Articles

Purification and Cloning of Toxins from Elapid Venoms that Target Cyclic Nucleotide-Gated Ion Channels[†,‡]

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ABSTRACT: In 1999, we purified pseudechetoxin (PsTx), the first peptide toxin known to block cyclic nucleotide-gated (CNG) ion channels, from the venom of *Pseudechis australis* [Brown, R. L., Haley, T. L., West, K. A., and Crabb, J. W. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 754−759]. Here we report the cloning of the cDNA encoding PsTx, as well as the discovery and cloning of pseudecin, a homologous toxin from the venom of *Pseudechis porphyriacus*. The mature proteins are 211 and 210 amino acids in length, and the amino acid sequences are 96.7% identical, differing in only seven residues. The purified toxins were applied to outside-out patches excised from *Xenopus* oocytes expressing CNG channels composed of the rod CNGA1 or olfactory CNGA2 channel subunits. Surprisingly, these patch-clamp studies revealed a 30-fold difference in affinity between PsTx and pseudecin for channels composed of CNGA2 subunits. The apparent K_i of PsTx was 15 nM, while the affinity of pseudecin was 460 nM. The difference in affinities for the CNGA1 subunit from rod photoreceptors was less pronounced, but the affinity of PsTx was 70 nM, compared with 1000 nM for pseudecin. This difference in affinity may be instructive as we attempt to identify the regions of the toxins that contact CNG channels. As the only known protein blockers of CNG channels, these toxins promise to be valuable tools to study the structure of the external face of these channels.

Over the past 30 years, a plethora of toxins have been isolated and characterized from venomous snakes. While some of these toxins affect blood coagulation factors (*1*−*6*), cellular proteins, and receptors (*7*−*9*), many toxins target ion channels. Elapid snake venoms are known to contain several peptide ion channel blockers, including α-neurotoxins (*10*−*16*), which inhibit nicotinic acetylcholine receptors, and dendrotoxins (*17*−*19*), which block voltage-gated potassium channels. These toxins have been proven to be extremely useful pharmacological tools for the study of these ion channels.

Cyclic nucleotide-gated (CNG) ion channels play a central role in signal transduction in retinal photoreceptors and olfactory neurons (*20*−*23*). In both of these cell types, CNG channels modulate the membrane potential of the cell and intracellular Ca^{2+} levels in response to stimulus-induced changes in cyclic nucleotide concentration. Functional CNG channels are formed when four subunits come together to form a cation nonselective pore that is opened by direct binding of cyclic nucleotides to a carboxy-terminal domain on each subunit (*24*, *25*). Most CNG channel α subunits (CNGA1−3) form functional CNG channels on their own when expressed in *Xenopus* oocytes or HEK-293 cells, but the properties of these homotetrameric channels are clearly distinct from CNG channels found in most native tissues (*26*, *27*). In contrast, the *β* subunits (CNGB1 and 3) do not form functional channels on their own, although they possess the same general transmembrane structure and cyclic nucleotide-binding site as α subunits (*28*−*31*). When coexpressed, the α- and *β*-subunits create heteromeric CNG channels with functional properties similar to those found in photoreceptors and olfactory neurons (*28*, *29*, *32*, *33*). More recently, CNG channels have been found in tissues throughout the body, including the brain (*34*), heart (*35*), kidneys (*35*), liver (*36*), and intestines (*37*), but the subunit composition of these channels remains unknown.

Recently, we purified a potent CNG channel blocker, named pseudechetoxin (PsTx), from the venom of the Australian King Brown Snake (*Pseudechis australis*) (*38*). PsTx[1] blocked olfactory (CNGA2) and retinal (CNGA1) α-subunit homotetrameric channels with high affinity when

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[‡] GenBank accession numbers: pseudechetoxin, AY072695; pseudecin, AY072696.

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[1] Abbreviations: CRISP, cysteine-rich secretory protein; HLTX, helothermine; *P.*, *Pseudechis*; PsTx, pseudechetoxin; RACE, rapid amplification of cDNA ends.

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applied to the exterior face of membrane patches ($K_i = 5$ and 100 nM, respectively). More recently, we have shown that PsTx forms high-affinity contacts with the pore turret region and most likely inhibits CNG channel current by blocking the external entrance to the transmembrane pore (*39*). Because of its high affinity, specificity, and extracellular site of action, PsTx possesses some distinct advantages over small molecule blockers of CNG channels, including L-*cis*-diltiazem (*40*), tetracaine (*41*), pimozide (*42*), LY-58358 (*43*), polyamines (*44*), and cGMP analogues (*45*, *46*).

In this study, we report the molecular cloning of PsTx from the venom gland of *P. australis*. Furthermore, we have isolated and cloned a homologous toxin, which we named pseudecin, from the venom of *Pseudechis porphyriacus*. Despite the high homology between these toxins, they blocked CNG channels with quite different affinities. These toxins promise to be useful pharmacological tools for studying the structure and function of CNG channels. In addition, because the slight sequence differences translate into a pronounced difference in binding affinity, a comparison of the two toxins will facilitate the identification of regions that interact with CNG channels.

