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Tyrosine-rich Conopeptides Affect Voltage-gated K^+ Channels*

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Two venom peptides, CPY-Pl1 (EU000528) and CPY-Fe1 (EU000529), characterized from the vermivorous marine snails *Conus planorbis* and *Conus ferrugineus*, define a new class of conopeptides, the conopeptide Y (CPY) family. The peptides have no disulfide cross-links and are 30 amino acids long; the high content of tyrosine is unprecedented for any native gene product. The CPY peptides were chemically synthesized and shown to be biologically active upon injection into both mice and *Caenorhabditis elegans*; activity on mammalian Kv1 channel isoforms was demonstrated using an oocyte heterologous expression system, and selectivity for Kv1.6 was found. NMR spectroscopy revealed that the peptides were unstructured in aqueous solution; however, a helical region including residues 12–18 for one peptide, CPY-Pl1, formed in trifluoroethanol buffer. Clones obtained from cDNA of both species encoded prepropeptide precursors that shared a unique signal sequence, indicating that these peptides are encoded by a novel gene family. This is the first report of tyrosine-rich bioactive peptides in *Conus* venom.

The venom of marine gastropods in the genus *Conus* has yielded numerous structurally and functionally diverse peptidic components (1). The increasing variety of bioactive peptides identified in cone snail venoms has provided insight into the seemingly endless variety of directions taken by *Conus* species in evolving neuroactive molecules to suit their diverse biological purposes.

The bioactive peptides in *Conus* ("conopeptides") are classified into two broad groups: the non-disulfide-rich and the disulfide-rich (1). The latter are conventionally called conotoxins. The non-disulfide-rich class includes conopeptides with no cysteines (contulakins (2), conantokins (3), and conorfamides (4)) and conopeptides with two cysteines forming a single disulfide bond (conopressins (5) and contryphans (6)). The conopeptides that compose the disulfide-rich class have two or more disulfide bonds (1); among the major classes of molecular targets identified for these structurally diverse conopeptides are members of the voltage-gated and ligand-gated ion channel superfamilies.

In this work, we present the discovery and characterization of a novel class of peptides, which we designate the conopeptide Y (CPY)[2] family, from the marine snails *Conus planorbis* and *Conus ferrugineus* (see Fig. 1). Both of these species belong to a distinct clade of *Conus* (7, 8). The first two peptides (9) belonging to the CPY family have a high frequency of tyrosine residues, in addition to basic and hydrophobic residues. The peptides are shown to be biologically active when injected into mouse and the nematode *Caenorhabditis elegans*. Activity on the Kv1 channel subfamily is demonstrated, and the subtype selectively within the subfamily is defined.

EXPERIMENTAL PROCEDURES

Extraction and Fractionation of C. planorbis Venom—Snails were collected in Cebu and Marinduque Islands, Philippines, and dissected. Venom was pressed out of freshly dissected venom ducts that were kept on ice. Venom pooled from several snails was lyophilized and stored at −70 °C. A 500-mg portion was resuspended in 35 ml of 30% acetonitrile and 0.2% trifluoroacetic acid using a Vortex mixer for 2× 1 min with an interval of 5 min on ice. The mixture was sonicated using a Branson LS-75 probe for 3× 0.5 min on ice with 1-min rest periods, and the sediment was pelleted in a Beckman Avanti centrifuge with an F650 rotor. Centrifugation was done for 30 min at 37,500 × *g*. The supernatant was diluted with 0.1% trifluoroacetic acid, centrifuged again to remove all residual particles, and applied to a preparative Vydac C_{18} high pressure liquid chromatography (HPLC) column (2.5 × 25 cm). Venom peptides were eluted from the column with a linear gradient of 4.5–90% acetonitrile and 0.1% trifluoroacetic acid at 0.9% acetonitrile/min. The flow rate was 20 ml/min, and the absorbance of the eluate was monitored at 220 nm. An analytical Vydac C_{18} HPLC column (4.6 × 250 mm) with a linear gradient at 0.45% acetonitrile/min in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min was used for subfractionation.

Bioassay of HPLC Fractions on Nereis virens—Specimens (usually within 10–16 cm long) of *N. virens* were maintained in seawater at 0–4 °C in a cold room. During injections, the worms were taken outside the cold room in individual beakers

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[2] The abbreviations used are: CPY, conopeptide Y; HPLC, high pressure liquid chromatography; TFE, trifluoroethanol; RACE, rapid amplification of cDNA ends.

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containing cold seawater. The temperature was maintained close to 4 °C by keeping the beakers immersed in iced water. Each worm was laid out on a block of Styrofoam right before injection and returned to cold seawater within a few seconds after injection. Lyophilized samples resuspended in 10–12 μl of normal saline solution were injected using an insulin syringe at the anterior end of the worm. A needle lid with the tip cut off was used to obtain a consistent depth of needle puncture of ~1.5 mm. The initial effects of injections were evaluated by transferring the worms to seawater at room temperature for 5–15 s at ~1 h after injection. These were observed side by side with saline-injected controls continuously for at least 4 h and checked every day for at least 2 weeks.

Characterization of Peptides—Samples were completely reduced with 10 mM dithiothreitol at 65 °C for 15 min and completely alkylated using 0.7% 4-vinylpyridine for 20 min in the dark at room temperature. Alkylation reactions were diluted 10-fold with 0.1% trifluoroacetic acid and fractionated in an analytical C_{18} HPLC column. Elution was done with a linear gradient of 1.8% acetonitrile/min in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

Peptide sequencing by Edman degradation chemistry was done on alkylated samples after HPLC purification. Uncertain readings of C-terminal residues were verified by sequencing of fragments obtained after endopeptidase digestion with Arg-C. The reaction conditions for enzyme digestion were as recommended by the supplier (Roche Diagnostics).

