FUNCTIONAL HYPERVARIABILITY AND GENE DIVERSITY OF CARDIOACTIVE NEUROPEPTIDES

Carolina Möller\(^1\), Christian Melaun\(^2\), Cecilia Castillo\(^3\), Mary E. Díaz\(^4\), Chad M. Renzelman\(^5\), Omar Estrada\(^6\), Ulrich Kuch\(^2\), Scott Lockey\(^5\), and Frank Marí\(^4\)\(^*\)

From the \(^1\)Department of Chemistry & Biochemistry, Florida Atlantic University, 777 Glades Rd., Boca Raton, FL 33431, USA. \(^2\)Biodiversity and Climate Research Centre (BiK-F), Senckenberganlage 25, 60325 Frankfurt am Main, Germany. \(^3\)Centro de Biociencias y Medicina Molecular, Instituto de Estudios Avanzados-IDEA, Apartado 17606, Caracas 1015-A, Venezuela. \(^4\)Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, UK. \(^5\)Department of Chemistry and Biochemistry, University of California at Santa Cruz, Santa Cruz, CA, 95064, USA. \(^6\)Instituto Venezolano de Investigaciones Científicas, Biophysics and Biochemistry Center, Apartado 21827, Caracas 1020-A, Venezuela.

Address correspondence to: Prof. Frank Marí, Tel: +1 561 2973315, Fax: +1 561 2972759, E-mail: mari@fau.edu

Crustacean cardioactive peptide (CCAP) and related peptides are multifunctional regulatory neurohormones found in invertebrates. We isolated a CCAP-related peptide (conoCAP-a, for cone snail CArdioactive Peptide) and cloned the cDNA of its precursor from venom of \textit{Conus villepinii}. The precursor of conoCAP-a encodes for two additional CCAP-like peptides: conoCAP-b and conoCAP-c. This multi-peptide precursor organization is analogous to recently predicted molluscan CCAP-like preprohormones, and suggests a mechanism for the generation of biological diversification without gene amplification. While arthropod CCAP is a cardio-accelerator, we found that conoCAP-a decreases the heart frequency in \textit{Drosophila} larvae, demonstrating that conoCAP-a and CCAP have opposite effects. Intravenous injection of conoCAP-a in rats caused decreased heart frequency and blood pressure in contrast to the injection of CCAP, which did not elicit any cardiac effect. Perfusion of rat ventricular cardiac myocytes with conoCAP-a decreased systolic calcium, indicating that conoCAP-a cardiac negative inotropic effects might be mediated via impairment of intracellular calcium trafficking. The contrasting cardiac effects of conoCAP-a and CCAP indicate that molluscan CCAP-like peptides have functions that differ from those of their arthropod counterparts. Molluscan CCAP-like peptides sequences, while homologous, differ between taxa and have unique sequences within a species. This relates to the functional hypervariability of these peptides as structure activity relationship studies demonstrate that single amino acids variations strongly affect cardiac activity. The discovery of conoCAPs in cone snail venom emphasizes the significance of their gene plasticity to have mutations as an adaptive evolution in terms of structure, cellular site of expression and physiological functions.

Physiological processes such as neurotransmission, metabolism and cardiac function are regulated by peptides. The structural diversity of these regulatory peptidic scaffolds varies from small linear peptides such as enkephalins to more elaborate disulfide-constrained peptide hormones such as insulin. Related to these peptides, endogenous-like toxins are utilized exogenously by venomous animals as evident by their frequent discovery in venom (1). For example, sarafotoxins are potent mammalian vasoconstrictors found in snake venom (2), and are members of the endogenous hormone endothelin-family (~60% sequence homology). Sequence differences between the endogenous and their exogenous counterparts can account for their change of activity. Posttranslational modifications can transform endogenous peptides for exogenous use as in the cases of the epimerization of C-type natriuretic peptide in platypus venom (3), glycosylation of neurotensin-related peptides (4) and carboxylation of vasopressin/oxytocin-related peptides in cone snail venom (5).
Among venomous animals, cone snails are exquisitely adapted to immobilize their prey (fish, worms, and mollusks), since their venom is composed of a complex multi-targeting concoction of highly modified peptides (conopeptides) (6,7). Conopeptides are typically expressed by exogenes with characteristic signal sequences followed by a pre-pro region and a hypervariable toxin region (8). Additionally, cone snail venom includes several families of endogenous-like peptides, such as the conopressins (vasopressin/oxytocin) (5,9) contulakins (neurotensin) (4), conophysin (neurophysin) (10), conorfamides (RFamides) (11,12), conomaps (myoactive tetradecapeptide) (13) and conkunitzins (Kunitz-type proteins) (14). Here, we report the discovery and characterization of a new class of endogenous-like conopeptides in the venom duct of *Conus villepinii* that belongs to the superfamily of cardioactive peptides (CAPs).