EXPERIMENTAL PROCEDURES

Materials. The lyophilized venoms and venom glands of *P. porphyriacus* and *P. australis* were purchased from Venom Supplies Pty. Ltd. (Tanunda, South Australia). Superdex 75 pg and SP-Sepharose High Performance columns were from Amersham-Pharmacia Biotech. Vydac Protein & Peptide C18 HPLC columns and COSMOSIL 5C18 AR-300 HPLC columns were purchased from Jasco (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Endoproteinase Lys-C was purchased from Seikagaku Corporation (Tokyo, Japan). The chemicals used in this study were purchased from Sigma, Amersham-Pharmacia Biotech., Wako Pure Chemical Industries, and Kanto Chemical Co.

Toxin Purification. Pseudechetoxin (PsTx) was purified by two successive rounds of RP-HPLC as described previously (*38*). In addition, we developed an alternative, nondenaturing purification scheme as follows: Five hundred milligrams of *P. australis* venom was dissolved in 3 mL of a buffer containing 50 mM Tris−HCl (pH 8.0) and 0.2 M NaCl on ice. After centrifugation to remove insoluble materials, the venom components were separated by gel-filtration chromatography on a Superdex 75pg column (ø = 2.6 × 60 cm), and 2-mL fractions were collected. Fractions containing PsTx were detected by ELISA using anti-pseudecin antiserum and dialyzed against 20 mM imidazole−HCl (pH 6.0) before being loaded onto an SP-Sepharose High Performance column (ø = 1.6 × 11 cm) equilibrated with the same buffer. Venom components were eluted with a linear gradient of NaCl from 0 to 1.0 M (developed through 8 column volumes at a flow rate of 2 mL/min). PsTx eluted at a concentration of 0.95 M NaCl, and we typically obtained 0.2 mg from 500 mg of crude venom.

For the purification of pseudecin, 100 mg of the *P. porphyriacus* venom was dissolved in 500 μL of Tris buffer and centrifuged to remove insoluble material as described above. The venom was then applied to a Superdex 75pg column (ø = 1.6 × 60 cm) equilibrated with the same buffer. One-milliliter fractions were collected, and pseudecin-containing fractions were identified by an ELISA. After dialysis against a buffer containing 20 mM imidazole−HCl (pH 6.0) and 0.5 M NaCl, the pooled fractions were applied to a SP-Sepharose High Performance column (ø = 1.6 × 11 cm) and eluted by a linear gradient to 1.0 M NaCl (developed through 4 column volumes). Pseudecin eluted at the concentration of 0.75 M NaCl, and we routinely obtained 0.5 mg from 100 mg of crude venom. All purification steps were carried out at 4 °C using AKTA explorer 10S (Amersham-Pharmacia Biotech), and column effluent was monitored by absorbance at 280 nm.

Amino Acid Sequence Analysis. PsTx and pseudecin were reduced for 3 h at room temperature with 20 mM dithiothreitol in 0.5 mL of buffer solution, containing 0.5 M Tris−HCl (pH 8.5), 6 M guanidine hydrochloride, and 2 mM EDTA. Three microliters of 4-vinylpyridine was added, and alkylation was allowed to proceed for 3 h at room temperature. The *S*-pyridylethylated proteins were separated from the reagents by C18 reverse-phase HPLC, and their amino acid sequences were determined by sequencing the peptides obtained by digestion with endoprotease Lys-C. All the samples were analyzed on Applied Biosystems protein sequencers (models 473A and 477).

Cloning of the cDNA Encoding PsTx and Pseudecin. Complementary DNAs encoding PsTx and pseudecin were cloned by RT-PCR. Typically, total RNA was isolated from the venom gland of *P. australis* and *porphyriacus* with ISOGEN (Wako Pure Chemical Industries, Japan), according to the manufacturer's instructions. 5′ and 3′ rapid amplification of cDNA ends (RACE) was carried out to determine the nucleotide sequence of 5′- and 3′-end cDNA using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). This procedure used degenerate primers that were designed based on the cDNA sequences of helothermine-like neurotoxins (Yamazaki, Y., and Morita, T. Unpublished data. Genbank accession numbers: ablomin, AF384218; triflin, AF384219; latisemin, AF384220). The sequences of primers were 5′-CTT TTG TAC CAA ACT ATC TG(A/G) GT(A/G) (A/T)AA TGG CC-3′ for 5′-RACE (corresponding to amino acid 106−115 in PsTx) and 5′-CTT GCT GCA GTG CTG C(A/G)A CAG TCT T for 3′-RACE (corresponding to putative signal peptide). The RACE products were subcloned into the pUC19 vector and sequenced using a DSQ 2000L DNA sequencer (Shimadzu, Japan).

