Direct Mass Spectrometric Peptide Profiling and Sequencing of Single Neurons Reveals Differential Peptide Patterns in a Small Neuronal Network[†]

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Received July 29, 1997; Revised Manuscript Received November 6, 1997

ABSTRACT: Mass spectrometry (MS) was employed to detect and structurally characterize peptides in two functionally related neurons, named VD1 and RPD2, which form a network involved in the modulation of heartbeat in *Lymnaea.* Matrix-assisted laser desorption/ionization MS, directly applied to single neurons VD1 and RPD2, showed overlapping yet distinct mass profiles, with a subset of putative peptides specifically present in neuron VD1. Direct tandem MS of a single VD1 neuron revealed the primary structures of the VD1-specific peptides, which were identified as members of the family of small cardioactive peptides. Based on the tandem MS data, a degenerate oligonucleotide was made for use in a polymerase chain reaction strategy to isolate the cDNA encoding the precursor to the small cardioactive peptides from a brain-specific cDNA library. The calculated masses of the mature, posttranslationally modified peptides, as predicted from the corresponding cDNA, agreed with the measured masses of the actual peptides, as detected in single-cell MS analysis. *In situ* hybridization studies showed that the transcript encoding the precursor is present in VD1, but not in RPD2, thus corroborating the single-cell MS analysis. Finally, the small cardioactive peptides were shown to enhance the contractions of the auricle *in vitro.*

Neuropeptides form the largest and most diverse class of signaling molecules in the brain (*1*, *2*) that are involved in the integration of complex physiological processes and behaviors (*3*, *4*). Functionally related neurons commonly express overlapping yet different sets of peptides, which enables the coordination of intricate spatiotemporal patterns of activity in target cells (*5*−*8*). Due to the high complexity in vertebrate neuronal systems, invertebrate systems have been exploited as alternative model systems to investigate the significance of the use of multiple peptide messengers for neuronal communication. We studied the two giant identifiable neurons VD1 and RPD2 in the brain of the mollusc *Lymnaea stagnalis* (*9*). VD1 and RPD2 are electronically coupled and form a simple neuronal network that is involved in the modulation of cardiorespiratory activity (*10*, *11*). Previous cDNA studies revealed that both VD1 and RPD2 contain transcripts encoding two peptide precursors, named the α1 and α2 peptide precursors, that are derived from alternative splicing of a single pre-mRNA (*12*, *13*). The mature peptides have been isolated from an extract of several hundred pooled singly dissected VD1 and RPD2 and structurally characterized by Edman sequencing. Two of the peptides, the α1 and α2 peptides, are cardioactive (*12*). The pharmacological and immunocytochemical data (*11*) together indicate that the α1 and α2 peptides are the messengers that VD1 and RPD2 use to modulate heartbeat.

Recently, we demonstrated the feasibility of the use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[1] to detect peptides contained in single neurons (*14*, *15*). Analysis of VD1 revealed the presence of molecular ion species with masses corresponding to the peptides derived from the two α peptide precursors (*14*). Unexpectedly, a number of molecular ion species of unknown identities were also detected (*14*), suggesting that the peptide complexity of these neurons is higher than that indicated by previous reports (*12*, *13*). In analogy to VD1, the functionally related RPD2 may also have a more complex peptide profile than previously described. In the present study, we extended the MALDI-MS analysis to include both VD1 and RPD2. Comparison of the MALDI mass spectra of single VD1 and RPD2 revealed that VD1 contains, in addition to the (putative) peptides that are also present in RPD2, a subset of unique (putative) peptides. As these hitherto not reported VD1-specific molecules define the

[†] We acknowledge the Netherlands Organization of Research (NWO) for an apparatus grant to K.W.L. and W.P.M.G. A.L.B. received financial support from NIH NCRR Grant RR 01614.

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[1] Abbreviations: MALDI-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SCP, small cardioactive peptide; MS, mass spectrometry; CID, collision-induced dissociation.

S0006-2960(97)01848-5 CCC: $15.00
Published on Web 02/06/1998

unique phenotype of VD1, we focused first on this set of peptides.

In the present study, we employed the innovative MALDI tandem double focusing magnetic−orthogonal acceleration time-of-flight MS technique (*16*, *17*) to structurally characterize the VD1-specific molecules of low molecular weight directly from a single VD1. Sequence informative fragments from collision-induced dissociation (CID) mass spectra elucidated the complete primary structure of one of the peptides as *Lymnaea* small cardioactive peptide a (SCPa), and the partial sequence of the other peptide as SCPb. Based on the tandem MS data, a degenerate oligonucleotide was constructed that was used in a polymerase chain reaction (PCR) strategy aimed at isolating the cDNA encoding the SCP prohormone. We demonstrated by *in situ* hybridization that the SCP transcript is specifically present in VD1 but not in RPD2. Finally, we showed that the *Lymnaea* SCPs modulate heart contraction *in vitro* with activities distinct from that of α peptides. Together, we demonstrated the colocalization of at least four cardioactive peptides derived from three different precursors in a single neuron, thereby illustrating the potentially high information handling capacity of VD1.