The first CAP was isolated from the shore crab *Carcinus maenas* (15) and described as crustacean cardioactive peptide (CCAP). Subsequently, this peptide was also isolated from several other arthropod species (16-18). In mollusks, two related CCAP-like peptides, M-CCAP1 and M-CCAP2, were isolated from the pulmonate snail *Helix pomatia* (19,20). Sequences encoding CCAP-like peptides have also been found in the genome of the sea snail *Lottia gigantea* and in ESTs from the California sea hare *Aplysia californica* and the Pacific oyster *Crassostrea gigas* (21) (Table 1). CCAP was originally described as a cardioaccelerator in arthropods (22-27). However, later studies found that CCAP to be multifunctional neuropeptide that is involved in several physiological processes such as producing gut and oviduct contractions (28,29), as well as inducing the release of adipokinetic hormone from the corpora cardiaca (30), modulating the cardiac ganglion (31), and regulating \( \alpha \)-amylase and protease activity (32,33). CCAP is also involved in cuticle sclerotization, ecdysis and circadian timing processes (34-38).

The *Conus* CAPs (conoCAP-a,b,c) reported herein have up to 78% sequence homology with CCAP. Remarkably, they decrease the heart rate, which is the opposite to the effect of CCAP. We found no cardiac effect of CCAP in rats; however, we determined that conoCAP-a decreases the heart rate and blood pressure. These effects agree with the reduced systolic calcium amplitude and contraction recorded in single ventricular myocytes from rats’ hearts.

The precursor of the conoCAPs encodes for all three conoCAPs (a-c), in an arrangement analogous to the CCAP-like precursor predicted from the genome of *Lottia gigantea* (21). In addition to defining a new class of conopeptides with unusual cardioactivity, our discovery of conoCAPs suggests the recruitment of an extremely ancient group of peptides into the venom duct of an extant predatory mollusk.

**MATERIALS AND METHODS**

Specimen collection, conoCAP-a isolation and characterization—Specimens of *Conus villepinii* were collected off the Florida Keys using a manned submersible vehicle operating at depths of 100-200 m (5). Venom extraction and its fractionation (SE-HPLC and RP-HPLC) were carried out as previously described (39). NMR spectroscopy of peptides was acquired on a Varian Inova 500 MHz instrument equipped with PFG, 3xRF channels and waveform generators. Nanomolar quantities of the native conopeptide directly isolated from the venom were dissolved in 40 \( \mu \)l of water with 10% D\(_2\)O (used for locking purposes) with 4 nmol of TSP and placed in 1.7 mm NMR tubes (Wilmad WG-1364-1.7). The pH was adjusted using 0.01 M solutions of HCl and NaOH and a Thermo micro-pH probe. Spectra were obtained using a Varian gHCN (generation 5) high performance 3 mm probe (pw90 = 3 \( \mu \)s, at the upper limit of the linear range of the RF amplifier) with a 1.7 mm capillary adaptor (Wilmad V-GFK-10/1.7). For 1D NMR experiments, the water signal was suppressed by using either double spin-echo (40) or preseturation. Peptide concentrations were evaluated by integrating the NMR signals of selected methyl groups and using the known concentration of TSP as an internal standard or the signal of selected methyl groups from peptides with known concentration as external standards. NMR of the synthetic peptides was acquired using similar conditions in a 3 mm NMR tube.

**Nomenclature**—In this publication we adopted a nomenclature that differs from that of the previously reported CCAP (crustacean cardioactive peptide). As the novel peptides and its precursor were isolated from the venom of a cone
Hypervariability and Gene Diversity of Cardioactive Neuropeptides

snail, we named them conoCAP (Conus cardioactive peptide) and used lower case letters to denote their chronological order of discovery.