Mass determinations on both native and synthetic peptides were done by matrix-assisted laser desorption ionization or electrospray ionization mass spectrometry at the University of Utah Mass Spectrometry and Proteomic Core Facility, the Salk Institute Peptide Biology Lab, and The University of Queensland Institute for Molecular Bioscience.

Chemical Synthesis and Purification of CPY-Pl1 and CPY-Fe1—CPY-Pl1 and CPY-Fe1 were synthesized on an ABI 433 peptide synthesizer (Applied Biosystems, Foster City, CA) following the FastMoc chemistry procedure from ABI. Standard protecting groups for the amino acid side chains were used. The cleaved peptide was further purified by HPLC on a semipreparative C_{18} column with a linear gradient of 0.18% acetonitrile/min in 0.1% trifluoroacetic acid. The flow rate was 2.5 ml/min, and absorbance at 220 and 280 nm was monitored.

NMR Spectroscopy—Samples for ^{1}H NMR measurements contained ~2 mM peptide in 90% H_2O and 10% D_2O (v/v) at ~pH 3 or 20% TFE, 80% H_2O, and 10% D_2O. Spectra were recorded at 288–298 K on a Bruker ARX-600 spectrometer equipped with a shielded gradient unit. Two-dimensional NMR spectra were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the t_1 dimension as described previously for other disulfide-rich peptides (10, 11).

Spectra were processed on a Silicon Graphics Indigo Workstation using XWINNMR software (Bruker). The t_1 dimension was zero-filled to 1024 real data points, and 90° phase-shifted sine bell window functions were applied prior to Fourier transformation. Chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate. NMR spectral assignments for CPY-Pl1 were made using established techniques (12).

Bioassay of Synthetic Samples in Mice and C. elegans—Intracranial injections were administered to mice (Swiss Webster) that were 15–17 days old. Peptide samples in HPLC solutions were lyophilized, resuspended in 12 μl of normal saline solution, and administered intracranially to mice using an insulin syringe. The peptide-injected mice were observed side by side with saline-injected controls continuously for 2–4 h and checked the next day. Microinjection of peptide samples into the pseudocoelomic region of the nematode *C. elegans* was done as described (13).

Electrophysiology—The *Xenopus* oocyte expression system was used to study the effect of the CPY peptides on K^+ and Na^+ channels (14) and the *N*-methyl-D-aspartate receptor (15). In K^+ channel assays, oocytes were treated, and channels were expressed as described (16). Whole cell currents were recorded at room temperature (19–22 °C) under two-electrode voltage-clamp control using an 0C-725C amplifier (Warner Instruments, Hamden, CT) or a Turbo-Tec amplifier (npi electronic GmbH, Tamm, Germany). Current records were low pass-filtered at 3 kHz and sampled at 10 or 1 kHz (−3 db) and sampled at 4 kHz. The bath solution was normal frog Ringer's (17) containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes-NaOH (pH 7.2). Bovine serum albumin (0.1 mg/ml) was added to the bath solution in all the dose-response experiments. The IC_{50} values for block of the Kv1.6 channel were calculated from the peak currents at a test potential of 20 or 0 mV according to $IC_{50} = fc/(1 - fc) \times [Tx]$, where *fc* is the fractional current and [Tx] is the toxin concentration.

Cloning—Total RNA preparations from a single duct each of *C. planorbis* and *C. ferrugineus* (Fig. 1) were obtained using the Qiagen RNeasy mini prep protocol for isolation of total RNA from animal tissues. Each RNA preparation (4.5 μg for *C. planorbis* and 5.1 μg for *C. ferrugineus*) was used in cDNA synthesis (18). Degenerate primers derived from CPY-Pl1 and CPY-Fe1 sequences were utilized in 3′-RACE (18) and 5′-RACE (SMART RACE, Clontech) experiments on *C. planorbis* cDNA. Oligonucleotide primers designed from the signal sequence identified for CPY-Pl1 were used in 3′-RACE (18) on *C. planorbis* and *C. ferrugineus* cDNAs. All PCR runs were done in a PTC-200 Peltier thermal cycler (MJ Research, Inc., Waltham, MA) using high fidelity Platinum *Taq* DNA polymerase (Invitrogen). The TOPO TA cloning kit (Invitrogen) was used for all transformations. DNA sequencing was done at the University of Utah Huntsman Cancer Institute Protein/DNA Core Facility using samples prepared following the Qiagen mini prep kit protocol.

RESULTS

Isolation of CPY Peptides from the Venom of C. planorbis (9)—Crude *C. planorbis* venom was fractionated by HPLC as described under "Experimental Procedures." Biological activity on the marine worm *N. virens* was used to identify active fractions from the preparative HPLC column (Fig. 2*A*) and to guide further subfractionation (Fig. 2*B*). Aliquots of the fractions and subfractions were pooled, lyophilized, and assayed on *N. virens* as described under "Experimental Procedures"; if a pool showed bioactivity as described below,

