 72 °C for 15 s).

The PCR product was gel-purified and recovered from agarose using the Bio-Rad Prep-A-Gene kit according to the manufacturer’s protocol. The eluted DNA fragment was ligated to SalI-digested PUC plasmid pTZ18U by first generating blunt ends (Smal from Life Technologies, Inc.). The purified PCR product (44 ng) was mixed with SalI-digested pTZ18U DNA (40 ng) and 0.2 μM of each of the four dNPs in a buffer containing 25 mM Tris- HCl, pH 7.4, 5 mM MgCl₂, 5 mM dithiothreitol, 0.2 μM each of 1 mM ATP, 1.25 mM ammonium acetate, and 10 μM/ml bovine serum albumin in a final volume of 16 μl. Three units of T₄ polymerase (New England Biolabs) were added, and the reaction incubated for 5 min at 37 °C. The enzyme was inactivated by incubation for 15 min at 75 °C and the reaction cooled to 25 °C. Ligation was carried out in the above reaction mixture, to which 5 units of T₄ ligase (Promega) and 2 μl of 1 mM ATP were added at room temperature overnight.

Competent DH5αF’IQ cells (0.1 ml; Life Technologies, Inc.) were transformed with the entire ligation mix and spread on B agar plates (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside) containing 50 μg/ml each of ampicillin and kanamycin (all from Sigma). After incubation overnight at 37 °C, colorless colonies produced by recombinant plasmids were screened for the presence of the correct sized insert via PCR with vector-targeting primers in an Idaho Technology air thermocycler. Colonies that contained the correct sized inserts were prepared for DNA sequencing.

Sequencing—Single-stranded DNA was prepared from putative ω-PVIIA clones for sequencing by PCR amplification of 50 ng of plasmid with a pair of vector primers, one of which is biotinylated, and binding the resulting PCR product to streptavidin-bound magnetic polylysine beads (Dynal, Dynabeads M-280 streptavidin). Material for solid-phase sequencing was prepared according to the manufacturer’s suggested protocol, generating single-stranded nucleic acid, which was sequenced using the Sequenase version 2.0 DNA sequencing kit, the non-biotinylated vector primer, and [35S]ATP, according to standard Sequenase protocol. The nucleotide sequence of the ω-PVIIA was confirmed using a Beckman 8 (7) gene sequencer cloned in a Bluescript vector. The Kv1.1 (RCK1; Ref. 8) and the chimeras were in pSGEM. Unique restriction sites, which left the primary sequence unchanged, and point mutations were introduced in Shaker and RCK1 channels by PCR mutagenesis, and the amplified sequences were controlled by sequencing. These silent changes added a XbaI site at the coding sequence for Leu186 (after S1), a Nhel site at Ala3268 (in front of S5), and a DraIII site at Ser3290 (beginning of S6) in RCK1. In Shaker, a

RESULTS

Precursor Sequence of ω-Conotoxin PVIIA—A tentative amino acid sequence for ω-PVIIA was assigned previously (1). An analysis of the purified peptide by mass spectrometry gave a mass (monoisotopic MH⁺ = 3268.4; theoretical = 3268.42) consistent with the predicted sequence if all Cys residues were disulfide-bonded and the C terminus was not amidated. Both the sequence assignment and the free C terminus were confirmed by chemical synthesis of the peptide (see following section) and by analyzing the sequence of a cDNA clone encoding ω-conotoxin PVIIA.

The nucleic acid sequence and predicted amino acid sequence of the prepropeptide precursor of ω-PVIIA is shown in Fig. 1. As is standard for Conus peptides, the 27-amino acid mature toxin is found at the C-terminal end of the 72-amino acid precursor; a typical signal sequence is seen at the N terminus. The stop signal that follows the C-terminal valine confirms that the C terminus is free and not amidated. The proteolytic signal immediately N-terminal to the first amino acid residue (Cys) of the mature peptide, Thr-Arg, is typical of proteolytic signals seen in other Conus peptide precursor sequences.