Cloning and sequencing of the precursor of conoCAPs - One venom duct from C. villepinii was removed from the freshly killed animal on dry ice and stored at -70°C. Approximately 1 cm of the duct was used for mRNA isolation with the Dynabeads® mRNA DIRECT™ Kit (Invitrogen). An appropriate toxin-based or signal sequence-based primer was used for PCR. 5'-RACE was performed using first strand cDNA transcribed with the smart race system (Clontech). The sequence of the primer used for the 5'-RACE was 5'-RTT RTT RTA RCA NCC RAA NSW RTT RCA RAA NGG-3' (based on the peptide sequence PFCNSFGCYN). The PCR conditions were: 94 °C, 1 min; 72 °C, 1 min (5 cycles); 94 °C, 30 sec; 69 °C, 30 sec; 72 °C, 2 min (5 cycles); 94 °C, 30 sec; 67 °C, 30 sec; 72 °C, 2 min (27 cycles); 72 °C, 5 min (1 cycle). 5'-RACE PCR products were ligated into the T-tailed plasmid vector pGEM®-Teasy (Promega) which was then used to transform NEB 5-alpha competent E. coli. Transformed colonies were screened using blue-white selection. Plasmids isolated using the QIAprep Spin Miniprep kit (Qiagen) and analyzed by agarose gel electrophoresis. Plasmids containing inserts of the expected size were selected for Sanger nucleotide sequencing of both strands using primers and BigDye Terminator v3.1 chemistry (Applied Biosystems). Based on the sequences obtained a primer binding to the signal peptide encoding region was designed (5'- ATG GTC TCG CTC GGC CAT GTG CTT T -3') and used for 3'-RACE PCR. 3'-RACE PCR amplification was carried out as follows: 95 °C, 2 min (1 cycle); 95 °C, 1 min; 48 °C, 1 min; 72 °C, 1 min (35 cycles); 72 °C, 7 min (1 cycle). PCR products were cloned as described above. Plasmids DNA of 100 positive clones were isolated and both strands of the insert were sequenced. Nucleotide sequences were analyzed using Bioedit and MEGA 4. The novel cDNA sequences have been deposited in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession numbers FN868446 and FN868447.

Peptide synthesis of conoCAPs and analogs - Peptides were synthesized on a solid phase using Fmoc chemistry (41), starting from Fmoc-RinkAmide-MBHA resin with the C-terminal amino acid attached to a solid support matrix. For this purpose we used a Protein Technologies Inc. peptide synthesizer, model PS3™. The peptides were cleaved from the resin with 94% TFA, 2.5% H2O, 2.5% EDT, and 1% TIS. Subsequently, the correctly folded and disulfide-bridged peptides and analogs were obtained by oxidation in DMSO by dissolving 0.5 mg/ml of the crude peptide in an aqueous solution with 5% acetic acid, 15% DMSO at pH 6 at room temperature for 48 hours (42). The folding process was followed by RP-HPLC and MALDI-TOF/MS. Peptide purifications were performed on RP-HPLC and the correct peptide sequence, folding, and purity were confirmed by Edman degradation, mass spectrometry, and NMR. CCAP were obtained from American Peptide Company, INC.

Cardiac frequency measurements in Drosophila melanogaster larvae - Cardiac frequency in D. melanogaster larvae preparations were performed as previously described with slight modifications (43). Early third instars wild type D. melanogaster larvae were restrained dorsal side up by placing a pin on each end on a sylgard plate. The semi-intact animals were dissected ventrally and pinned on four corners. All visceral organs were removed and the heart was kept intact and functioning. Using a microscope, heart and trachea movements were used for visual count of the heart rate. Trachea movements were a consequence of the heart pulling on the ligament attachments. The dissected larva was first stabilized in Schneider Drosophila medium (Lonza) for 10 min after dissection. The heartbeats were counted for a minute, every five minutes. The basal heart rate was determined during the first 20 min in Schneider Drosophila medium and then the solution was exchanged with one that contained the dissolved peptide (1 μM) in the same buffer. Heart rates in the presence of the peptide tested were counted every 5 min for 40 min. The basal heart rate for each larva was determined by obtaining an average of the measurements over the first 20 min in Drosophila medium. Subsequently, this value was utilized for normalizing the measured heart frequencies in the presence of peptide during the rest of the assay.

Intravenous injection of conoCAP-a and CCAP in rats - Sprague Dawley rats were anaesthetized with an intraperitoneal (i.p.)
injection of sodium pentobarbital (50 mg/kg). The trachea was exposed and an i.v. line was placed in the femoral vein using a polyethylene catheter, avoiding ventilation disturbances, to facilitate the intravenous administration of the compound. Arterial blood pressure was recorded from the femoral artery through a catheter connected to a blood pressure transducer (MLT844; PowerLab) and a bridge amplifier (ML110, PowerLab) from which blood pressure and heart rate were continuously recorded using a 4/20 High Performance Data Recording System (PowerLab). Changes on MABP and HR induced by conoCAP-a and CCAP were evaluated once basal conditions remained constant for 30 min. Samples in the indicated doses were administered as a single bolus of 100 μl (PBS with 5% DMSO as vehicle) injected over 30 sec, and their effects were continuously recorded for 120 minutes. Statistical analysis was performed for a mean difference between basal values and maximal responses to peptide samples.