MATERIALS AND METHODS

Sample Preparation. Individual neurons were dissected from adult laboratory-bred *L. stagnalis* by using tiny hooks and a microscope (*14*). They were directly transferred to 0.5 μL matrix (10 mg of 2,5-dihydroxybenzoic acid and 0.5 mg of 2-hydroxy-5-methoxybenzoic acid in 1 mL of 7.5 mM trifluoroacetic acid containing 30% acetonitrile) on a metal sample target, using a fine glass pipette (tip diameter 30−50 μm). The matrix/analyte preparation was dried by a cool air stream.

Mass Spectrometry. MALDI-MS for the generation of peptide profiles of single neurons was performed by using a laboratory-built laser desorption reflectron time-of-flight mass spectrometer equipped with a pulsed nitrogen laser (337 nm; pulse width 5 ns). In general, 50 spectra were accumulated to improve the signal-to-noise ratio (*14*). The mass accuracy, which was measured by external calibration using a synthetic peptide (amino acid sequence SADSAPSS ANEVQRF) and insulin, was ∼0.05%.

For peptide sequencing by high-energy collision-induced dissociation (CID) tandem MS, a MALDI hybrid mass spectrometer (Auto Spec TOF, Micromass, U.K.) was used, as previously described (*16*). MS1 possesses trisector ion optics in an electric−magnetic−electric configuration. The ion beam from MS1 passes into the collision gas cell with xenon after retardation to a collisional energy of 800 V. Fragment ions are collimated and passed into an orthogonal acceleration chamber. Here, the ion beam is subjected to a rapidly pulsed orthogonal electric field, pushing out a segment of the ion beam toward the time-of-flight detector.

Cloning of PreproSCP. A degenerate sense primer SCP (5′-CCAAGCTTAA(T C)TA(CT)(CT)TNGCNTT(TC)CCN-(AC)GNATGGG-3′) (Isogen Bioscience, The Netherlands), containing a 5′-*Hin*dIII restriction site was designed, based on the SCP sequence YLAFPRMG. Amplification was performed on cDNA of a λZAP-II library of the cerebral ganglia (*18*), using the SCP primer in combination with either λZAP II primer T33 (5′-GCGCAATTAACCCTCACT-AAAGG-3′) or T77 (5′-GCGTAATACGAC TCACTAT-AGGGCGA-3′) for 40 cycles: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. Amplified cDNA was digested with *Eco*RI and *Hin*dIII and then cloned in an M13mp18 vector, sequenced, and used as a probe in a screening of the cDNA library. SCP cDNA from pBluescript II, generated by *in vivo* excision, was subcloned into M13mp18 and 19 vectors. PCR products, subclones, and pBluescript II cDNA were sequenced in both orientations, according to the dideoxy chain-termination method (*19*), using T7 DNA polymerase (U.S. Biochemical Corp., U.K.).

Cellular Localization of the SCP mRNA. Brains were fixed overnight at 4 °C in 1% paraformaldehyde and 1% acetic acid. After dehydration, the tissue was embedded in paraffin. Parallel 7 μm sections were adhered to 0.5% gelatin/0.5% chromalum-coated slides. Sections were either hybridized with a digoxigenin-labeled SCP cRNA probe or incubated with an antiserum specific for the α peptides (*11*). The cRNA probe was made on 100 ng of a PCR fragment of the SCP cDNA, including T3 and T7 RNA polymerase promoters. Hybridization, washing and visualization were done as described by Smit *et al.* (*20*).

Heart Bioassay. The dissected auricle or ventricle of the heart was suspended in an organ bath and attached to a displacement transducer (*21*). The preparation was superfused with snail saline (*21*). Synthetic peptides (HPLC pure) were added as bolus injections of 10 μL before the perfusion opening in the bath. Contractions were recorded with a pen recorder.

RESULTS

Mass Spectrometric Peptide Profiling of Single Neurons Reveals the Presence of Overlapping yet Distinct Profiles of Putative Peptides in VD1 and RPD2. To compare the peptide content of single neurons VD1 and RPD2, individual cell bodies were dissected and directly subjected to MALDI mass analysis. The mass spectra of VD1 (Figure 1A) and RPD2 (Figure 1B) indicate the presence of molecules with masses corresponding to those of previously identified peptides α1, α2, and β, which are derived from two related precursors (*12*, *13*). Interestingly, a subset of putative peptides (mass peaks A, B, and I) is exclusively present in VD1 (Figure 1A). The identities of these VD1-specific putative peptides are of particular interest, because they may give clues to new modulatory functions of VD1. For these reasons, we focus here on the VD1-specific molecules, with the exclusion of other molecules of unknown identities that are shared by VD1 and RPD2 (*cf.*, peaks C−H in the mass spectra of VD1 and RPD2 in Figure 1, panels A and B, respectively). The unidentified molecules that are shared by VD1 and RPD2 are currently under investigation (*22*) and their identity will be described elsewhere.