Expression of CNG Channels in Xenopus Oocytes. Complementary DNA encoding the rod CNGA1 and the olfactory CNGA2 subunits were gifts of Dr. William Zagotta (University of Washington, Seattle, WA). The cDNAs had been modified to include convenient restriction sites (*47*) and were inserted into the pGEMHE vector (*48*), which promotes high expression by incorporating flanking sequences from the *Xenopus* β-globin gene. For in vitro transcription, plasmids containing CNGA1 and CNGA2 were linearized with Pst I and Nhe I. Complementary RNA was transcribed from the T7 promoter and capped using the mMessage mMachine kit (Ambion, Austin, TX). *Xenopus* oocytes were prepared and injected with cRNA (∼10−20 ng) by standard methods (*49*). Following injection, oocytes were maintained at 18 °C for 3−7 days in ND96 media containing 50 μg/mL gentamycin.

Electrophysiological Methods. Recording electrodes were pulled from borosilicate glass (World Precision Instruments) on a P97 Brown-Flaming puller (Sutter, Novato, CA) and

polished on a microforge (Life Science Resources) to a resistance of 1−2 MΩ. Recordings were made in symmetrical solutions containing 130 mM NaCl, 3 mM HEPES (pH 7.4), and 0.2 mM EDTA. To activate channel currents, the patch pipet contained 1 mM cGMP. Currents were recorded from patches in the outside-out configuration with an Axopatch 200A amplifier driven by pClamp 8.0 using a Digidata 1200 interface (Axon Instruments, Foster City, CA). Two hundred millisecond voltage pulses, ranging from −100 to 100 mV, were given from a holding potential of 0 mV. Solutions were applied to the extracellular face of the patch via the "sewer pipe" method, using an RSC-100 rapid perfusion system (Molecular Kinetics, Pullman, WA). The seal resistance and "leak" current were estimated following application of solution containing 10 mM $MgCl_2$ to the extracellular face of the patch. This concentration of Mg^{2+} blocks > 98% of the current at −50 mV in both channels studied (*50*). Solutions containing toxins were supplemented with 50 μg/mL BSA to prevent nonspecific surface adsorption. Toxin concentrations were estimated on the basis of their calculated molar extinction coefficients: ε (10 mg/mL) = 21.6 for PsTx and 21.5 for pseudecin.

Data Analysis. Data analysis was performed using the Clampfit program in the pClamp 8.0 suite. Dose-response curves for inhibition by the two toxins were generated using SigmaPlot 2000, and fits were generated using a modified form of the Hill equation:

$$\text{Fractional current} = 1 - ([\text{Tx}]^n/([\text{Tx}]^n + K_D{}^n))$$

RESULTS

Our previous study demonstrated that the Australian King Brown Snake (*P. australis*, Elapidae) venom contains a potent blocker of CNG ion channels, which we named pseudechetoxin (PsTx) (*38*). PsTx blocked CNG channels formed by the retinal and olfactory α-subunits with 10−100 nM affinity. In this study, we cloned the cDNA encoding PsTx from the venom gland of *P. australis* using the 5′ and 3′-RACE method. The cloned PsTx cDNA is 1319 base pairs in length, encoding a product of 238 amino acid residues with a predicted molecular weight of ∼26.5 kDa (Figure 1). The N-terminal sequence of intact PsTx, as determined by protein sequencing, was SNKKNYQK, starting at amino acid 28 from the initial methionine. This is likely due to cleavage of a signal peptide from an intracellular precursor of secreted PsTx. Analysis of the pro-PsTx N-terminal sequence using the signal peptide prediction program PSORT II revealed that pro-PsTx could be cleaved between Gly and Thr in the sequence QQSSG/TADFA. This result suggests that pro-PsTx possesses a 19-residue signal peptide, and it may be further cleaved at amino acid 8 by a sequence-specific protease after secretion. The predicted amino acid sequence of the mature protein is 211 residues (molecular mass of 23.7 kDa) and includes a large number of basic amino acid residues (predicted pI of 10.0). The sequences of the enzymatic-digested fragments of PsTx were found in the predicted amino acid sequence with 100% identity (Figure 1, *underlined*).

Upon searching for other PsTx-like molecules in the venom of related snakes, we isolated a homologous toxin, named pseudecin, from the venom of Red-bellied Black

