

Rapid Communication

Neuropeptide Expression and Processing as Revealed by Direct Matrix-Assisted Laser Desorption Ionization Mass Spectrometry of Single Neurons

C. R. Jiménez, *P. A. van Veelen, K. W. Li, W. C. Wildering, W. P. M. Geraerts,
*U. R. Tjaden, and *†J. van der Greef

Graduate School Neurosciences Amsterdam, Research Institute Neurosciences, Vrije Universiteit, Faculty of Biology, Amsterdam; *Leiden/Amsterdam Centre for Drug Research, Centre for Bio-Pharmaceutical Sciences, Division of Analytical Chemistry, Leiden; and †TNO-IBC, Centre for Structure Elucidation and Instrumental Analysis, Zeist, The Netherlands

Abstract: Neuropeptides were directly detected in single identified neurons and the neurohemal area of peptidergic (neuroendocrine) systems in the *Lymnaea* brain by using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The samples were placed in matrix solution and ruptured to allow mixing of cell contents with the matrix solution. After formation of matrix crystals, the analytes were analyzed by MALDI-MS. It was surprising that clean mass spectra were produced, displaying extreme sensitivity of detection. In one of the neuroendocrine systems studied, we could demonstrate for the first time, by comparing the peptide patterns of soma and of neurohemal axon terminals, that processing of the complex prohormone expressed in this system occurs entirely in the soma. In the other system studied, novel peptides could be detected in addition to peptides previously identified by conventional molecular biological and peptide chemical methods. Thus, complex peptide processing and expression patterns could be predicted that were not detected in earlier studies using conventional methods. As the first MALDI-MS study of direct peptide fingerprinting in the single neuron, these experiments demonstrate that MALDI-MS forms a new and valuable approach to the study of the synthesis and expression of bioactive peptides, with potential application to single-cell studies in vertebrates, including humans. **Key Words:** Matrix-assisted laser desorption mass spectrometry—Direct neuropeptide fingerprinting—Single neurons—Peptide processing. *J. Neurochem.* 62, 404–407 (1994).

Peptides are widely used by the nervous system both as neuromodulators/neurotransmitters and as neurohormones to regulate different processes such as reproduction, growth, metabolism, and behavior. The peptides are synthesized in the form of larger prohormones, from which they are proteolytically cleaved, further modified, and stored in secretory granules in axon terminals, from which they are released in response to depolarization. Neuropeptides occur in a large diversity, which is generated by various mechanisms, including gene duplications, intragenic duplications, alternative splicing of a single gene, posttranslational modifications, and tissue-specific prohormone processing. A peptide is often coexpressed with other peptides and/or classic transmitters (for review, see Hökfelt, 1991). Moreover, the

peptide expression and processing patterns may be differentially regulated, both during development and in response to various stimuli. As a result, the spatiotemporal expression patterns of neuropeptides are often very complex, even in closely apposed and functionally related neurons. To examine the significance of the intrinsic peptide patterns for brain functions, it is important to investigate the peptide contents in single neurons. However, such studies are hindered by the lack of appropriate methodology.

We demonstrate here that matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), pioneered by Karas et al. (1987) and recently shown to be useful in the analysis of biological tissue (van Veelen et al., 1993), can be used for direct mass analysis of intact peptides in single neurons. MALDI-MS can detect high-molecular-weight substances, is extremely sensitive, and can tolerate more impurities in the sample than other mass spectrometric methods. Laser desorption mass spectrometry without matrix has been applied in studies on low-molecular-weight compounds in cells using microprobe instruments (reviewed by Fresenius, 1981), thus making it possible to investigate very small sample spots. We analyzed peptides in single identified large neurons of the mollusc *Lymnaea stagnalis*. First, we examined various types of neuron of the neuroendocrine caudodorsal cell (CDC) network, which uses the intercerebral commissure (COM) as the neurohemal area for release of multiple peptides involved in the control of the stereotyped egg-laying behavior of *Lymnaea* (for review, see Geraerts et al., 1991). In addition, we ana-

Resubmitted manuscript received October 13, 1993; accepted October 13, 1993.