Chemical Synthesis—The sequence assignment for ω-PVIIA and the free C terminus was confirmed by chemical synthesis (see "Experimental Procedures"). The synthetic peptide was biologically active when injected into fish and mice, and proved to have an elution time identical to that for the native material upon high performance liquid chromatography (see Fig. 2). The peptide elicited the same fin-popping syndrome as did native ω-PVIIA when injected into goldfish, and caused the same hyperactivity syndrome seen with native material when injected intracerebroventricularly into mice. Thus, the successful chem-
ical synthesis of biologically active \( \kappa \)-conotoxin PVIIA validates the sequence assignment of Terlau et al. (1). The synthesis protocol described routinely yields 5–10 mg of pure peptide.

**Disulfide Bridging Pattern of \( \kappa \)-Conotoxin PVIIA**—The disulfide bridge connectivity of \( \kappa \)-PVIIA was analyzed, using the partial reduction strategy of Gray (10). The spectrum of products obtained after partial reduction using tris(2-carboxyethyl)phosphine is shown in Fig. 3. The partially reduced intermediates (labeled PR1 and PR2 in Fig. 3) were further characterized using the double alkylation strategy described previously (4). Basically, reduced cysteine residues were alkylated using iodoacetamide under rapid alkylation conditions, followed by full reduction and pyridylethylation (using 4-vinylpyridine) of the remaining cysteine residues. The doubly alkylated products were then sequenced for pairwise deduction of the disulfide connectivity.

The results of this analysis for two of the partially reduced peptides shown in Fig. 3 provides a consistent data set indicating that the disulfide bridging pattern for \( \kappa \)-PVIIA is: Cys\(^1\)-Cys\(^{18}\), Cys\(^2\)-Cys\(^{20}\), Cys\(^{15}\)-Cys\(^{26}\). The disulfide linkage pattern is homologous to that seen for all \( \omega \)-conotoxins analyzed so far, as well as for the two \( \delta \)-conotoxins that have been examined, \( \delta \)-conotoxins TXVIA and OmVIA (4, 11).

**Electrophysiological Studies**—A comparison of the activity of \( \kappa \)-conotoxin PVIIA on the Shaker and rat brain Kv1.1 \( K^+ \) channels is shown in Fig. 4. The Shaker \( K^+ \) channel shows fast inactivation, whereas the Kv1.1 channel exhibits non-inactivating currents. It is evident from this figure that, whereas the Shaker \( K^+ \) channel is sensitive to \( \kappa \)-conotoxin PVIIA, the cloned Kv1.1 channel from rat brain is not. To define the region of the channel that has the most important determinants for toxin binding, complementary chimeras of the Shaker and Kv1.1 channels were constructed.

The chimeric constructs shown (Fig. 5, bottom row) were expressed in oocytes. The effects of \( \kappa \)-conotoxin PVIIA on these chimeras demonstrate that if the region between S5 and S6 is from the Shaker potassium channel, the resultant hybrid channel is toxin-sensitive. The region between the first four transmembrane domains of the channel protein appears to have little effect on toxin sensitivity; when this domain of Shaker was introduced to the Kv1.1 channel, the chimeric channel remained toxin-insensitive (middle panel). Fast, or N-type, inactivation is dependent on the inactivation particle (“ball”) at the N terminus of the Shaker channel. The chimera in Fig. 5 (left panel) containing this N-terminal region is expected to exhibit inactivation. However, this chimera is also toxin-insensitive since it contains the SS-S6 region of Kv1.1 potassium channel. The complementary chimeric channel (Fig. 5, right panel) is non-inactivating (since it has the Kv1.1 N terminus) and toxin-sensitive (since it has the Shaker S5-S6 region). The latter chimeric channel could be more convenient for structure-function and kinetic studies on \( \kappa \)-PVIIA using electrophysiological techniques than is the original Shaker potassium channel, because the chimeric construct is toxin-sensitive but non-inactivating.