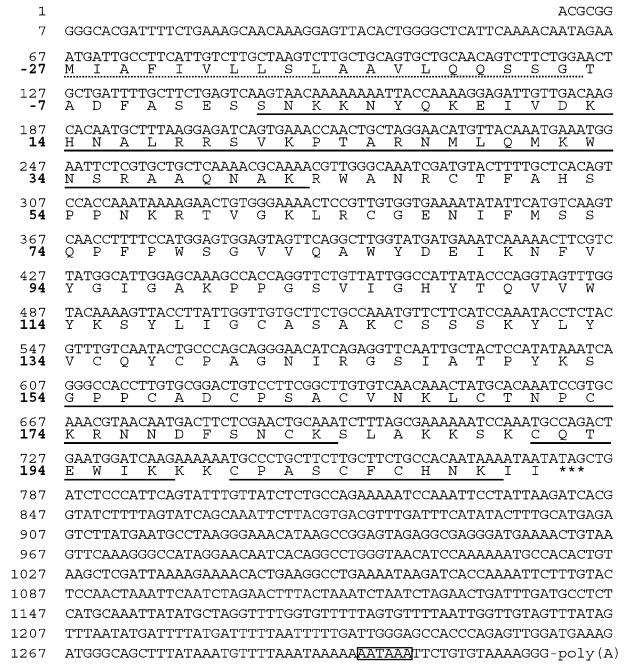

FIGURE 1: cDNA cloning of PsTx from the venom gland of *P. australis*. The amino acid sequence is shown in single-letter code beneath the nucleotide sequence. Nucleotide and amino acid (bold) numbers are shown in the column on the left side. The N-terminus was determined by the sequencing of purified PsTx and is designated as amino acid **1**. Translation is depicted as starting at nucleotide 67. The putative signal peptide is identified by a dotted underlined (from −27 to −9 in amino acid number). The underlined amino acids indicate the amino acid sequences identified from sequencing *S*-pyridylethylated peptides from enzymatic digests. The putative polyadenylation signal is boxed.

Snake (*P. porphyriacus*, Elapidae). After gel-filtration of *P. porphyriacus* venom, the fractions containing pseudecin were identified by ELISA and subjected to cation-exchange chromatography (Figure 2). Pseudecin eluted at a concentration of ∼0.75 M NaCl, indicating that it was a highly basic protein, similar to PsTx. In this purification procedure, we obtained 0.5 mg of purified protein from 100 mg of crude venom. Purified pseudecin as a 23 kDa protein on nonreducing SDS−PAGE and 24.5 kDa under reducing conditions (Figure 2 *inset*). The complementary DNA encoding pseudecin was then cloned by the RACE method. The nucleotide sequence was 1,280 base pairs and revealed an open reading frame of 238 amino acids (Figure 3). Analysis with PSORT II suggested that the deduced amino acid sequence includes a predicted signal peptide (amino acids 1−19). N-terminal sequence analysis of the purified protein revealed the secreted protein started with NKKNYQ; therefore, it seems likely that pseudecin, like PsTx, is also digested by a protease at the position of amino acid 9 and becomes a protein of 210 residues (molecular mass, 23.6 kDa; predicted pI = 9.69). The deduced amino acid sequence was confirmed by the peptide sequencing (Figure 3, underlined). The amino acid sequence identity between PsTx and pseudecin is extremely high (96.7%); in fact, they differ in only seven of 211 residues (Figure 6).

The masses of PsTx and pseudecin were determined by matrix-associated desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a Voyager-DE (Applied Biosystems). The average molecular mass of intact PsTx and pseudecin were 23 815.7 and 23 651.9, respec-

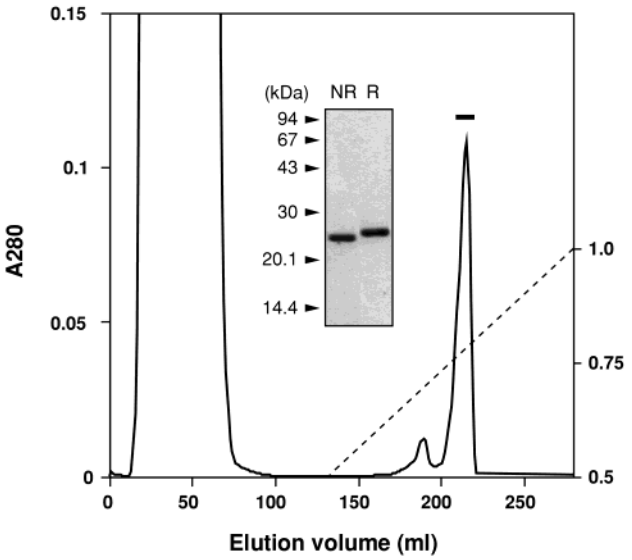

FIGURE 2: Isolation of pseudecin from the venom of *P. porphyriacus*. After gel filtration of the venom of *P. porphyriacus* (100 mg) by Superdex 75pg column (ø = 1.6 × 60 cm), the reactive fractions with anti-pseudecin antiserum were subjected to a SP-Sepharose High Performance column (ø = 1.6 × 11 cm) and developed with a linear gradient of NaCl (dotted line). The pooled fraction (bar) contained purified pseudecin. The result of SDS−PAGE of the purified pseudecin is shown in inset (NR: nonreduced, R: reduced). Five milligrams of pseudecin was obtained from 1 g of crude venom. For detailed purification procedures, see Experimental Procedures.