Characterization of VD1-Specific Peptides by Tandem MS. To determine directly the primary structures of the small molecules at mass peaks A and B in Figure 1A from a single VD1, we employed a newly developed tandem MS methodology for peptide sequencing (*16*). The molecular ions at *m/z* 1040.6 and 1121.4 corresponding to the molecular ions A and B, respectively, in the spectrum of Figure 1A were selected to obtain the CID mass spectra shown in Figure

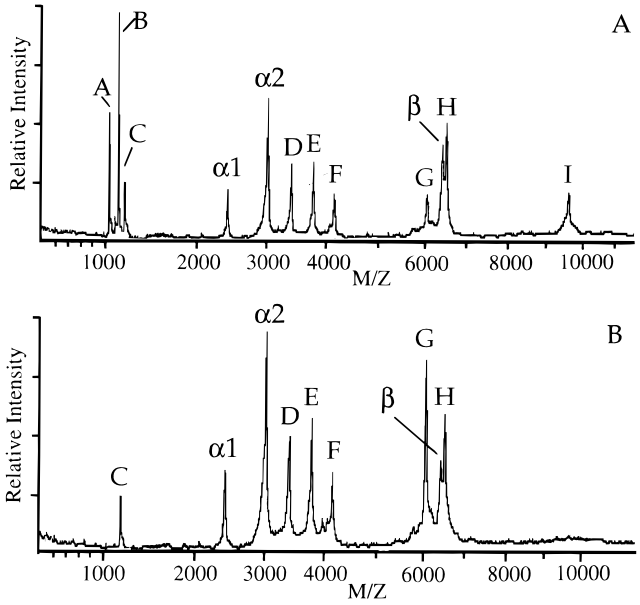

FIGURE 1: Profiles of putative peptides generated by direct MALDI-MS of single VD1 and RPD2 neurons. The mass spectra of VD1 (A) and RPD2 (B) reveal the presence of several molecular ions, which partly overlap in VD1 and RPD2. Peptides with masses corresponding to those of previously identified α1, α2, and *β* peptides (*12*) are indicated. Molecular ions of unidentified molecular species are labeled A−I. The *x*-axis shows *m/z*, mass to charge ratio; the *y*-axis shows intensity in arbitrary units.

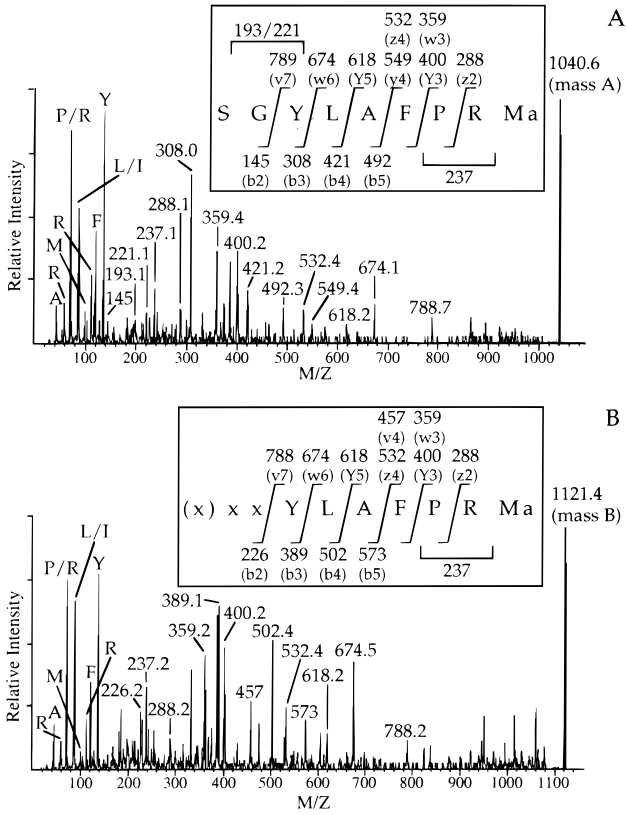

FIGURE 2: Characterization of unidentified molecules by tandem MS analysis of material obtained from a single VD1 neuron. (A) CID mass spectrum of the molecular ion A at *m/z* 1040.6 in Figure 1A; (B) CID mass spectrum of the molecular ion B at *m/z* 1121.4 in Figure 1A. Insets in the mass spectra indicate the assignments of the fragment ions. Overlapping series of backbone fragment ions are present of the b-, y-, Y-, and z-types, as well as side-chain specific ions of the v- and w-types. The Y-type ions are y-ions minus 2 Da. For further explanations, see text.

2, panels A and B. These CID mass spectra contain information that reveals the amino acid composition as well as the sequence of the peptides. In both of these spectra, the occurrence of immonium ions at *m/z* 44.1, 59.1 (and 112.1), 70.1, 86.1, 104.1, 120.2, and 136.2 indicate the presence of the amino acid residues Ala, Arg, Arg/Pro, Leu/Ile, Met, Phe, and Tyr, respectively. Arg is represented by several immonium ions, of which the ion at *m/z* 70.1 overlaps with the immonium ion of Pro (*23*). Manual interpretation of the carboxyl-terminal sequence and satellite ion series (*23*) observed in these high-energy CID mass spectra (see assignments in Figure 2) reveals that these two peptides have an identical carboxyl-terminal partial sequence bearing carboxyl-terminal amidation, *viz.*, ...YLAFPRMa. The presence of b4 and w6 ions permits the assignment of the isobaric amino acid in this partial sequence as Leu. The y, z, v, and w ion series define the carboxyl-terminal amino acid sequence as YLAFP (RM)a, where z2 ion at *m/z* 288.1 indicates the presence of two amino acid residues Arg and Met, with a carboxyl-terminal amidation. The internal fragment ion PR-17 at *m/z* 237.1 establishes the order of the carboxyl terminus as RMa. This partial sequence is confirmed by the presence of an amino-terminal sequence ion series as well, namely, b2−b5 in Figure 2A at the anticipated mass values. So, in the case of peptide A the mass value remaining unexplained defines the composition of the amino-terminal residues as Ser and Gly. The order is established as SG from the presence of two internal ions GY and GY − 28 at values *m/z* 221.2 and 193.1, respectively, yielding the complete sequence as SGYLAFPRMa. In comparison, a previous attempt to identify this peptide by using pools of hundreds of singly dissected neurons, followed by liquid chromatography fractionation of the peptides and Edman sequencing, yielded only a partial amino-terminal sequence (*14*). In the case of peptide B, the remaining unexplained mass value (226.2 Da) could correspond to several possibilities that cannot be discriminated by the spectrum shown in Figure 2B, namely, PK/Q (Q/KP) or the isobaric PGA (AGP or GAP) or pyro-EN. Subsequent cDNA studies indicate that the mass value corresponds to pyro-EN (see below). Screening of the EMBL database revealed that the peptide SGYLAFPRMa shares high sequence similarity with other members of the SCP family (*24*). The SCPs are originally defined by their low molecular weight and their ability to stimulate the activity of gastropod hearts (*25* and see below). In addition, they are potent modulators of central and peripheral synapses of the molluscan nervous system [reviewed by Lloyd (*26*) and Weiss *et al*. (*27*)].