**Effect of conoCAP-a on cardiac myocytes activity**- Single cardiac myocytes were isolated from adult male Wistar rats by collagenase/protease digestion (44). The cells were loaded with 5 μM fluo-3 (AM) for 10 min, followed by at least 30 min deesterification. Myocytes were placed in a superfusion chamber on the stage of an inverted microscope adapted for epifluorescence (Nikon Diaphot 300). Voltage-clamp was imposed by using the perforated patch technique (45) with amphotericin-B. Low resistance pipettes (1–3 MΩ) were filled with the following solution (in mmol/L) KCl 125, KCl 20, NaCl 10, HEPES 10, and MgCl₂ 5; titrated to pH 7.2 with KOH and amphotericin-B (added to a final concentration, 240 μg/mL). Cells were superfused with control tyrode solution (in mmol/L) NaCl 135, Glucose 11, CaCl₂ 1, HEPES 10, MgCl₂ 1, and KCl 4; titrated to pH 7.4 with NaOH. To avoid interference from outward currents 5 mmol/L 4-aminopyridine and 0.1 BaCl₂ were also added to the control solution. conoCAP-a was added to the tyrode solution at 1 μmol/L. An Axoclamp 2A (Axon Instruments, Foster City CA, U.S.A.) was used. The membrane potential was held at -40 mV and stimulation consisted of 100 ms duration pulses from -40 to 0 mV (to enable LTCC measurements).

**RESULTS**

**Discovery, characterization and synthesis of conoCAP-a**- Fractionation of the venom of *C. villepinii* (SE and RP-HPLC) produced a pure peptide with a monoisotopic molecular mass of 1148.6 Da (Figure 1). The amino acid sequence of the peptide (conoCAP-a) was obtained by Edman degradation (Figure 1, Table 1), and corresponds to a 10-residue/1-disulfide bond hydrophobic conopeptide with three aromatic residues, which is highly homologous to other CAPs (Table 1). ConoCAP-a was synthesized with all L-amino acids. NMR comparison (Figure 1) and RP-HPLC coelution of native material with the synthetic one confirmed the identity of conoCAP-a, including its amidated C-terminus.

**Cloning and sequence analysis of the cDNA of the precursor of conoCAPs**- The cDNA sequence precursor of conoCAP-a is shown in Figure 2. This precursor encodes 207 amino acids and has a 21-residue signal sequence. A sequence similarity search (46) detected homologies with the precursors of CCAP from arthropods as well as *L. gigantea* (21)(Table 1S). The conoCAP-a precursor contained two other related CAPs: conoCAP-b and conoCAP-c. All conoCAP peptides are predicted to be C-terminally amidated, since Gly is present at the loci preceding the C-terminal cleavage site (47).

**Cardiac frequency studies in Drosophila melanogaster larvae**- We performed in vivo studies with *Drosophila melanogaster* larvae (Figure 3). Peptide concentrations from 10 nM to 1 mM were tested for cardiac response (Fig. 1S). As peptide solubility was limited, the optimal concentration of conoCAP-a was 1 μM. Larger concentrations of peptide caused severe arrhythmia and killed the larvae. The CCAP positive control produced a 14 ± 4% increase in heart rate (HR). In contrast, the three peptides from the venom of *C. villepinii* tested resulted in a decreased HR: 26 ± 5% for conoCAP-a, 23 ± 3% for conoCAP-b and 12 ± 2% for ConoCAP-c. In addition to the HR decrease by conoCAP-a, we observed arrhythmia after 20 min of conoCAP-a application. Similarly, M-CCAP-1 addition resulted in a HR decrease of 13 ± 6%.

**Intravenous injection of conoCAP-a in rats**- ConoCAP-a, being the most active conoCAP in *Drosophila* larvae, was used for further cardiovascular tests in vertebrates. Table 2 shows
the effect of conoCAP-a in mean arterial blood pressure (MABP) and HR in rats (n = 6). Intravenous injection of conoCAP-a produced a 25 ± 9 % decrease in MABP and a decrease of 29 ± 9 % in HR; decreases in MABP and HR were observed only 21-37 min after peptide administration. Basal levels of MABP and HR were not recovered after 2 hrs of monitoring. While i.v. injection of conoCAP-a produced significant decreases in HR and MABP, no major changes were observed after i.v. injection of the same concentration of CCAP. No arrhythmia was observed during these experiments.