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  1                                                    ACGCGGGGGCTCATTCAAAACAATAGAA
 29  ATGATTGCCTTCATTGTCTTGCTAAGTCTTGCTGCAGTGCTGCAACAGTCTTCTGGAACT
-28  M  I  A  F  I  V  L  L  S  L  A  A  V  L  Q  Q  S  S  G  T
 89  GTTGATTTTGCTTCTGAGTCAAGTAACAAAAAAAATTACCAAAAGGAGATTGTTGACAAG
 -8  V  D  F  A  S  E  S  S  N  K  K  N  Y  Q  K  E  I  V  D  K
149  CACAATGCTTTAAGGAGATCAGTGAAACCAACCGCTAGGAACATGTTACAAATGAAATGG
 13  H  N  A  L  R  R  S  V  K  P  T  A  R  N  M  L  Q  M  K  W
209  AATTCTCATGCTGCTCAAAATGCAAAACGTTGGGCAGATCGATGTACTTTTGCTCACAGT
 33  N  S  H  A  A  Q  N  A  K  R  W  A  D  R  C  T  F  A  H  S
269  CCACCAAATACAAGAACTGTGGGAAAACTCCGTTGTGGTGAAAATATATTCATGTCAAGT
 53  P  P  N  T  R  T  V  G  K  L  R  C  G  E  N  I  F  M  S  S
329  CAACCTTTTCCATGGAGTGGAGTAGTTCAGGCTTGGTATGATGAAATCAAAAATTTCGTC
 73  Q  P  F  P  W  S  G  V  V  Q  A  W  Y  D  E  I  K  N  F  V
389  TATGGCATTGGAGCAAAGCCACCCGGTTCTGTTATTGGCCATTATACCCAGGTAGTTTGG
 93  Y  G  I  G  A  K  P  P  G  S  V  I  G  H  Y  T  Q  V  V  W
449  TACAAAAGTCACCTTATTGGTTGTGCTTCTGCCAAATGTTCTTCATCCAAATACCTCTAC
113  Y  K  S  H  L  I  G  C  A  S  A  K  C  S  S  S  K  Y  L  Y
509  GTTTGCCAATACTGCCCAGCAGGGAACATCAGAGGTTCAATTGCTACTCCATATAAATCA
133  V  C  Q  Y  C  P  A  G  N  I  R  G  S  I  A  T  P  Y  K  S
569  GGGCCACCTTGTGCGGACTGTCCTTCGGCTTGTGTCAACAGACTATGCACAAATCCTTGC
153  G  P  P  C  A  D  C  P  S  A  C  V  N  R  L  C  T  N  P  C
629  AATTATAACAATGACTTCTCGAACTGCAAATCTTTAGCGAAAAAATCCAAATGCCAGACT
173  N  Y  N  N  D  F  S  N  C  K  S  L  A  K  K  S  K  C  Q  T
689  GAATGGATCAAGAAAAAATGCCCTGCTTCTTGCTTCTGCCACAATAAAATAATATAGCTG
193  E  W  I  K  K  K  C  P  A  S  C  F  C  H  N  K  I  I  ***
749  ATCTCCCATTCAGTATTTGTTATCTCTGCCAGAAAAATCCAAATTCCTATTAAAATCATG
809  GCATCTTTTAGTATCAGCAAATTCTTACGTGACATTTGATTTCATATACTTTGCATGAGA
869  GTCTTATGAATGCCTAAGGGAAACATAAGCCAGAGTAGAGGCGAGGGATGAAAACTGTAA
929  GTTCAAAGGGCCATAGGAACAATCACAGGCCTGGGTAACATCCAAAAAATGCCACACTGT
989  AAGCTCGATTAAAAGAAAACACTGAAGGCCTGAAAATAAGATCACCAAAATTCTTTGTAC
1049 TCCAACTAAATCCAATCTAGAACTTTACTAAATCTAATCTAGAACTGATTTGATGCCTTT
1109 CATGCAAATTATATGCTAGGTTTTGGTGTTCTTAGTGTTTTAATTGGTTGTAGTTTATAG
1169 TTTAATATGATTTTATGATTTTTAATTTTTGATTGGGAGCCACCCAGAGTTGGATGAAAG
1229 ATGGGCAGCTTTATAAATGTTTTAAATAAAAAAATAAATTCTGTGTAAAAGC-poly(A)
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FIGURE 3: Nucleotide sequence of pseudecin. Pseudecin cDNA was cloned from the venom gland cDNAs of *P. porphyriacus* using 5′- and 3′-RACE. Cloned pseudecin cDNA is depicted as the same as Figure 1. Dotted underline, putative signal peptide (from −28 to −10 in amino acid number); underline, the amino acid sequence identified from enzymatic-digested peptides; boxed, putative poly adenylation signal. N-terminal was determined by the sequencing of purified pseudecin.

tively. The molecular masses were identical to the molecular masses of the processed toxins (23 706.09 and 23 568.78) predicted by the cDNA sequences, within the experimental errors of 0.46% and 0.35%. These results confirm that PsTx and pseudecin are the appropriate gene products of our cloned cDNAs and suggest that there are no further posttranslational modifications beyond the proteolytic processing described above.