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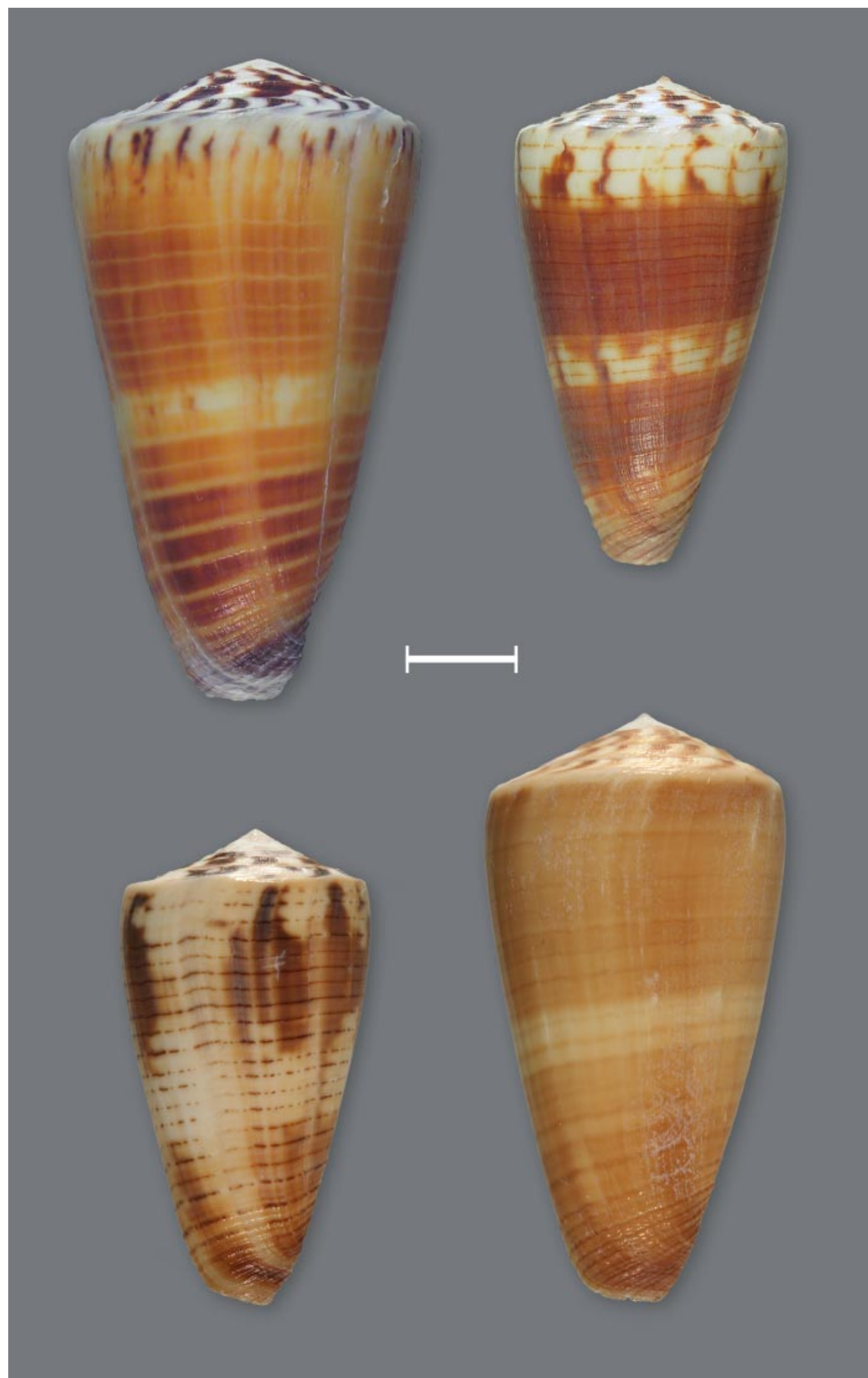

FIGURE 1. **Shells of *C. planorbis* (*upper row*) and *C. ferrugineus* (*lower row*).** The *bar* represents 1 cm. Note that freshly collected specimens of *C. ferrugineus* could easily be mistaken for specimens of *C. planorbis* (photograph by Kerry S. Matz).

individual fractions were assayed. The fraction indicated in Fig. 2*A* gave characteristic symptoms when injected into *N. virens*. Worms that had not been injected or those injected with the control solution (normal saline solution) would move in energetic S-like patterns when transferred to seawater at room temperature. In contrast, affected worms did not move in S-like patterns, but would just glide or move very slowly. All injected worms were moved back to cold seawater after 5–15 s of observation in seawater at room temperature; worms that manifested the initial symptomatology eventually became weak or died in 1–5 days. Uninjected and control worms survived for months.

The subfractions in Fig. 2*B* were assayed individually, and one peak exhibited the activity on *N. virens* described above. The active peak in Fig. 2*B* was completely reduced and alkylated as described under "Experimental Procedures." The reduced and alkylated material was fractionated by HPLC (Fig. 2*C*); the earlier eluting peak indicated by the *arrow* is CPY-Pl1, and the later eluting peak is CPY-Fe1.

The peptides were sequenced using standard Edman methods. The peptide sequences obtained had a most striking sequence feature: an unusually high frequency of tyrosine residues, in addition to basic and hydrophobic residues. The sequences of the two peptides are shown in Fig. 3; the monoisotopic masses of 4092.1 and 4040.1 Da that were obtained corresponded to the respective calculated masses for CPY-Pl1 and CPY-Fe1. Sixteen of the 30 residues are identical in the two peptides, including five tyrosine residues.

The high content of tyrosine in CPY-Pl1 and CPY-Fe1 is reminiscent of the vertebrate neuropeptide Y family (see Fig. 3); we refer to these peptides as the conopeptide Y or CPY family. We propose to name these CPY family peptides from *Conus* venoms using the following nomenclature. The individual peptides will be indicated by the first two letters of the species name, followed by a number representing the numerical sequence of peptides that belong to this family from each species. The two Tyr-rich peptides isolated from what was presumed to be *C. planorbis* venom are referred to as CPY-Fe1 (Fe for *ferrugineus*) and CPY-Pl1 (Pl for *planorbis*). Results from cloning, described below, suggest that both *C. ferrugineus* and *C. planorbis* venoms were present in the sample labeled "pooled *C. planorbis* venom" that was used. Because of the close resemblance of shells of the two species (Fig. 1), the mistaken identification of one species for the other was highly probable during the period that specimens were accumulated to prepare the pooled crude venom that yielded the purified peptides.