**Effect of conoCAP-a on ventricular cardiac myocyte activity** - We evaluated the effects of conoCAP-a at the level of rat cardiomyocyte cell function by monitoring intracellular calcium ([Ca\(^{2+}\)]\(_i\)) changes with fluo-3 (acetoxymethyl ester form). Figure 4 shows [Ca\(^{2+}\)]\(_i\) transients of a ventricular myocyte recorded before and during exposure to conoCAP-a (1 μM). Application of conoCAP-a to the superfusing solution induced a time-dependent decrease in the [Ca\(^{2+}\)]\(_i\) transient amplitude and subsequent contraction by 58% in 6 out of 8 myocytes tested. Both effects were irreversible.

**Effects of conoCAP-a analogs on the cardiac frequency of D. melanogaster larvae** - Analogs of conoCAP-a (Ala-scan, peptide truncation, S-blocked analogs) were used to determine the structure-function relationships. Figure 5 shows the HR effects of these analogs on Drosophila larvae preparations. Substitution or truncation of the N-terminal amino acids Pro-1 or Phe-2 abrogated the reduction in HR produced by conoCAP-a, while substitution or deletion of the C-terminal amino acids, Tyr-9 and Asn-10 had no effect. The reduction in HR was lost when the Cys residues were substituted by Ala or when they were S-methylated. Less significant changes were observed when the intracystine amino acids were replaced by Ala.

**DISCUSSION**

Finding peptides belonging to the CCAP superfamily in animal venom is unprecedented. ConoCAPs (a-c) define a new class of conopeptides in the ever-expanding molecular arsenal of cone snails. The conoCAPs sequences are analogous to other molluscan CCAP-like peptides (Table 1) in having conserved Phe-2, Cys-3 and Asn-4 residues as well as the Gly that preceding the second Cys residue. As with all CAPs, conoCAPs are C-terminally amidated. C-terminal amidation is known to be essential for activity of neuropeptides hormones since it improves target binding and enhances *in vivo* stability (48).

ConoCAPs expand the library of CCAP-like peptides in mollusks. These peptides can be grouped in subclasses according to their N-terminal amino acid, Pro, Val or Leu (Table 1). The N-terminal residue can determine the *in vivo* peptide stability (49); accordingly, Pro-1 and Val-1 containing CCAP-like peptides, which include CCAP, are predicted to be the most stable subclasses. Pro-1 is functionally critical as shown by the loss of activity observed for the P1A analog of conoCAP-a (Fig. 5). The Val CCAP-like subclass was not found in the venom duct of *C. villepinii* or in the CNS of *H. pomatia* (19,20). The Leu CCAP-like subclass is the least stable; it was not found in the venom duct of *C. villepinii* either. However, it was found in the CNS of *H. pomatia* (19). The absence of such peptides in dissected venom might be due to lower levels of expression when compared to conoCAP-a or their potential expression in other tissues of the snail for different purposes.

Cloning the precursor of conoCAP-a revealed that it contains sequences encoding two other CCAP-like peptides: conoCAP-b and conoCAP-c. Thus, the conoCAPs precursor organization is analogous to the predicted CCAP-like preprohormone of *L. gigantea*, *A. californica*, and *C. gigas* (21) (Fig.6). Our discovery of conoCAP-a and its precursor highlight the evolutionary diversification of the CAPs gene superfamily into multipeptide for functionally different peptides. In *Lottia* and *Aplysia* the precursor encodes three such peptides vs. only two in *Crassostrea*. Additional linear peptides can be predicted from the precursors of conoCAPs and the CCAP-like peptides from *Aplysia* (Fig. 6, Table 1S). Two molluscan CCAP-like peptides were directly isolated from the CNS of *H. pomatia* (Table 1). However, their precursor has not been reported. Here we present the first example of a paired CCAP-like multipeptide precursor as well as its natural peptide in the dissected venom of a cone snail suggesting an expansion of the functional diversity of these compounds.
All conopeptide precursors described so far encode a canonical three-domain structure of signal peptide, propeptide and mature peptide (8). Hence, the conoCAP precursor arrangement is unprecedented for peptides expressed in the cone snail venom duct. Instead, this precursor resembles the molluscan CCAP-like precursors in having an organization reminiscent of preprohormone gene structures encoding a varying number of multiple neuropeptides. This multipeptide arrangement within the same gene seems to provide a mechanism for biological diversification without a need for gene duplication. In the case of other conopeptide families, multiple peptides per family are expressed in single venom presumably to increase targeting coverage (50). The presence of the three conoCAPs encoding sequences in a single precursor might either be a different mechanism to maintain such coverage.