Elucidation of the Primary Sequence of the Lymnaea SCP Precursor by cDNA Cloning. Since the SCP precursor of the related mollusc *Aplysia* contains a large carboxyl-terminal peptide domain (*24*), we hypothesized that mass peak I, which is uniquely present in VD1 but not in RPD2 (compare Figure 1, panels A and B), may represent the *Lymnaea* carboxyl-terminal peptide counterpart. Because this molecule is too large for tandem MS analysis, and also to elucidate the amino terminus of *Lymnaea* SCPb, we turned to molecular biological techniques to identify the *Lymnaea* SCP precursor. On the basis of the identical part YLAFPRMa present in the two *Lymnaea* SCP sequences, we

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          5'-cgccaggccatctcttagcgcccctcgagttgagcagaaccgcagtaaaaacgtagaccacgtttagaagctgcacgtctagggac    86
aggaaacatatccaagcaccgaagtttagctaagtaattgtctcctcaagcccacccttgtgttgtcgctcaacttcaaacaagaacttaggatagagt   185
ccgtctgctgtcaagaaacggtcgaggtcaaggcgacgtcacgaaacaagaaccgacgtggagtctttcactgccaaaatccaagaaaacacaaatcaa   284
agctaggggagataacatccatttcagggagacaaccagacatactactctaagcgccacctaaccaaccaaccaattaaaaactaaaacagaacaaaa   383

-24 signal peptide ──►                                                                  -1 ▼ +1
atg gaa atc aca ttg cca cgt gtc tca ctc tca ttg gct gtc ctg ctg gtc atc gtc tgc agc gta gac gcc cag   458
Met Glu Ile Thr Leu Pro Arg Val Ser Leu Ser Leu Ala Val Leu Leu Val Ile Val Cys Ser Val Asp Ala Gln
SCPb                                  ──► SCPa                              20      ──► C-terminal
aat tac ctg gcc ttt cca aga atg ggc aga tcg ggt tat ctc gcc ttt cca cgc atg ggc cgt tca cat ttc aag   533
Asn Tyr Leu Ala Phe Pro Arg Met Gly Arg Ser Gly Tyr Leu Ala Phe Pro Arg Met Gly Arg Ser His Phe Lys
                                                  40
tct gaa act tcc gct gac gtc acc gga tgt tgc ggg gtg ggg atc aag aac gag ttc ctg att ggt cag gac gga   608
Ser Glu Thr Ser Ala Asp Val Thr Gly Cys Cys Gly Val Gly Ile Lys Asn Glu Phe Leu Ile Gly Gln Asp Gly
                                  60
aag gaa gag atc aga tcc gct tgc ggg gcg cgt gct gac tgc tgc gag gga ctg aag gaa gtg gtc gac cag aaa   683
Lys Glu Glu Ile Arg Ser Ala Cys Gly Ala Arg Ala Asp Cys Cys Glu Gly Leu Lys Glu Val Val Asp Gln Lys
            80                                                                          100
aat gac ggc gtc tac ttc tcc atg tgt gtc cca gac att acc ttt gcc cag gct tca agt gtc cgt tct tcg gaa   758
Asn Asp Gly Val Tyr Phe Ser Met Cys Val Pro Asp Ile Thr Phe Ala Gln Ala Ser Ser Val Arg Ser Ser Glu
                                      112
gtc ttc aac aag ttg aag agc ctc ttg gag aag tga tgacctcgccaaggcagtcgtttcattccatctttcgcgttgtgcaatagt   845
Val Phe Asn Lys Leu Lys Ser Leu Leu Glu Lys ***

gggggatactaaagatcatcatgaacttatcatacagactatcatttgaaattgttataccgttagtacttttcacagacaaatagaacttcgtaattt   944
tttaaaaaatattttcccggaagctatctgggggcgggtgaaatgtgggctaaaataattcaaataattatatatatatatttcaaacaaacaaaaaac  1043
tcaatatacataaaaaaggttttttgtataattcattagtgtattatgcattgtgtttgaatatttaaatataaaata[aataaa]agttttcataactac  1142
aaaaaaaaa-3'                                                                                          1151
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FIGURE 3: Nucleotide sequence and derived amino acid sequence of *Lymnaea* preproSCP. The proteolytic processing sites (Arg) are boxed and the various peptide domains are indicated: signal peptide, SCPa, SCPb, and carboxyl-terminal peptide (C-terminal). Vertical arrow shows the predicted signal cleavage site. The number of nucleotides is indicated at the end of each line. The number of amino acids is indicated above lines. The signal for polyadenylation is indicated in boldface type.