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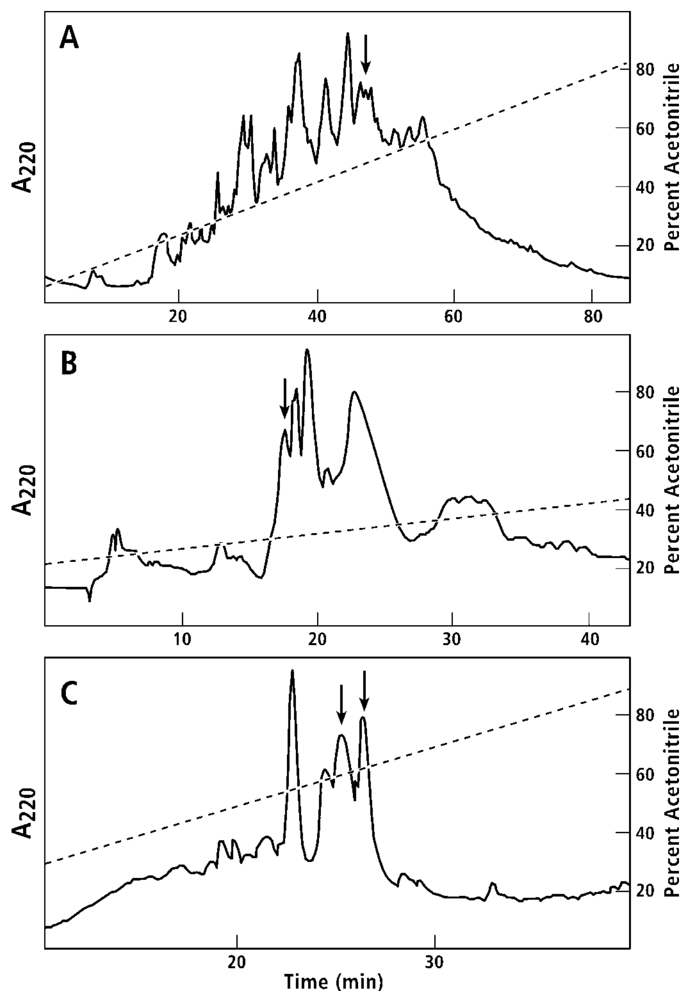

FIGURE 2. **Isolation of CPY-Pl1 and CPY-Fe1 from an extract of *C. planorbis* venom.** *A*, preparative HPLC chromatogram of the extract showing the absorbance profile at 220 nm. The preparation of extract and the HPLC parameters used were as described under "Experimental Procedures." The peak indicated by the *arrow* was potent on the marine worm *N. virens* as described under "Results." *B*, analytical HPLC chromatogram showing the absorbance profile of subfractionation of the active peak in *A*. The *arrow* marks the active subfraction. *C*, analytical HPLC chromatogram of the alkylation reaction mixture of the active subfraction in *B*. Peptide sequencing of the peaks denoted by *arrows* yielded the sequences for CPY-Pl1 (peak on the *left*) and CPY-Fe1 (peak on the *right*).

CPY-Fe 1	GT**Y**L**Y**PFS**YY**RLWR**Y**FTRFLHKQP**YYY**VHI
CPY-Pl 1	ARFLHPFQ**YY**TL**Y**R**Y**LTRFLHR**Y**PI**YY**IR**Y**
NPY	**Y**PSKPDNPGEDAPAEDMAR**YY**SALRH**Y**INLITRQR**Y**-NH_2

FIGURE 3. **Alignment of CPY peptides with neuropeptide Y (*NPY*) (29).**

Chemical Synthesis of CPY Peptides—The chemical synthesis of the CPY peptides was carried out as described under "Experimental Procedures." The HPLC chromatograms for the coelution of synthetic CPY-Fe1 and CPY-Pl1 with the respective native samples are shown in Fig. 4 (*upper* and *lower*) (9). The isotopic peaks in the mass spectra of both synthetic peptides matched those in the spectra of the native samples.

NMR Spectroscopy—The secondary shifts of CPY-Pl1 in aqueous solution and in 20% TFE are shown in Fig. 5. In general, analysis of αH chemical shifts of peptides can provide a strong indication of the type of secondary structure present. For CPY-Pl1 in aqueous solution, the chemical shifts suggest that no regular secondary structure is present based on the lack of stretches of consistent positive or negative secondary shifts. However, in the presence of TFE, a series of significant (*i.e.* >0.1 ppm) negative deviations from random coil shifts between residues 12 and 18 suggest the presence of helical structure in this region. TFE is commonly reported to stabilize helical structures in peptides having an intrinsic helical tendency, and this appears to be the case for CPY-Pl1.

Bioassay of CPY-Fe1 Using Mice and C. elegans—Mice were injected intracranially with CPY-Fe1. Mice injected with ~0.7 nmol of peptide/g of mouse exhibited only mild symptoms such as slightly more than usual grooming, climbing, or hyperactivity. An attempt to elicit more definitive symptomatology by dropping the metal lid onto the cage, thereby creating a loud acoustic input, caused a 30-s seizure in a mouse injected with ~1.5 nmol of peptide/g of mouse. A surprising effect was elicited when one peptide-injected mouse was accidentally dropped while being transferred between two cages; not only was a seizure elicited, but death occurred within 10–15 s after the seizure was initiated. The seizures were observed within 2–3 min after the drop. Thus, we routinely carried out a test referred to as "the mouse-drop assay" after all succeeding mouse intracranial injections of the CPY peptides. Injected mice were dropped from a height of ~1.5 ft onto the cage bottom starting at ~3–5 min after injection. This was repeated every 5–10 min up to ~30 min after injection. The seizure response to the mouse drop was usually observed after the first drop. No symptoms were observed in normal saline solution-injected controls or unaffected mice, even after four to six drops within the 30-min period after injection. Both peptides induced seizures with the mouse-drop assay. The dose response was determined for the CPY-Fe1 peptide. At a dose of 5 nmol, ~50% of the mice responded with mild to moderate seizures. With 20 nmol injected per mouse, most of the mice developed severe seizures and died.