CCAP genes in arthropods, while diverse, only encode a single peptide per gene. CCAP-like genes in mollusks are also diverse; however, they encode multiple copies of CCAP-like peptides, whose sequences differ between taxa. These findings suggest that genes encoding for CCAP-like peptides underwent an internal repetitive duplication to acquire their present structure. This gene organization is also observed in enkephalins and FMRFamides, where duplication of the peptide-coding exons resulted in several peptides sequences within a precursor; presumably, in response to adaptive pressures and mutations and yielding related but distinct peptides (51).

CCAP plays a variety of functional roles in crustaceans and insects. As previously shown (26), we found that CCAP increases the basal HR in Drosophila larvae preparations. Unexpectedly, we found that conoCAPs elicit a delayed HR decrease. ConoCAP-a produced the most pronounced decrease in HR accompanied by strong arrhythmia. Remarkably, these effects were also observed in mammalian cardiac system as conoCAP-a decreased the MABP and HR of rats by 25% and 29% respectively (Table 2). In contrast, the injection of CCAP did not elicit any cardiac effect in rats. As CCAP-like peptides are only found in invertebrates, previous to this study their effects on mammalian systems had not been described. Thus, it was surprising that small variations in the sequence of CCAP, such as the ones found in conoCAPs, elicited such distinct physiological responses in mammals.

Application of conoCAP-a on rat cardiac myocytes gradually decreased systolic \([\text{Ca}^{2+}]_{i}\) transient amplitude and contractile activity. The systolic \(\text{Ca}^{2+}\) transient in cardiac muscle is triggered by \(\text{Ca}^{2+}\) entry via the L-type \(\text{Ca}^{2+}\) channel (LTCC), which stimulates further \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum (SR). SR calcium release takes place via ryanodine receptors (RyR) by a calcium-induced calcium release (CICR) process (52). Since decreased systolic calcium could arise from effects of conoCAP-a on LTCC, we investigated this possibility. Surprisingly, voltage-clamp experiments to measure LTCC showed that conoCAP-a had no effect on LTCC current, ruling out LTCC as a target of the peptide. Likewise, conoCAP-a did not affect other cell membrane channels and membrane receptors involved in the cardiovascular physiology (\(\beta_1\)-AR, \(\beta_2\)-AR, HCN1, HCN2, Kv1.4, Nav1.5, hERG, V1a, V1b and, V2). ConoCAP-a did produce a reduction of HR and decreased the systolic calcium in a peculiar time-dependent way. This together with the lack of effects at the cell membrane level suggest that conoCAP-a may require binding to an intracellular target protein that is essential for intracellular calcium handling. Such a target could be at the mitochondrial or SR levels. This could be, at least in part, explained by the fact that conoCAP-a shows passive membrane permeability, whereas CCAP does not (Table 2S). Furthermore, the effects may also involve secondary modification.

CCAP is a nanomolar ligand of a GPCR in Drosophila (53). This receptor does not cross-react with other Drosophila neuropeptides. Binding of CCAP to this GPCR is predicted to occur at the extracellular domain of the receptor (54). However, despite the sequence homology of conoCAPs with CCAP, the HR decrease elicited by conoCAPs suggests that their target receptor is different from the CCAP-sensitive GPCR. In this context, it is noteworthy that the cardiac regulation of mammals, arthropods, and mollusks might involve non-overlapping phylum-specific receptors, and that this could affect the cardiac modulation by these peptides (55).

The opposed cardiac effects of conoCAP-a and CCAP indicates that molluscan CCAP-like
Hypervariability and Gene Diversity of Cardioactive Neuropeptides

peptides might also have functions that differ from those of their arthropod counterparts. Furthermore, the presence of several mature peptide coding sequences in the same precursor is indicative of multiple functional roles encoded in the same gene. As here shown, subtle sequences differences can have dramatic functional consequence. This results in a great potential for functional hypervariability of these peptides whose cardiac activity is profoundly affected by single amino acid substitutions (Fig. 5). Similar cases have been reported for other related peptides such as conopressins, oxytocin and vasopressin in which changes in amino acids at position 7, 8 and 9 define their affinity to the oxytocin and vasopressin 1a and 2 type receptors and ultimately their physiological effects (9).

While our functional studies concentrated on evaluating the effects of conoCAPs in cardiac systems, the presence of these peptides in venom as toxins cannot be ruled out given the source within the snail (dissected venom) from which their precursor was amplified. In this regard it is also intriguing that conoCAP-a was present in *C. villepinii* dissected venom in sufficient quantities to allow its chromatographic purification and peptide sequence determination. *Conus villepinii* is a worm-hunting cone snail species that might use conoCAPs for targeting a specific receptor in their prey and facilitate their capture.