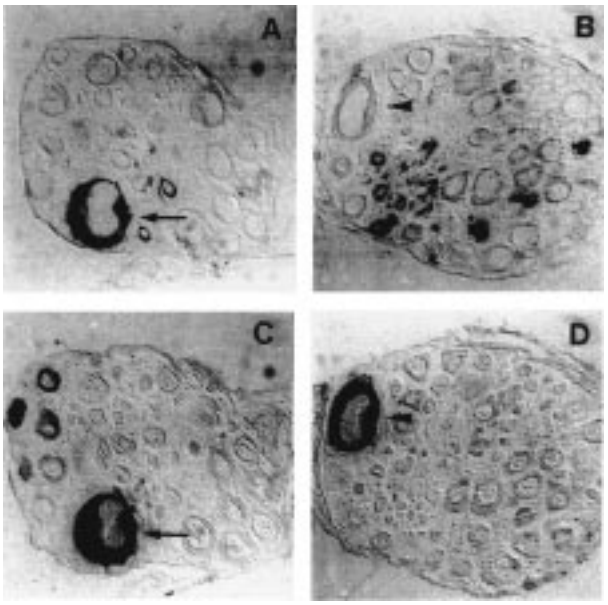

FIGURE 4: Cellular localization of the SCP transcript in VD1: *in situ* hybridization (A and B) of the *Lymnaea* brain with a SCP cRNA probe and SCP transcripts and immunostaining (C and D) on alternate sections with a polyclonal anti-α peptide antibody that recognizes both α1 and α2 peptide. The SCP transcript is present in VD1 (arrow in A) but not in RPD2 (arrowhead in B), whereas both cells are immunoreactive to the anti-α peptide antibodies (arrow in C for VD1 and arrow head in D for RPD2). The SCP gene is not exclusively expressed in VD1, since several small neurons in the ganglia also show hybridization signals. Magnification 200×.

synthesized a degenerate oligonucleotide, which was subsequently used in a PCR strategy on cDNA from a brain-specific library. The obtained PCR product, encoding the identical SCP sequence, was used to screen the cDNA library. This yielded several candidate cDNA clones, and sequence analysis in both directions of the longest cDNA clone revealed a single open reading frame that predicts a preprohormone of 136 amino acids (Figure 3). Cleavage of the signal peptide is predicted to occur after Ala at position −1 (*28*), generating a prohormone of 112 amino acids, which can be endoproteolytically cleaved at two monobasic (Arg) cleavage sites, giving rise to a single copy each of the SCPb sequence QNYLAFPRMG, the SCPa sequence SGYLAFPRMG, and a Cys-rich carboxyl-terminal peptide of 90 residues. Both immature SCPa and SCPb contain carboxyl-terminal Gly residues that serve as an amidation signal for peptidylglycine α-amidating monooxygenase, which transforms carboxyl-terminal glycine residues into carboxyl-terminal α-amides (*29*, *30*). Furthermore, the amino-terminal Gln of SCPb is modified to a pyroglutamate, as can be deduced from the amino-terminal CID fragment ions b2−b5 (see inset of Figure 2B). As this conversion makes peptides more resistant to aminopeptidase activity (*31*), released SCPb might be relatively stable and therefore can reach target sites at a relatively large distance from the release site.

The measured mass of the VD1-specific molecule of 9576 Da (peak I in Figure 1A, mass accuracy ≈0.05%) is in agreement with the calculated mass (9580 Da) of the carboxyl-terminal peptide having three disulfide bridges, and with the carboxyl-terminal Lys residue trimmed off, presumably by carboxylpeptidase E that is present in the secretory granules. The physiological function of the enzyme is to produce peptides from propeptides by trimming off the carboxyl-terminal basic amino acids, *i.e.*, Arg and Lys residues representing the processing sites that are generated via processing of precursor protein by prohormone convertases. Together, we tentatively propose that the VD1-specific molecule of 9576 Da is the carboxyl-terminal peptide. Nevertheless, in contrast to the two small cardioactive peptides, structural elucidation of this big peptide by MALDI methodology was not successful (data not shown). Future work will require the fractionation of this peptide from single VD1 by a microcolumn HPLC system (*cf* refs *32*, and *33*) and the digestion of the peptide with carboxylpeptidase/aminopeptidase to obtain carboxyl/amino-terminal sequence information (see ref *34*). Furthermore, derivatization/reduction/derivatization experiments with reagents for sulfhydryl