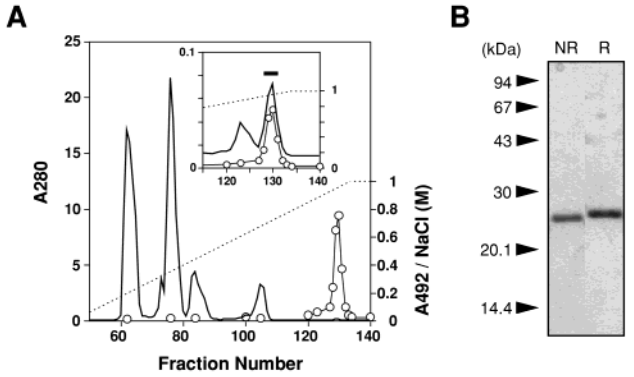

FIGURE 4: Alternative purification procedure of PsTx from *P. australis* venom. (A) After gel-filtration of *P. australis* venom (500 mg) by Superdex 75pg column (ø = 2.6 × 60 cm), the PsTx fraction was loaded onto a SP-Sepharose High Performance column (ø = 1.6 × 11 cm) and eluted with a NaCl gradient (0−1.0 M, dotted line). Two-milliliter fractions were collected. The reactivity of anti-pseudecin is indicated as open circles. The inset shows the expanded chromatogram from fraction 115−140. The pooled fractions (bar) contained purified PsTx. (B) The result of SDS−PAGE of the purified PsTx (NR, nonreduced; R, reduced). The relative molecular weight of purified PsTx was 24 kDa on nonreducing SDS−PAGE and 25 kDa under reducing conditions.

In the previous study, we isolated PsTx using an HPLC system. For a better comparison of the biological activity of PsTx and pseudecin, we developed a nondenaturing method for the purification of PsTx. The venom of *P. australis* was passed over a gel filtration column, and the PsTx-containing fractions were pooled and subjected to cation-exchange chromatography (Figure 4). PsTx was eluted at a higher concentration of NaCl (~0.95 M) than pseudecin, and 0.4 mg of purified protein was obtained from 1 g of lyophilized crude venom. The venom content of PsTx is considerably lower (0.04% of total venom protein) than that of pseudecin (0.5%).

To compare their inhibitory activities, the purified toxins were applied to outside-out patches excised from *Xenopus* oocytes expressing the CNG channel α-subunits from rod photoreceptors (CNGA1) and olfactory neurons (CNGA2). Fractional block was measured at potentials ranging from −100 to +100 mV (Figure 5A). This revealed only a slight voltage-dependence of toxin inhibition, which was more pronounced with pseudecin. When applied to CNGA2, 500 nM PsTx blocked 93% ± 1% ($n = 3$) of the current at −80 mV and 89% ± 2% ($n = 3$) of the current at +80 mV. In contrast, when 5000 nM pseudecin was applied to CNGA2, it blocked 80% ± 5% ($n = 2$) of current at −80 mV and 56% ± 7% ($n = 2$) at +80 mV. This voltage-dependence of block is evident in Figure 5A as a time-dependent decrease in the current magnitude at negative potentials and an increase at positive potentials. The dose-response relation shown (Figure 5B,C) was measured at −60 mV.

On the basis of their sequence similarity (96.7% identity), we expected the two toxins to block the CNG channels with similar affinities. Surprisingly, we found that these modest sequence differences translated into profoundly different blocking affinities (Figure 5B,C), with PsTx being a much