Microinjections in *C. elegans* were made at a dose of ~10 pmol of CPY-Fe1/worm. Partial paralysis, which lasted for ~20 min, was observed in four of four worms injected. In three of the four worms, paralysis occurred from the midsection to the posterior end; the fourth worm showed paralysis from the midsection to the anterior end. An initial test on the effect of CPY-Fe1 on the biphasic contractile response of electrically stimulated rat prostatic vas deferens (19) did not show any effect on both noradrenaline- and ATP-mediated responses with 10 μM CPY-Fe1.

Electrophysiology—Initial screening for activity using CPY-Fe1 on channels expressed in *Xenopus* oocytes included Kv1.1 to Kv1.6, Kv2.1, Kv3.4, and Nav1.2. In these experiments, the oocytes used were free of the vitelline membrane ("skinned") during the clamping experiments. CPY-Fe1 was found to be active at 1 μM on Kv1.6. When attempts were made to determine IC_{50} values with skinned oocytes, evidence for high affinity block of Kv1.6 was obtained for both peptides. However, there were technical problems using skinned oocytes; these invariably developed a leak current after a variable length of time as the measurements were being made. Thus, it was not feasible to obtain reproducible quantitative measurements using skinned oocytes.

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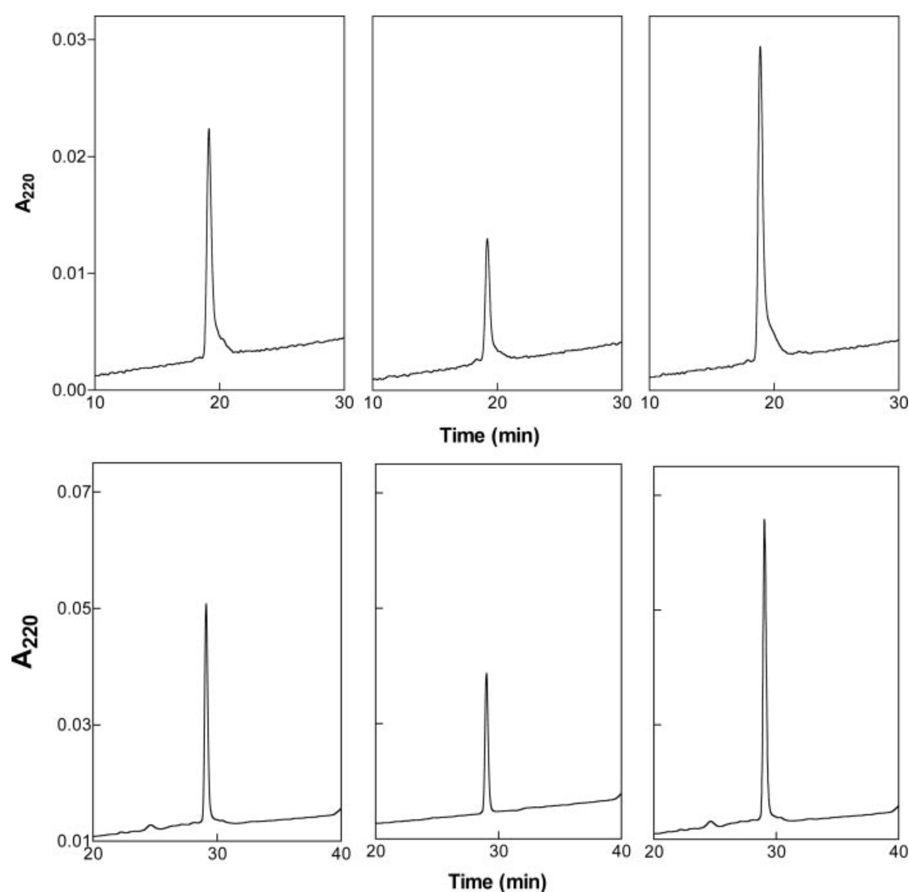

FIGURE 4. **Coelution (*right panels*) of CPY-Fe1 (*upper panels*) and CPY-Pl1 (*lower panels*) that were isolated from the venom (*left panels*) and the corresponding synthetic peptides (*middle panels*).** HPLC was performed in an analytical C_{18} column using a gradient of 0.18% acetonitrile/min in 0.1% trifluoroacetic acid in a Waters Millenium HPLC system with an autosampler, and data were transcribed into Prism.

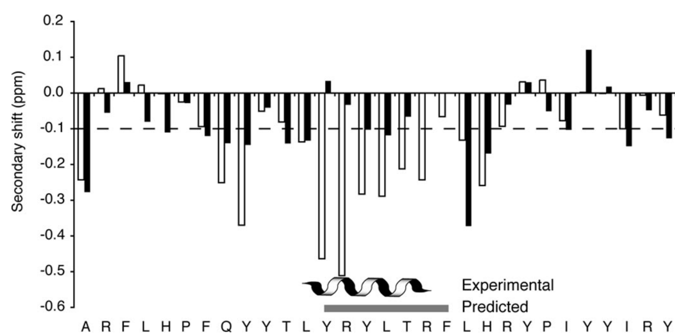

FIGURE 5. **Chemical shift analysis of CPY-Pl1.** The secondary shifts were determined by subtracting the random coil shifts (30) from the experimental αH shifts of CPY-Pl1. Values in aqueous solution are shown with *black bars*, and those in the presence of 20% TFE with *white bars*. The *dashed line* at −0.1 ppm indicates the threshold for significant deviations from the random coil. The helical region in the presence of TFE from residues 12 to 18 is highlighted schematically. The predicted helical region (NNPREDICT) is shown with a *gray bar* and overlaps significantly with the helical region deduced from the chemical shifts.

The dose responses for both CPY-Fe1 and CPY-Pl1 (Fig. 6) demonstrate that CPY-Pl1 is more potent on the Kv1.6 subtype. The data shown in Table 1 and Fig. 6 were obtained using unskinned *Xenopus* oocytes; the kinetics were slow and took tens of minutes to reach equilibrium. Table 1 presents the IC_{50} values obtained from dose-response experiments in oocytes expressing Kv1.2 to Kv1.6. CPY-Pl1 is more active than CPY-Fe1 on both Kv1.2 (>15-fold) and Kv1.6 (~50-fold). In further assays using unskinned oocytes, CPY-Pl1 (1 μM) inhibited ~15% of the *N*-methyl-D-aspartate receptor (NR1–3b/NR2A and NR1–3b/NR2B) activity and ~30% of the Nav1.2 activity.