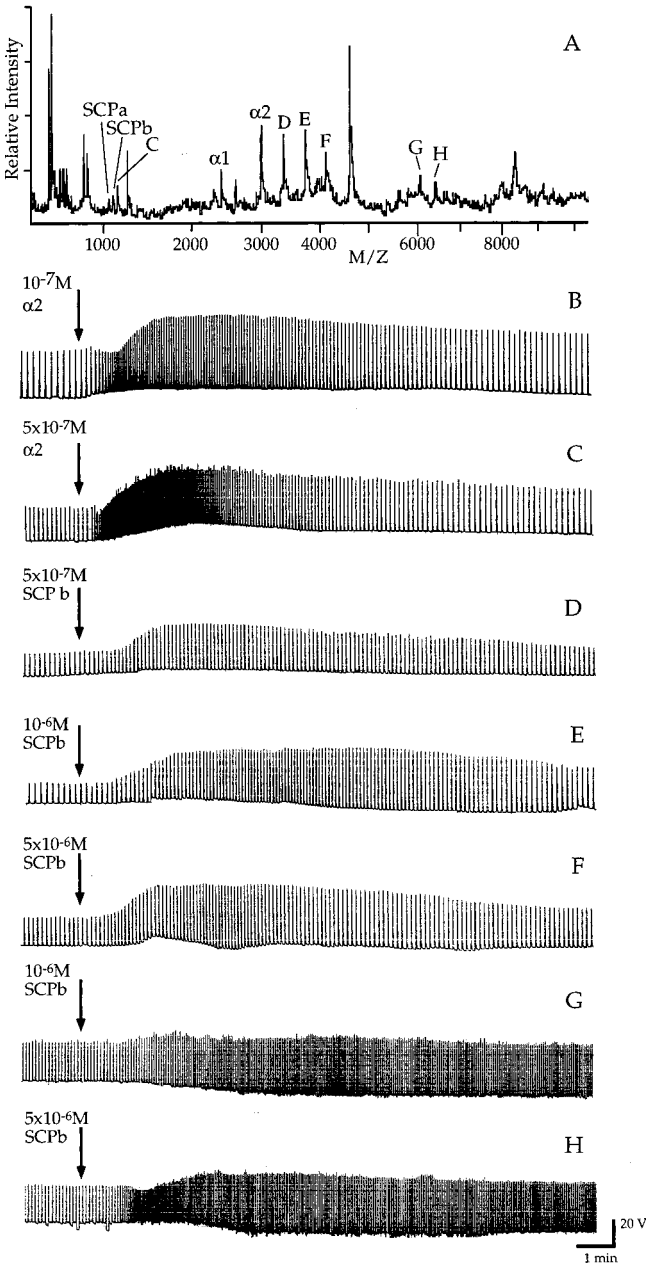

FIGURE 5: Peptide profiling of the auricle of the heart of *Lymnaea* and the responses of the isolated auricle to application of α2 peptide and SCPb. (A) MALDI mass analysis of a small piece of auricle tissue indicates that SCPs and α peptides are present in the auricle. (B, C) Two examples of responses obtained after application of α2 peptide (at arrow) to the isolated auricle, in end concentrations of 1×10^{-7} and 5×10^{-7} M. (D−H). Examples of the excitatory responses of two auricles (D−F and G−H, respectively) obtained after application of SCPb (at arrow) at the indicated end concentrations.

group modification can be used to confirm the presence of disulfide linkages, based on the mass shift before and after derivatizations.

Localization of SCP mRNA by in Situ Hybridization. We used an independent method, *in situ* hybridization, to confirm the expression pattern of the SCPs. *In situ* hybridization experiments on histological sections of the *Lymnaea* brain employing the SCPa cRNA probe confirm the MALDI-MS data (see Figure 1A) and show that the SCP transcript is indeed present in VD1 (Figure 4A) and not in RPD2 (Figure 4B). In addition, several neurons in the vicinity of VD1 and RPD2 express the SCP gene (Figure 4A, B), which further emphasizes the importance of the analysis of single neurons to avoid contamination from surrounding neurons. Figure 4C,D shows that both VD1 and RPD2 contain the α peptide immunoreactive molecules, which represent the α1 and α2 peptides derived from the two α peptide precursors that arise by alternative splicing of the pre-mRNA (*13*); indeed, molecules with masses corresponding to α1 and α2 peptides could be detected in the single-cell MALDI-MS of neurons VD1 and RPD2 (see Figure 1).

Differential Modulation of the Auricle by α *Peptides and SCPs.* To investigate the presence of VD1/RPD2 peptides in the auricle, we performed MALDI mass analysis directly on a biopsy sample of the auricle. The auricle spectrum (an example is shown in Figure 5A) revealed many mass peaks and indicated that the VD1/RPD2 peptides are present, including the SCPs and α peptides. However, the carboxyl-terminal peptide of the SCP precursor was not detected, possibly due to the high background. Other mass peaks corresponded to different classes of identified cardioactive peptides, such as FMRFa, FLRFa, and related heptapeptides (*35*). In addition, unidentified molecular ion species were present.

To investigate the cardioexcitatory actions of both α peptides and SCPs, their effects on the isolated auricle *in vitro* were examined at several concentrations. In line with previous results (*12*), application of α2 peptide consistently induced, within half a minute, a long-lasting (up to 10 min) increase in both contraction rate (at 5×10^{-7} M, 200% ± 20%, mean ± SD; $n = 10$) and amplitude (at 5×10^{-7} M, 40% ± 5%, mean ± SD; $n = 10$). Examples of responses to 1×10^{-7} M and 5×10^{-7} M α2 peptide are shown in Figures 5, panels B and C, respectively. The responses of the auricle to SCP applications varied and differed qualitatively as well as quantitatively from those to α2 peptide applications. Figure 5D−H shows examples of auricle responses to several concentrations of SCPb in two different preparations. In general, the auricle responses to SCP application developed quite slowly, with an onset 2−3 min after application, and consisted of relatively variable increases in auricle contraction rate with thresholds that ranged from 10^{-8} to 10^{-6} M. In line with previous reports of the effects of SCPs on the heart of various molluscan species (*25*, *36*, *37*), the increase in contraction rate was sometimes accompanied by an increase in the contraction amplitude and a decrease in diastolic tone. In addition, in many preparations that developed a relative strong excitatory response after application of SCP, subsequent SCP application even after long (2 h) intervals failed to induce a new response, suggesting complete desensitization for SCP (data not shown). However, application of α2 peptide to such preparations could elicit a typical α2 peptide response, suggesting a receptor-specific desensitization (data not shown). Possibly, the variability in the auricle response to SCP applications is caused by desensitization that may depend on prior SCP actions. Desensitization of the auricle response in *Aplysia*, especially at higher concentrations of SCPs, have also been reported by others (*e.g.*, ref *25*).