**Interaction of \( \kappa \)-Conotoxin PVIIA with the TEA Binding Site on the Shaker Channel**—The Shaker potassium channel is sensitive to external TEA, and mutagenesis has demonstrated that
a specific threonine residue, Thr\(^{449}\), is a major determinant in the interaction of the channel with TEA (12). Mutation of the protein at this locus from threonine to tyrosine renders the Shaker \(K^+\) channel more TEA-sensitive (12), and makes it resistant to a number of channel-blocking toxins including charybdotoxin (13). Since the experiments on chimeras described above indicated that \(\kappa\)-PVIIA might be interacting as a channel blocker, we investigated the effects of the T449Y mutation on sensitivity to \(\kappa\)-PVIIA. As shown in Fig. 6, this mutation does in fact render the channel insensitive to \(\kappa\)-PVIIA, consistent with the toxin acting as a pore blocker directly interacting with the external TEA binding site. Introducing Lys or Gln in place of Thr\(^{449}\) also resulted in a channel that is insensitive to \(\kappa\)-PVIIA (data not shown).

\(\kappa\)-Conotoxin PVIIA and Charybdotoxin Differ in Their Interaction with the Shaker \(K^+\) Channel—Thus far, all evidence presented indicates that \(\kappa\)-conotoxin PVIIA and charybdotoxin interact very similarly with the Shaker \(K^+\) channel. However, in at least one case, the effect of a mutation in the Shaker \(K^+\) channel has opposite effects on the two toxins.

It has been shown previously that substitution of the residue Phe\(^{425}\) by glycine increases the affinity of charybdotoxin for the Shaker \(K^+\) channel by several orders of magnitude (14). We have assessed the F425G mutation as shown in Fig. 6; in contrast to the strikingly increased affinity of charybdotoxin for the mutant channel, this mutation instead abolishes Shaker potassium channel sensitivity to \(\kappa\)-conotoxin PVIIA. As a control, we also made the mutation F425Y, a conservative substitution. In contrast to the F425G substitution, the F425Y mutant is still sensitive to 1 \(\mu\)M \(\kappa\)-conotoxin PVIIA.

Together, these results indicate that both \(\kappa\)-conotoxin and charybdotoxin generally interact with the pore region between S5 and S6 on the Shaker \(K^+\) channel, as well as with the external TEA binding site. However, the effect of the F425G substitution is consistent with different “microsite” interactions (15) for these toxins when binding functionally to the Shaker channel.

**DISCUSSION**

The purification and preliminary amino acid sequence assignment for \(\kappa\)-conotoxin PVIIA has been described by Terlau et al. (1). In this work, we have rigorously established the sequence assignment in three ways: 1) the mass spectrometric analysis yielded a value consistent with the previous sequence assignment; 2) a cDNA encoding \(\kappa\)-conotoxin PVIIA was identified and sequenced; revealing a precursor consistent with the expected amino acid sequence of the mature toxin; and 3) biologically active peptide identical to the natural material was chemically synthesized and folded. The synthetic, oxidized peptide was shown to co-elute with the native material on HPLC. In addition, the three disulfide linkages have been assigned. All the data are consistent with the conclusion that \(\kappa\)-conotoxin PVIIA shares a common disulfide bridging pattern with the \(\omega\) and \(\delta\)-conotoxins.
The general biochemical features of \(\kappa\)-conotoxin PVIA are similar to the \(\omega\)-conotoxin family of peptides, which block certain subtypes of voltage-gated calcium channels. \(\kappa\)-Conotoxin PVIA has the same disulfide pattern as \(\omega\)-conotoxins and similarly sized intervals between cysteine residues. Furthermore, like the \(\omega\)-conotoxins, \(\kappa\)-PVIA has a large number of positive charges. However, compared with \(\omega\)-conotoxins as a group, \(\kappa\)-conotoxin PVIA has a greater preponderance of hydrophobic residues, as well as a greater number of negative charges leading to a smaller net positive charge.

The prepropeptide sequence of the \(\kappa\)-conotoxin PVIA precursor establishes that it belongs to the same superfamily of \(\kappa\)-conotoxin: the \(\delta\)-conotoxins, and the \(\mu\)-O-conotoxins; this superfamily of peptides has previously been referred to as the \(\omega\)-conotoxin superfamily. Apart from the shared disulfide bonding pattern, homology within this \(\kappa\)-conotoxin superfamily may not be obvious when the individual mature toxin sequences are compared. However, Table I demonstrates that all members of the superfamily share conserved regions in their prepropeptide sequences, particularly in the signal sequence region. A comparison of precursor sequences indicates that the \(\kappa\)-conotoxins are significantly more closely related to the \(\omega\)-conotoxins than to the \(\delta\)- and \(\mu\)-O-conotoxins.