ConoCAPs discovery defines a new class of conopeptides related to the CAPs superfamily and reveals for the first time the presence of such peptides in the venom apparatus of an animal. The diversity of genes of this invertebrate peptide superfamily emphasizes the significance of their plasticity to utilize mutations as an adaptive evolutionary force in terms of structure, cellular site of expression and physiological functions. This suggests that their functions may not only define by the peptide sequence but also determined by the organ or cell system in which they are expressed.
REFERENCES

Hypervariability and Gene Diversity of Cardioactive Neuropeptides

* Address correspondence to: Prof. Frank Mari, Department of Chemistry & Biochemistry, Florida Atlantic University, 777 Glades Rd., Boca Raton, FL 33431, USA. Tel: 1-561-297-3115, E-mail: mari@fau.edu

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1 The abbreviations used are: CCAP, crustacean cardioactive peptide; CAP, cardioactive peptide; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; IAM, iodoacetamide; DTT, dithiothreitol; SPPS, solid phase peptide synthesis; DMSO, dimethyl sulfoxide; MABP, mean arterial blood pressure; HR, heart rate; [Ca$^{2+}$], intracellular calcium; LTCC, L-type calcium channel; RyR, ryanodine receptor; CICR, calcium-induced calcium release; SR, sarcoplasmic reticulum; β1-AR and β2-AR, beta 1 and beta 2- adrenergic receptor; HCN1 and HCN2, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 and 2; Kv1.4, voltage-gated potassium channel 1.4; Nav1.5, voltage-gated sodium channel 1.5; hERG, human ether-à-go-go related gene; V1a, V1b, V2 vasopressin receptor type 1a, 1b and 2; GPCR, G protein-coupled receptors; PAMPA, parallel artificial membrane permeability assay; TIC, total ion current detection.
Hypervariability and Gene Diversity of Cardioactive Neuropeptides

FIGURE 1. Isolation of conoCAP-a from the venom of C. villepinii. (A) (B): Venom separation by SE HPLC (Superdex 30) showing the absorbance profile at \( \lambda = 220 \) nm. (C): UV trace of the analytical RP-HPLC separation. The conoCAP-a fraction is labeled with an arrow. (D): MALDI-TOF MS of the HPLC peak corresponding to conoCAP-a showed an m/z value of 1148.55. C-terminal amidation was determined by the difference of 1 Da between the calculated and experimental masses. (E): Amino acid sequence of conoCAP-a obtained by Edman degradation. (F): 1D \(^1\)H-NMR of native and synthetic conoCAP-a confirmed the identity and proper folding of the synthetic peptide.

FIGURE 2. Nucleotide and amino acid sequences of the precursor of conoCAPs. The mature conopeptides conoCAP-a, conoCAP-b and conoCAP-c are shadowed; the signal peptide sequence is underlined. Red color indicates the posttranslational cleavage sites for processing and green color shows the Gly residue needed for C-terminal amidation.

FIGURE 3. Heart rate (HR) measurements on Drosophila melanogaster larvae preparations. For the first 20 min, larvae were exposed to Schneider Drosophila medium. This solution was then exchanged with 1 \( \mu \)M of peptide: CCAP (◊), M-CCAP-1 (Δ), conoCAP-a (■), conoCAP-b (○), conoCAP-c (□) or the Schneider buffer as control (♦). Heart rates were measured every 5 minutes. (\( P < 0.005, n=8 \)).

FIGURE 4. Effects of conoCAP-a on systolic calcium. (A) Continuous record taken from the same cell showing seven systolic \([Ca^{2+}]_i\) transients in control (left; black) and decrease after adding 1 \( \mu \)M of conoCAP-a (right; red). The cell was stimulated every 3 seconds throughout. (B) Average of 20 systolic \( Ca^{2+}\) transients for the control (black) and in the presence of conoCAP-a (red).

FIGURE 5. Effect of conoCAP-a Ala scan analogs on the heart rate (HR) of Drosophila melanogaster larvae preparations. Other analogs tested: (P1F2) = truncated N-terminus Pro-1 and Phe-2, (P1) = truncated N-terminal Pro-1, (N10) = truncated C-terminal Asn-10, (Y9N10) = truncated C-terminal Tyr-9 and Asn-10, and (no S-S) = S-methylated analog. Heart rates were measured every 5 minutes. Values shown are after 40 minutes of peptide application. (0.001 < \( P < 0.05, n=8 \)).