Address correspondence and reprint requests to Dr. C. R. Jiménez at Graduate School Neurosciences Amsterdam, Research Institute Neurosciences, Vrije Universiteit, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

Abbreviations used: CDC, caudodorsal cell; COM, intercerebral commissure; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

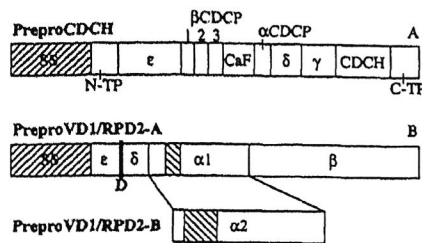


FIG. 1. Schematic drawing of the organization of three preprohormones of *Lymnaea*. The preprohormones are not drawn to scale. Thin vertical bars in all preprohormones represent proteolytic processing sites. **A:** The complex prepro-CDCH is expressed in the CDC system (Geraerts et al., 1991). After removal of the signal sequence in the rough endoplasmic reticulum, pro-CDCH is loaded into secretory granules and further processed to yield 11 mature peptides that are released during CDC discharges to induce egg laying and associated behaviors. The nominal peptide is the ovulation-inducing caudodorsal cell hormone (CDCH) [cf. Geraerts et al. (1991)]. Also indicated are the carboxy-terminal peptide (C-TP); the γ peptide (γ); the δ peptide (δ); the α -caudodorsal cell peptide (α CDCH), which together with CDCH acts as an autoexcitatory peptide inducing electrical discharges in all CDCs; calfluxin (CaF), which induces calcium fluxes in the female sex organs; β_{1-3} CDCP, three related β peptides that act as transmitters; the ϵ peptide (ϵ); and the amino-terminal peptide (N-TP). **B:** The two precursors, prepro-VD1/RPD2 A and B (Bogerd et al., 1993), expressed in identified neurons VD1 and RPD2, arise by alternative splicing. The prohormones are identical, except for the α -peptide domain, which consists of 21 amino acids in the case of α_1 peptide and 28 amino acids in the case of the α_2 peptide. The difference between α_1 and α_2 is restricted to the amino acid stretches indicated by shaded boxes. Both α peptides have inotropic as well as chronotropic effects on the *Lymnaea* heart (Bogerd et al., 1993); however, there are clear differences in potencies. The thick bar in prepro-VD1/RPD2 A and B represents the single aspartic acid residue (D) that is located between two putative processing sites. Also indicated are the ϵ , δ , and β peptides.

lyzed the peptides stored in the COM. Furthermore, we studied the single VD1 neuron that together with the RPD2 neuron forms a simple peptidergic (neuroendocrine) network that is involved in the regulation of respiratory behavior (van der Willt et al., 1988) and heartbeat (Bogerd et al., 1993). Because the peptides and corresponding cDNAs encoding the prohormones (Fig. 1) expressed in these peptidergic systems have previously been studied by conventional molecular biological and peptide chemical methods, we could easily assess the reliability of MALDI-MS. We find that the cellular compartments of prohormone processing can be studied by using the MALDI-MS technique and, moreover, that this method can predict novel neuropeptide expression patterns that are difficult to detect using the conventional methods.

EXPERIMENTAL PROCEDURES

Preparation of samples

Mature specimens (shell height, 30–35 mm) of the freshwater gastropod snail *L. stagnalis* were used. They were bred under laboratory conditions in tanks with a continuous water supply, at 20°C and a 12-h light/dark cycle. Food consisting of lettuce was supplied ad libitum. The brain was dissected, and neurons of interest were isolated under a microscope using tiny hooks. To prevent precocious rupture of

the cell wall, 1,2-propanediol (20%) was added. The neurons were transferred to a small container with Ringer solution to wash off the 1,2-propanediol, as this compound prevents a good matrix preparation. A glass pipette (tip diameter smaller than the cell diameter) was used to rupture and transfer the neurons individually to 0.5–1- μ L drops of matrix solution (2,5-dihydroxybenzoic acid, pH 2, 10 g/L) on a stainless steel target. Of the COM, a piece ~0.5 mm long was dissected, put in the matrix solution, and crushed using small hooks to free the peptides from the tissue. The solution was dried by a gentle stream of cold air. The target was inserted into the mass spectrometer immediately afterward.

Mass spectrometry

MALDI-MS was performed on a Finnigan MAT Vision 2000 laser desorption time of flight mass spectrometer, equipped with a pulsed nitrogen laser (337 nm; pulse width, 3 ns). The sample was irradiated just above the threshold for obtaining ions (10^6 – 10^7 W/cm²). The acceleration voltage was 6.5 kV. Ions were postaccelerated to a conversion dynode at –10 kV in front of the electron multiplier. A camera was mounted on the microscope for inspection of the sample. After initial external calibration with a known standard on the same target, the spectra were calibrated internally on previously identified peaks. Usually 30–100 individual spectra were accumulated to increase the signal-to-noise ratio. The mass accuracy of the instrument is 0.1%.