Thus, there may be two main branches of the \(\kappa\)-conotoxin superfamily: peptides targeting \(K^+\) and \(Ca^{2+}\) channels (i.e. the \(\kappa\)- and \(\omega\)-conotoxins) and peptides that target \(Na^+\) channels (i.e. the \(\delta\)-

| Table I |
| --- |
| A comparison of \(\kappa\)-superfamily precursor sequences |
| The sequence of \(\kappa\)-conotoxin PVIA, obtained from \(C.\) purpurascens is compared to other precursor sequences of the \(\kappa\)-superfamily. Both \(\kappa\)-conotoxins PVIA and \(\delta\)-conotoxins PVIA are from \(C.\) purpurascens, while \(\omega\)-conotoxin PVIA is from \(C.\) geographus; both are fish-hunting cone snails. \(\delta\)-Conotoxin TXVIA and \(\mu\)-O-conotoxin MhVIA is from \(C.\) textile and \(C.\) marmoreus, respectively, both snail-hunting species. Note that \(\delta\)-conotoxin PVIA is much more similar to \(\mu\)-O-conotoxin MhVIA than it is to \(\kappa\)-conotoxin PVIA found in the same venom. This suggests that the divergence of the two branches of the \(\kappa\)-superfamily predates the divergence of fish-hunting and snail-hunting cone snails. Although \(\kappa\)-conotoxin PVIA is the first potassium channel-targeted toxin so far described from \(C.\) venoms, preliminary results indicate that peptides targeted to potassium channels are likely to be widely distributed among different \(C.\) venoms.2

\(\kappa\)-Conotoxin PVIA should be a useful probe for the Shaker potassium channel. We have defined a region that contains the major determinants for \(\kappa\)-conotoxin PVIA binding in the Shaker channel complex. For both charybdotoxin and \(\kappa\)-conotoxin PVIA, the interaction sites that confer high affinity binding are within the S5-S6 loop (for \(\kappa\)-PVIA, see results on chimeras above; for charybdotoxin, see Ref. 2). However, although the two toxins interact with the same general region, there are clearly different microsite interactions. Thus, when \(Phe^{244}\) of Shaker is mutated to a glycine, the \(K_p\) for block by charybdotoxin increases by over 1000-fold, from 120 nM to about 70 pm (16). In contrast, the same mutation resulted in a channel insensitive to 1 \(\mu\)-\(\kappa\)-conotoxin PVIA (see Fig. 6). The effects of the F425G mutation on charybdotoxin were interpreted to be due to a reduction in steric hindrance; the loss of \(\kappa\)-PVIA sensitivity by the mutant channel clearly indicates strikingly different interactions with this locus by the two toxins.

The two toxins are similar in their interaction with a residue known to be a strong determinant of external TEA binding, threonine 449 (12). The mutation T449Y results in a channel insensitive to 1 \(\mu\)-\(\kappa\)-conotoxin PVIA. Introducing lysine at the same position (T449K) also results in a channel insensitive to \(\kappa\)-PVIA (data not shown). Thus, although the two toxins clearly do not interact with the channel target site in the same way, they may both block functionally important loci for channel function such as the TEA binding site.

The remarkable amount of work that has been done using charybdotoxin as a probe for the Shaker channel serves as a useful paradigm for defining the \(\kappa\)-conotoxin:Shaker \(K^+\) channel complex, as well as for evaluating structural proposals of the outer channel vestibule based on scorpion toxin data. Clearly, an essential requirement before such structure-function work can proceed productively is a determination either by multidimensional NMR techniques or by \(x\)-ray crystallography of the three-dimensional structure of \(\kappa\)-conotoxin PVIA. Such a structural analysis has been initiated.3

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