FIGURE 6. Scaled schematic representation of CAPs precursors. Encoding conoCAPs (from the cDNA of Conus villepinii) (this work), molluscan CCAP-like peptides (from the genome of Lottia gigantea, ESTs of Aplysia californica and Crassostrea gigas) (21), crustaceans (from the cDNA of Carcinus maenas) (56) as well insect CCAP (from the cDNA of Periplaneta americana) (33).
TABLE 1. Sequences of known and predicted conoCAPs and CCAP-like peptides.
* indicates an amidated C-terminal. The conoCAP-b C-terminal cleavage site was assumed to be monobasic (57) to preserved C-terminal amidation as in all other CCAP-like peptides. CCAP-like peptides were divided in three subclasses depending on the identity of their first amino acid: (Pro, Leu, and Val). Cystines are shaded in black, amino acid residues that are conserved within subclasses amino acids are shaded in dark gray and conservative substitutions in light gray. (a) this work, (b) refs (19, 20), (c) ref (21), (d) refs (15-18).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Found in</th>
</tr>
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<tbody>
<tr>
<td>conoCAP-a</td>
<td>PFCNSF-CCFN*</td>
<td>Dissected venom*</td>
</tr>
<tr>
<td>M-CCAP1</td>
<td>PFCNYS-SYNS*</td>
<td>CNSA</td>
</tr>
<tr>
<td>Aplysia-1</td>
<td>PFCNTL-CYNG*</td>
<td>ESTSc</td>
</tr>
<tr>
<td>Lottia-1</td>
<td>PFCNW-CGNS*</td>
<td>Genomic DNAc</td>
</tr>
<tr>
<td>CCAP</td>
<td>PFCAPT-CG*</td>
<td>CNS, visceral &amp; pericardial organs, genomic DNAd</td>
</tr>
<tr>
<td>Arthropods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>conoCAP-c</td>
<td>PFCNGGYCCRG*</td>
<td>Venom duct cDNAa</td>
</tr>
<tr>
<td>M-CCAP2</td>
<td>LFCNGYGGC2NL*</td>
<td>CNSB</td>
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<tr>
<td>Aplysia-2</td>
<td>LFCNGYGGC2NG*</td>
<td>ESTSc</td>
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<td>LFCNYGCGRG*</td>
<td>Genomic DNAc</td>
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<td>Crassostrea-1</td>
<td>LFCNT-GGCF*</td>
<td>ESTSc</td>
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<td>conoCAP-b</td>
<td>VFCNGFT-CG*</td>
<td>Venom duct cDNAa</td>
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<td>Aplysia-3</td>
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<td>VFCNGFTCGGRHR*</td>
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<tr>
<td>Crassostrea-2</td>
<td>VFCNGFTCGGS*</td>
<td>ESTSc</td>
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TABLE 2. Cardiovascular effects of conoCAP-a after intravenous injection in rats (1 mg/Kg). 0.01<p<0.05, n=6, ↓=decrease. MABP = mean arterial blood pressure, HR = heart rate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MABP (mmHg)</th>
<th>HR (bpm)</th>
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<tbody>
<tr>
<td></td>
<td>Basal values</td>
<td>Maximum response</td>
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<tr>
<td>Control (PBS)</td>
<td>120±10</td>
<td>115±10</td>
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<tr>
<td>ConoCAP-a</td>
<td>124±10</td>
<td>92±10</td>
</tr>
<tr>
<td>CCAP</td>
<td>125±10</td>
<td>121±8</td>
</tr>
</tbody>
</table>
Hypervariability and Gene Diversity of Cardioactive Neuropeptides

Figure 1

(A) Conus villopinii

(B) SE-HPLC

(C) RP-HPLC

(D) MALDI-TOF-MS

(E) conoCAP-a Sequence

PFQNSFGCVN-NH₂

(F) 1D 'H-NMR of conoCAP-a

Native

Synthetic
Figure 2
Figure 3
Figure 4

A

Control
conoCAP-a

B

Control
conoCAP-a

Fluorescence (AU Units)

5 sec

Fluorescence (AU Units)

1 sec
Figure 5
Figure 6
Functional hypervariability and gene diversity of cardioactive neuropeptides.

Carolina Möller, Christian Melaun, Cecilia Castillo, Mary E. Diaz, Chad M. Renzelman, Omar Estrada, Ulrich Kuch, Scott Lokey, and Frank Mari

The legend for Fig. 3 (page 40677) should read as follows.

FIGURE 3. Heart rate measurements on D. melanogaster larval preparations. For the first 20 min, larvae were exposed to Schneider Drosophila medium. This solution was then exchanged with 1 μM peptide (CCAP (○), M-CCAP-1 (△), conoCAP-a (●), conoCAP-b (○), or conoCAP-c (■)) or with the Schneider buffer as a control (●). Heart rates (HR) were measured every 5 min (p < 0.005, n = 8).