Molecular Cloning: Definition of the CPY Family—A cDNA library made from *C. planorbis* venom ducts was used to identify the precursors of the CPY peptides. The predicted open reading frame including signal sequence, propeptide, and mature peptide is shown in Fig. 7. The cleavage site between the signal peptide, MMSKLGVVLFVFLLLLPLAAP, and the propeptide was predicted through the SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/).

Initial 3′-RACE experiments utilizing *C. planorbis* cDNA and oligonucleotide primers derived from both peptides yielded clones (7/7) for CPY-Pl1 but no clones for CPY-Fe1. Clones (6/6) obtained in 5′-RACE experiments for both CPY peptides on *C. planorbis* cDNA using primers from both peptides gave the CPY-Pl1 precursor even with the primer derived from CPY-Fe1, which has a region homologous to the primer derived from CPY-Pl1. A 3′-RACE experiment on *C. planorbis* cDNA utilizing oligonucleotide primers derived from the signal sequence gave CPY-Pl1 clones (7/7) up to the poly(A) tail. A screen for CPY peptides in *C. ferrugineus* cDNA using one of the primers derived from the signal sequence and the poly(T)-containing Q_T primer (18) gave clones (4/4) for CPY-Fe1.

The signal and propeptide sequences are completely shared by the two peptides up to the nucleotide level. Sixteen (Fig. 7) of the 30 residues of the mature peptide are also shared up to the nucleotide level. There is 91% homology in the 3′-untranslated regions of CPY-Fe1 (EU000529) and CPY-Pl1 (EU000528). The unique signal sequence revealed from clones encoding precursors of these peptides defines a new gene family of conopeptides, which we refer to as the conopeptide Y or CPY family.

DISCUSSION

The peptides characterized in this study are unusual in their high content of tyrosine, normally an uncommon amino acid. Most *Conus* venom peptides are stabilized by multiple disulfide bonds. As a rule, the *Conus* venom peptides that lack disulfide cross-links tend to be smaller in size than the disulfide-rich conotoxins. The CPY peptides that are characterized here are the longest conopeptides without disulfides shown to be biologically active. In addition to the high tyrosine content, these

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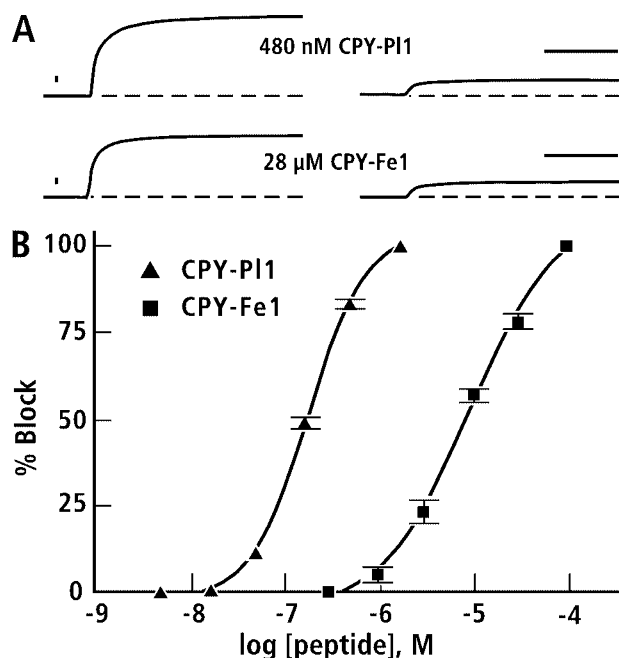

FIGURE 6. **Activity of CPY-Fe1 and CPY Pl1 on the Kv1.6 channel.** *A*, the *horizontal bars* correspond to 50 ms, and the *vertical bars* correspond to 1 μA. *B*, each data point is an average of responses obtained from at least three oocytes as described under "Experimental Procedures," and the dose-response curves were generated using Prism. The IC_{50} for CPY-Fe1 is 8.8 μM, and that for CPY-Pl1 is 170 nM.

TABLE 1

Activity of CPY peptides on mammalian Kv1 isoforms expressed in *Xenopus* oocytes

Peptide	IC_{50} (95% confidence interval)				
	Kv1.2	Kv1.3	Kv1.4	Kv1.5	Kv1.6
			μM		
CPY-Fe1	>30	>50	>50	>50	8.8 (6.5–12.0)
CPY-Pl1	2.0 (1.0–3.9)	>50	>50	>5	0.17 (0.16–0.19)

Signal Sequence:

CPY-Pl 1 **MMSKLGVVLFVFLLLLPLAAP**

CPY-Fe 1 **MMSKLGVVLFVFLLLLPLAAP**

Propeptide:

CPY-Pl 1 **QPVGDQPADQPADRNAEAR**

CPY-Fe 1 **QPVGDQPADQPADRNAEAR**

Mature Peptide:

CPY-Pl 1 AR**F**L**H**P**F**Q**YY**T**L**Y**RY**L**TRFLH**RY**P**V**YY**IRY

CPY-Fe 1 GTY**L**Y**PF**S**YY**R**L**W**RY**F**TRFLH**KQ**P**Y**YY**VHI

FIGURE 7. **CPY-Pl1 and CPY-Fe1 precursors.** Complete homology between the two clones occurs in the signal sequence and propeptide regions; 91% homology is observed in the 3′-untranslated regions (not shown). The residues shared by the mature peptides are in *boldface*. Polymorphism was found in the CPY-Pl1 clones at the sixth residue from the C terminus of the mature peptide where isoleucine was substituted with valine in ~30% of the sequences obtained; the peptide with the isoleucine residue was identified in the venom (Fig. 3) and subsequently synthesized.

peptides are enriched in arginine and large hydrophobic residues (Phe, Leu, and Ile); in CPY-Pl1, these five amino acids account for >70% of the total.