DISCUSSION

A frequent observation is that multiple distinct peptides are contained in a single neuron; together, they can elicit

effects that a single messenger cannot produce. To identify the peptide messengers contained in neurons of interest is generally tedious due to their high structural diversity. In the present study, we used a combined strategy to investigate the peptide messengers that are contained in neuron VD1 but not in the functionally related neuron RPD2. First, direct MALDI-MS screening of peptide profiles of single VD1 and RPD2 revealed a set of peptides contained specifically in VD1. The small VD1 specific peptides were then structurally characterized by tandem MS. Finally, molecular biological techniques were used to elucidate the prohormone structure on the basis of the primary peptide sequence generated by tandem MS. We show that the VD1-specific peptides share high sequence similarity to the molluscan SCPs and that *Lymnaea* SCP precursor has the same organization as that of the *Aplysia* SCP precursor. Previous immunocytochemical studies, using *Aplysia* SCP antiserum, did not indicate VD1 as a SCP-containing neuron (*38*). It is likely that this antiserum does not specifically recognise the epitope of *Lymnaea* SCPs. This further demonstrates the importance of single-cell MALDI-MS peptide profiling as a complementary method to existing techniques.

Obligatory for a physiological role of a given peptide transmitter is the demonstration of its presence in target tissue. Previous immunocytochemical studies have shown that one of the peripheral targets of VD1 and RPD2, the auricle of the heart, is innervated by α-immunopositive fibers terminating on auricle trabeculae (*11*). In line with this, the MALDI-MS profiling data of auricle tissue (Figure 5A) indicated the presence of the VD1 peptides, including the α peptides and SCPs.

SCPb and α2 peptide had clearly different qualitative and quantitative effects on the contractions of the isolated auricle. The effects of the α2 peptide (at 5×10^{-7} M) on the contraction rate were consistent and occurred relatively fast, with a delay of ∼0.5 min, reaching a maximum in the second minute. In contrast, the effects of SCPb (at $5 \times 10^{-7} - 10^{-6}$ M) developed more slowly, with maximum rates occurring after several minutes. Moreover, the desensitization characteristics of the auricle response to SCPs and α peptides differed considerably (Jiménez *et al*., unpublished data). Thus, we could now demonstrate the colocalization in a single VD1 neuron of at least four cardioactive peptides that belong to two distinct classes with different effects on the auricle. The effects of other peptides contained in VD1 are also being investigated (*22*). Preliminary results indicate that some of these peptides are cardioactive. In order to understand the physiological significance of the presence of multiple cardioactive peptides in VD1 it will be necessary to examine the interactive effects of a mixture of VD1 peptides on the activity of the heart. In principle, the ratio of peptides released in the target organ can be modified through differential expression of the transcripts, differential targeting of the peptides to distinct granules, and differential exocytosis of the different granules. Theoretically, each peptide ratio may represent a distinct message converging on a target organ and, accordingly, could elicit a distinct effect. We, therefore, speculate that VD1 can modulate heart beat in subtle ways, by using different ratios of cocktails of cardioactive peptides.

ACKNOWLEDGMENT

We thank Dr. Z. Yu for discussion of the CID spectra and Miss T. Laan for typing of the manuscript.