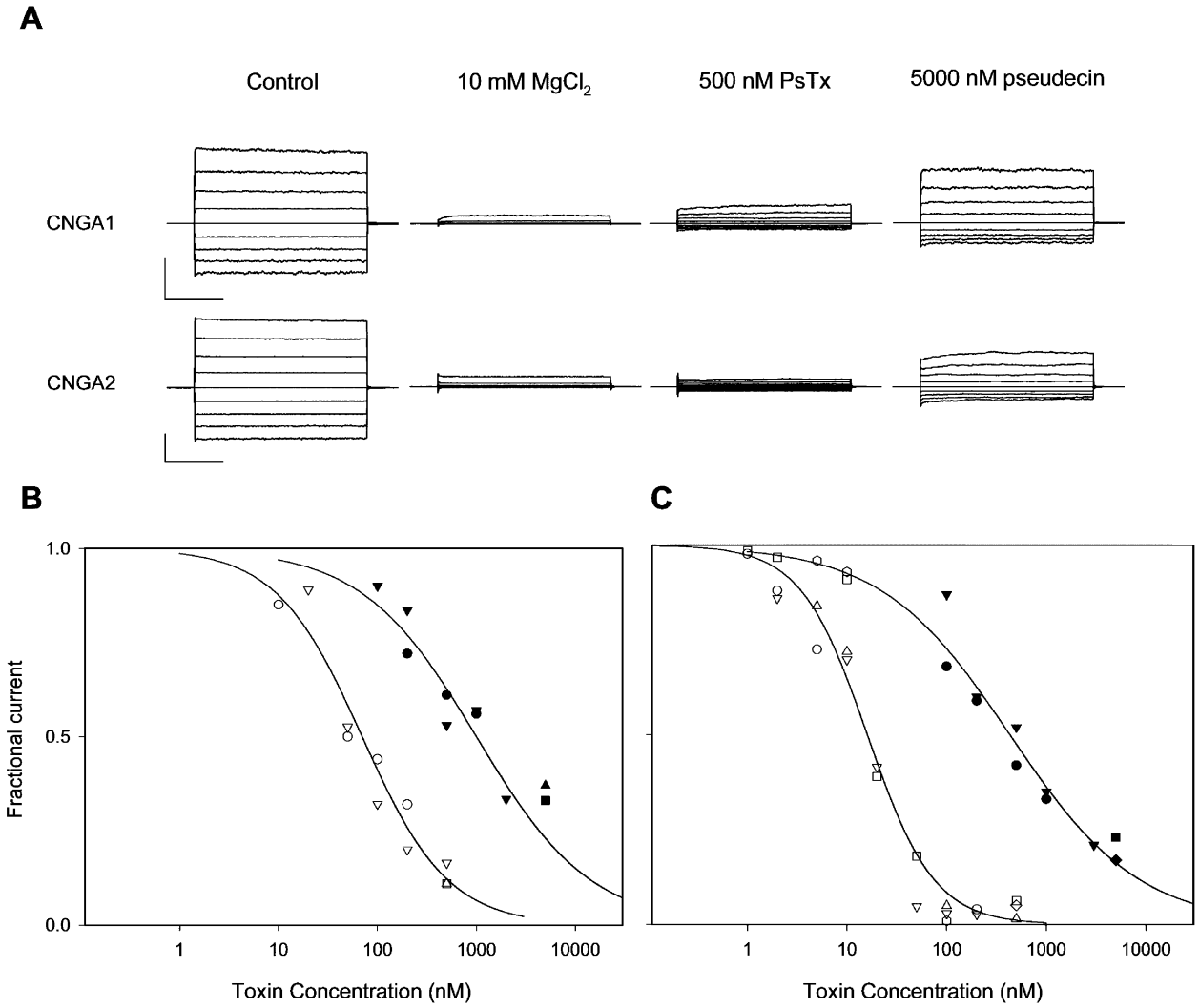

FIGURE 5: PsTx blocks CNGA1 and CNGA2 channels with higher affinity than pseudecin. (A) Current traces demonstrating inhibition of CNG channel currents by 500 nM PsTx and 5000 nM pseudecin. Outside-out patches were held at 0 mV and stepped to potentials ranging from −100 to +100 mV in 20 mV increments. Scale bars indicate 1 nA on the vertical axis and 50 ms on the horizontal axis. Dose-response curves were measured at −60 mV on outside-out patches excised from oocytes expressing either CNGA1 (B) or CNGA2 (C). Open symbols illustrate block by PsTx; filled symbols illustrate block by pseudecin. Data were pooled from 4−6 patches for each channel isoform. Different symbol shapes represent data from different patches. Data points were fit using a modified form of the Hill equation, and the fits are shown as smooth lines. Fit parameters are as follows: panel B (CNGA1), PsTx $K_D = 70$ nM and $n = 1.0$, pseudecin $K_D = 1000$ nM and $n = 0.75$; panel C (CNGA2), PsTx $K_D = 15$ nM and $n = 1.3$, pseudecin $K_D = 460$ nM and $n = 0.7$.

more potent blocker of both CNG channel isoforms. The affinity difference between PsTx and pseudecin was >10-fold when tested on CNGA1 channels being 70 nM for PsTx versus 1000 nM for pseudecin. The affinity difference was a striking 30-fold when tested on channels formed by CNGA2, being 15 nM for PsTx versus 460 nM for pseudecin. Similar K_i values were determined for at least four different preparations of each toxin. The values obtained for PsTx were similar for both purification methods and are in good agreement with those reported in our earlier study (*38*).

DISCUSSION

The venoms of snakes, cone shells, and spiders contain an abundance of toxins that target a variety of ion channels. Most of the well-known ion channel blockers, such as α-neurotoxins, dendrotoxins, conotoxins, and agatoxins, are small peptide molecules (<9 kDa). One known exception to this rule is helothermine (25.5 kDa), which has been reported to block voltage-gated Ca^{2+} channels, K^+ channels, and ryanodine receptors (*51*−*54*). In 1999, we purified a potent blocker of CNG channels from the venom of the Australian King Brown Snake (*P. australis*), which we named pseudechetoxin (PsTx). PsTx migrated with a relative molecular weight of 25 kDa on reducing SDS−PAGE, and partial peptide sequences suggested some homology with helothermine. Here, we report the molecular cloning of PsTx, as well as the isolation and cloning of a homologous protein, designated pseudecin, from the venom of the Red-bellied Black Snake (*P. porphyriacus*). Our present study indicates that helothermine-like high molecular weight toxins are also found in the venom of Elapid snake venoms. PsTx and pseudecin are fascinating toxins from this structural standpoint, in addition to their unique biological activity.