Both CPY-Fe1 and CPY-Pl1 were isolated (9) from a batch of venom that had been assumed to have come from specimens of *C. planorbis*. The cloning results strongly indicated, however, that one of the CPY peptides that was isolated from the pooled venom, CPY-Fe1, could be cloned only from cDNA derived from a *C. ferrugineus* venom duct. Correspondingly, only CPY-Pl1 clones (20/20) were detected from *C. planorbis* cDNA. As shown in Fig. 1, there are shells of *C. ferrugineus* or *C. planorbis* that could easily be confused as one or the other; indeed, the precise taxonomy of the complex of species related to *C. planorbis* remains unresolved. Each cDNA preparation was obtained from a single venom duct of each species; thus, the cloning results seem more reliable than those obtained by extraction from venom pooled from 100 or so specimens.

We have demonstrated that these peptides are antagonists of voltage-gated K^+ channels belonging to the Kv1 subfamily. Both of the peptides characterized exhibited selectivity for Kv1.6 among the Kv1 subfamily channels tested, with CPY-Pl1 showing submicromolar affinity (IC_{50} = 0.17 μM). Conopeptides derived from venoms of worm-hunting *Conus* species (such as *C. planorbis*) generally tend to show lower affinity for mammalian homologs than do the corresponding conopeptides from venoms of fish-hunting cone snails; thus, the data are not inconsistent with the native target of this peptide being the Kv1.6 homolog in the polychaete worm prey of *C. planorbis*. Another possibility is that an even higher affinity K^+ channel target will be found in mammalian systems as well as in polychaetes. The data in Table 1 suggesting that these peptides have a higher affinity for Kv1.6 but also target Kv1.2 with lower affinity raise the possibility that the native target is a heteromeric combination of subunits (such as an interface between Kv1.6 and Kv1.2). Thus, selectivity for a heteromeric Kv1 target that has more than one type of subunit is also a viable possibility that needs to be considered.

The variable onset of leak currents in skinned oocytes suggests that, in addition to the K^+ channel blocking activity, the CPY peptides may also be membrane-active. Because these are hydrophobic peptides, the possibility that they can insert into membranes to form pores or destabilize the oocyte membranes in some other manner could explain the unpredictable leak observed; in contrast, the unskinned oocytes remained stable enough so that the reliable dose-response data shown in Fig. 6 and Table 1 could be obtained. A small peptide lacking disulfides that inhibited voltage-gated K^+ channels was reported previously from *Conus monile*; the subfamily selectivity was not reported (20). The CPY peptide that has been most well characterized, CPY-Pl1, is highly selective for the Kv1.6 subtype. It is notable that CPY-Pl1 is ~18-fold more potent on Kv1.6 than pl14a, a disulfide-rich peptide that was isolated previously from the same venom (9, 21).

Conus species have a structurally diverse group of peptides targeted to the Kv1 subfamily; the first such peptide characterized was κ-conotoxin PVIIA (22), from the venom of the fish-hunting cone snail *Conus purpurascens*; this peptide has three disulfide bonds and belongs to the O-gene superfamily. Surprisingly, when Kv1 channel-targeted peptides from two other clades of fish-hunting cones were elucidated, peptides structurally unrelated to κ-conotoxin PVIIA were found: κM-conotoxin RIIIK (23) from *Conus radiatus* also has three disulfide bonds but belongs to the M-gene superfamily, and conkunit-

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zin-S1 (24, 25) is a Kunitz domain K^+ channel inhibitor from *Conus striatus*. The investigation of *C. planorbis* venom revealed not only K^+ channel-targeted peptides unrelated to the three classes above but also two different toxins unrelated to each other that both preferentially target Kv1.6: κJ-conotoxin PlXIVA, a peptide with two disulfide bonds and previously called pl14a (9, 21), and CPY-Pl1, the peptide characterized in this study. It is not unprecedented for venom from one species to contain unrelated peptides that antagonize the same ion channel complex. It has been previously shown that different peptides antagonize the same nicotinic receptor subtype in *C. purpurascens* (26, 27) and *Conus imperialis* (28) venoms. In both cases, the peptides were structurally unrelated and were subsequently shown to target different pharmacological sites on the same receptor complex. This appears to be part of the sophisticated combination drug therapy strategy that cone snails employ for prey paralysis. We speculate that the natural prey of *C. planorbis* has a Kv1.6-like *Shaker*-type K^+ channel that may be an important molecular target in the snail's strategy for prey capture.

Multiple disulfide cross-links presumably stabilize most *Conus* peptides once they are injected into the body of the targeted animal. Because the CPY peptides are unstructured in aqueous solution, they would appear to be subject to rapid proteolytic degradation. However, a significant number of *Conus* peptide toxins may target axons right at the site of injection; in the case of fish-hunting cone snails, K^+ channel blockers are part of the "lightning-strike cabal" of toxins that cause a massive depolarization of axons in the immediate vicinity of the injection site, which in effect rapidly immobilizes the prey. It seems reasonable to speculate that the CPY peptides in *C. planorbis* and *C. ferrugineus* venoms may play an analogous role *in vivo*; if this were the case, the peptides may act right at the injection site and, once bound to their targets, would presumably become much more highly structured and resistant to attack by proteases.