REFERENCES

1. Hökfelt, T. (1991) *Neuron 7*, 867−879.
2. Reisine, T. D. (1994) in *Biological bases of brain function and disease* (Frazer, A., Molinoff, P., and Winokur, A., Eds.) pp 127−141, Raven Press, New York.
3. Angulo, J. A., and McEwen, B. S. (1994) *Brain Res. Rev. 19*, 1−28.
4. Leibowitz, S. F. (1994) in *Models of Neuropeptide Action* (Strand, F. L., Beckwith, B, Chronwall, B., and Sandman, C. A., Eds.), Annals of the New York Academy of Sciences, Vol. 739, pp. 12−35, The New York Academy of Sciences, New York.
5. Kupfermann, I. (1991) *Physiol. Rev. 71*, 683−731.
6. Van Golen, F. A., Li, K. W., De Lange, R. P. J., Van Kesteren, R. E., Van der Schors, R. C., and Geraerts, W. P. M. (1995) *Neuroscience 69*, 1275−1287.
7. Geraerts, W. P. M., Smit, A. B., Li, K. W., Vreugdenhil, E., and Van Heerikhuizen, H. (1991) in *Current Aspects of the Neurosciences* (Osborne, N. N., Ed.), Vol. 3, pp 255−305, MacMillan Press, London.
8. Brezina, V., Orekhova, I. V., and Weiss, K. R. (1996) *Science 273*, 806−810.
9. Boer, H. H., Schot, L. P. C., Roubos, E. W., Ter Maat, A., Lodder, J. C., Reichelt, D., and Schwaab, D. F. (1979) *Cell Tissue Res. 202*, 231−240.
10. Van der Wilt, G. J., Van der Roest, M., and Janse, C. (1988) *Symp. Biol. Hung. 36*, 377−386.
11. Kerkhoven, R. M., Croll, R. P., Van Minnen, J., Bogerd, J., Ramkema, M. D., Lodder, H., and Boer, H. H. (1991) *Brain Res. 556*, 8−16.
12. Bogerd, J., Li, K. W., Jiménez, C. R., Van der Schors, R. C., Ebberink, R. H. M., and Geraerts, W. P. M. (1994) *Mol. Brain Res. 23*, 66−72.
13. Bogerd, J., Van Kesteren, R. E., Van Heerikhuizen, H., Geraerts, W. P. M., Veenstra, J., Smit, A. B., and Joosse, J. (1993) *Cell. Mol. Neurobiol. 13*, 123−136.
14. Jiménez, C. R., Van Veelen, P. A., Li, K. W., Wildering, W. C., Geraerts, W. P. M., Tjaden, U. R., and Van der Greef, J. (1994) *J. Neurochem. 62*, 404−407.
15. Li, K. W., Hoek, R. M., Smith, F., Jiménez, C. R., Van der Schors, R. C., Van Veelen, P. A., Chen, S., Van der Greef, J., Parish, D. C., Benjamin, P. R., and Geraerts, W. P. M. (1994). *J. Biol. Chem., 269*, 30288−30292.
16. Medzihradszky, K. F., Adams, G. W., Burlingame A. L., Bateman, R. H., and Green, M. R. (1996) *J. Am. Soc. Mass Spectrom. 7*, 1−10.
17. Li, K. W., Kingston, R., Dreisewerd, K., Jiménez, C. R., van der Schors, R. C., Bateman, R. H., and Geraerts, W. P. M. (1997) *Anal. Chem. 69*, 563−565.
18. Smit, A. B., Jiménez, C. R., Dirks, R. W., Croll, R. P., and Geraerts, W. P. M. (1992) *J. Neurosci. 12*, 1709−1715.
19. Sanger, F., Nicklen, N., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
20. Smit, A. B., Spijker, S., Van Minnen, J., Burke, J. F., De Winter, F., Van Elk, R., and Geraerts, W. P. M. (1996) *Neuroscience 70*, 589−596.
21. Geraerts, W. P. M., Van Leeuwen, J. P. Th.M., Nuyt, K., and De With, N. D. (1981) *Experientia 37*, 1168−1169.
22. Jiménez, C. R. (1997) *Generation of complex peptide diversity in neuroendocrine cells of* Lymnasa stagnalis *and the rat*, Ph.D. Thesis, Free University, Amsterdam.
23. Medzihradszky, K. F., and Burlingame, A. L. (1994) *A Companion Methods Enzymol. 6*, 284−295.
24. Mahon, A. C., Lloyd, P. E., Weiss, K. R., Kupfermann, I., and Scheller, R. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3925−3929.
25. Lloyd, P. E., Kupfermann, I., and Weiss, K. R. (1985) *J. Comp. Physiol. A. 156*, 659−667.

26. Lloyd, P. E. (1986) *Trends Neural Sci. 9*, 428−432.
27. Weiss, K. R., Brezina, V., Cropper, E. C., Hooper, S. L., Miller, M. W., Probst, W. C., Vilim, F. S., and Kupfermann, I. (1992) *Experientia 48*, 456−463.
28. Von Heijne, G. (1986) *Nucleic Acids Res. 14*, 4683−4690.
29. Eipper, B. A., Stoffers, D. A., and Mains, R. E. (1992) *Annu. Rev. Neurosci. 15*, 57−85.
30. Ping, D., Monier, C. E., and May, S. W. (1996) *J. Biol. Chem. 270*, 29250−29255.
31. Burbach, J. P. H. (1987) in *Vasopressin: Principles and Properties* (Gash, D. M., and Boer, G. J., Eds.) pp 497−515, Plenum Press, New York.
32. Kennedy, R. T., Oates, M. D., Cooper, B. R., Nickerson, B., and Jorgenson, J. (1989) *Science 246*, 57−63.
33. Valaskovic, G. A., Kellcher, N. L., and McLafferty, F. W. (1996) *Science 273*, 1199−1202.
34. Bonetto, V., Bergman, A., Jörnvall, H., and Sillard, R. (1997) *Anal. Chem. 69*, 1315−1319.
35. Saunders, S. E., Kellet, E., Bright, K., Benjamin, P. R., and Burke, J. F. (1992) *J. Neurosci. 12*, 1033−1039.
36. Price, D. A., Lesser, W., Lee, T. D., Doble, K. E., and Greenberg, M. J. (1990) *J. Exp. Biol. 154*, 421−437.
37. Buckett, K. J., Dockray, G. J., Osborne, N. N., and Benjamin, P. R. (1990) *J. Neurophysiol. 63*, 1413−1424.
38. Santama, N., Wheeler, C. H., Burke, J. F., and Benjamin, P. R. (1994) *J. Comp. Neurol. 324*, 335−351.

BI971848B