The amino acid sequences of PsTx and pseudecin are highly homologous (96.7% identical); in fact, they differ in only seven residues (Figure 6, spotted residues). PsTx and pseudecin are both highly basic proteins (predicted pI of 10.0

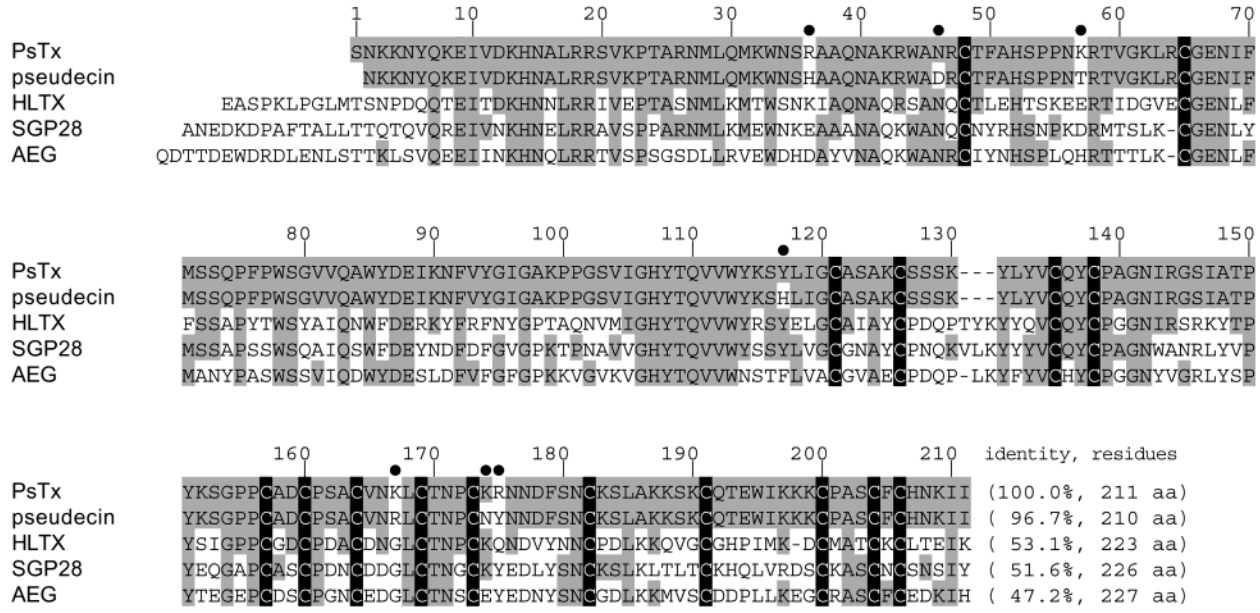

FIGURE 6: Sequence alignment of PsTx and pseudecin and their structurally related proteins. The residues conserved between the related proteins are shadowed. Gaps (−) have been inserted to maximize similarity. All cysteine residues are shown in reversed character. The number of residues corresponds to that of PsTx. Numbers on the end of each sequence are identity (%) vs PsTx and total amino acids (aa). The different residues between PsTx and pseudecin are marked with spots. HLTX, helothermine; SGP28, human specific granule protein of 28 kDa; AEG, rat acidic epididymal glycoprotein (protein D/E). Genbank accession numbers: PsTx, AY072695; pseudecin, AY072696; HLTX, U13619; SGP28, S68691; AEG, M31173.

and 9.69, respectively), with most of the lysine and arginine residues clustered near the N- and C-termini. Sequence alignment demonstrated that the amino acid sequences of PsTx and pseudecin have high identity with the cysteine-rich secretory proteins (CRISPs), such as the specific granule protein of 28 kDa (SGP28) in granules (*55*) and acidic epididymal glycoprotein (AEG) in epididymis (*56*), as well as helothermine (Figure 6). All cysteine residues are completely conserved with other members of this family. Although helothermine is known to block several types of ion channels, the functions of other members of the CRISP family are virtually unknown.

As PsTx and pseudecin are quite similar in sequence, the difference in their blocking affinity for CNG channels is surprising. PsTx blocked olfactory CNG channel (CNGA2) currents with a 30-fold higher affinity than pseudecin (15 nM to 460 nM) and also blocked the rod type channel (CNGA1) with a 15-fold higher affinity (70 nM versus 1000 nM). Of the seven residues that differ between PsTx and pseudecin, sequence comparisons suggest that Lys^{167} and two adjacent basic residues (Lys^{174} and Arg^{175}) of PsTx are likely to interact with CNG channels. The position of Lys^{167} in PsTx is replaced by a similar basic residue Arg^{166} in pseudecin; in contrast, the amino acid found in the corresponding position of other CRISPs, including helothermine, is glycine. Because helothermine did not affect CNG currents (R. Lane Brown, unpublished observation), the basic residue of this location may be specific for proteins that inhibit CNG channels. Furthermore, two adjacent basic residues (Lys^{174} and Arg^{175}) in PsTx are replaced by neutral amino acids (Asn^{173} and Tyr^{174}) in pseudecin, suggesting that the interaction between PsTx and CNG channels may be largely electrostatic in nature. These hypotheses will serve to guide future experiments as we seek to determine the sites of interaction by heterologous expression of mutant toxins. Both PsTx and pseudecin are useful pharmacological tools, which may provide new insights into the structure of the external face of CNG channels, as well as the molecular functions of other CRISP family members.

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