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REFERENCES

1. Terlau, H., and Olivera, B. M. (2004) *Physiol. Rev.* **84,** 41–68
2. Craig, A. G., Norberg, T., Griffin, D., Hoeger, C., Akhtar, M., Schmidt, K., Low, W., Dykert, J., Richelson, E., Navarro, V., Macella, J., Watkins, M., Hillyard, D., Imperial, J., Cruz, L. J., and Olivera, B. M. (1999) *J. Biol. Chem.* **274,** 13752–13759
3. Olivera, B. M., McIntosh, J. M., Clark, C., Middlemas, D., Gray, W. R., and Cruz, L. J. (1985) *Toxicon* **23,** 277–282
4. Maillo, M., Aguilar, M. B., Lopez-Vera, E., Craig, A. G., Bulaj, G., Olivera, B. M., and Heimer de la Cortera, E. P. (2002) *Toxicon* **40,** 401–407
5. Cruz, L. J., de Santos, V., Zafaralla, G. C., Ramilo, C. A., Zeikus, R., Gray, W. R., and Olivera, B. M. (1987) *J. Biol. Chem.* **262,** 15821–15824
6. Jimenez, E. C., Olivera, B. M., Gray, W. R., and Cruz, L. J. (1996) *J. Biol. Chem.* **281,** 28002–28005
7. Espiritu, D. J. D., Watkins, M., Dia-Monje, V., Cartier, G. E., Cruz, L. J., and Olivera, B. M. (2001) *Toxicon* **39,** 1899–1916
8. Duda, T. F., Jr., Kohn, A. J., and Palumbi, S. R. (2001) *Biol. J. Linn. Soc.* **73,** 391–409
9. Imperial, J. S. (2007) *Novel Peptides from Conus planorbis, Terebra subulata, and Hastula hectica*. Ph.D. thesis, The University of Queensland
10. Daly, N. L., Ekberg, J. A., Thomas, L., Adams, D. J., Lewis, R. J., and Craik, D. J. (2004) *J. Biol. Chem.* **279,** 25774–25782
11. Rosengren, K. J., Daly, N. L., Plan, M. R., Waine, C., and Craik, D. J. (2003) *J. Biol. Chem.* **278,** 8606–8616
12. Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York
13. Mello, C. C., Kramer, J. M., Stirchcomb, D., and Ambros, V. (1991) *EMBO J.* **10,** 2959–3970
14. Zhang, M., Green, B. R., Caitlin, P., Fiedler, B., Azam, L., Chadwick, A., Terlau, H., McArthur, J. R., French, R. J., Gulyas, J., Rivier, J. E., Smith, B. J., Norton, R. S., Olivera, B. M., Yoshikami, D., and Bulaj, G. (2007) *J. Biol. Chem.* **282,** 30699–30706
15. Teichert, R. W., Jimenez, E. C., Twede, V., Watkins, M., Hollmann, M., Bulaj, G., and Olivera, B. M. (2007) *J. Biol. Chem.* **282,** 36905–36913
16. Jacobsen, R. B., Koch, E. D., Lang-Malecki, B., Stocker, M., Verhey, J., van Wagoner, R. M., Vyazovkina, A., Olivera, B. M., and Terlau, H. (2000) *J. Biol. Chem.* **275,** 24639–24644
17. Horton, R. M., Manfredi, A. A., and Conti-Tronconi, B. M. (1993) *Neurology* **43,** 983–986
18. Frohman, M. A. (1990) in *PCR Protocols* (Innis, M. A., ed) pp. 28–45, Academic Press, San Diego, CA
19. Sharpe, I. A., Gehrmann, J., Loughnan, M. L., Thomas, L., Adams, D. A., Atkins, A., Palant, E., Craik, D. J., Alewood, P. F., and Lewis, R. J. (2001) *Nat. Neurosci.* **4,** 902–907
20. Sudarslal, S., Singaravadivelan, G., Ramasamy, P., Ananda, K., Sarma, S. P., Sikdar, S. K., Krishnan, K. S., and P., B. (2004) *Biochem. Biophys. Res. Commun.* **317,** 682–688
21. Imperial, J. S., Bansal, P. S., Alewood, P. F., Daly, N. L., Craik, D. J., Sporning, A., Terlau, H., Lopez-Vera, E., Bandyopadhyay, P. K., and Olivera, B. M. (2006) *Biochemistry* **45,** 8331–8340
22. Shon, K., Stocker, M., Terlau, H., Stühmer, W., Jacobsen, R., Walker, C., Grilley, M., Watkins, M., Hillyard, D. R., Gray, W. R., and Olivera, B. M. (1998) *J. Biol. Chem.* **273,** 33–38
23. Al-Sabi, A., Lennartz, D., Ferber, M., Gulyas, J., Rivier, J. E., Olivera, B. M., Carlomagno, T., and Terlau, H. (2004) *Biochemistry* **43,** 8625–8635
24. Imperial, J. S., Silverton, N., Olivera, B. M., Bandyopadhyay, P. K., Sporning, A., Ferber, M., and Terlau, H. (2007) *Proc. Am. Philos. Soc.* **151,** 185–200
25. Bayrhuber, M., Vijayan, V., Ferber, M., Graf, R., Korukottu, J., Imperial, J., Garrett, J. E., Olivera, B. M., Terlau, H., Zweckstetter, M., and Becker, S. (2005) *J. Biol. Chem.* **280,** 23766–23770
26. Hopkins, C., Grilley, M., Miller, C., Shon, K., Cruz, L. J., Gray, W. R., Dykert, J., Rivier, J., Yoshikami, D., and Olivera, B. M. (1995) *J. Biol. Chem.* **270,** 22361–22367
27. Shon, K., Grilley, M., Jacobsen, R., Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torres, J., Yoshikami, D., and Olivera, B. M. (1997) *Biochemistry* **36,** 9581–9587
28. Ellison, M., McIntosh, J. M., and Olivera, B. M. (2003) *J. Biol. Chem.* **278,** 757–764
29. Cerda-Reverter, J. M., and Larhammar, D. (2000) *Biochem. Cell Biol.* **78,** 371–392
30. Wishart, D. S., Bigam, C. G., Hodges, R. S., and Sykes, B. D. (1995) *J. Biomol. NMR* **5,** 67–81

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