RESULTS AND DISCUSSION

We first studied the CDC network that expresses the complex pro-CDCH (Fig. 1), from which 11 peptides may be derived that are all involved in the coordination of egg-mass production and associated egg-laying behaviors (Geraerts et al., 1991). Most of the matured CDC peptides contained in the COM have been isolated previously and amino acid sequenced (K. W. Li, C. R. Jiménez, P. A. van Veelen, and W. P. M. Geraerts, submitted). The CDC system consists of two clusters, one in each cerebral ganglion, of together 100 CDCs. The bioactive peptides are released during electrical discharges, which occur simultaneously in all CDCs and which last ~1 h. Two types of CDC are distinguished: dorsal CDCs (~45 cells per cluster), which send a single axon directly to the neurohemal area in the COM, and ventral CDCs (about five cells per cluster), which have two axons, one that runs directly to the COM and another that first crosses the COM, then turns and projects to the neurohemal area. Of both CDC types, the ventral CDCs receive by far most of the sensory input. The mass spectra obtained from single dorsal CDCs (Fig. 2A) and single ventral CDCs (Fig. 2B) of the same CDC system reveal similar peptide spectra for both types of cell. Moreover, spectra similar to the CDC somata were obtained from small pieces of the COM of the same CDC system (Fig. 2C). These results for the first time show that the complex pro-CDCH is fully processed already in the CDC soma. The reliability of the MALDI-MS technique is clear from spectra of CDCs and COM obtained from different animals ($n = 10$), which were all very similar to the ones shown in Fig. 2. To illustrate this, an example of a mass spectrum of a COM of an animal other than that shown in Fig. 2C is given in Fig. 2D. We could identify all peptides contained by pro-CDCH, except β_2 peptide, the amino-terminal peptide, and γ peptide, which suggests either that it is not possible to detect these peptides by using

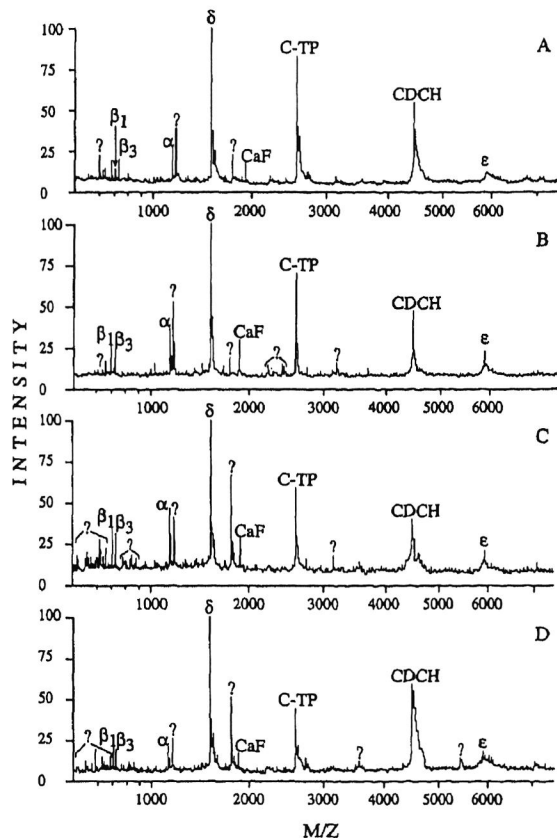


FIG. 2. Typical examples of spectra of single CDC somata and COM. Spectra were surprisingly clean and displayed neuropeptides present in the neuron and the axon terminals in the neurohemal area. **A:** Mass spectrum of a single dorsal CDC cell. The somata of 15 dorsal CDCs, randomly taken from the two clusters of the same CDC network, were examined individually in separate experiments; all yielded spectra that were very similar to the one shown. Peaks could be identified as CDCH [measured protonated mass, MH^+ , is 4,476 Da; the mass as calculated from previous studies (cf. Geraerts et al., 1991) is 4,476 Da], the ϵ peptide (5,960 vs. 5,962 Da), C-TP (2,590 vs. 2,590 Da), CaF (1,898 vs. 1,897 Da), δ peptide (1,566 vs. 1,566 Da), α CDCP (1,169 vs. 1,169 Da), β_1 CDCH (705 vs. 706 Da), and β_3 CDCH (729 vs. 729 Da). For abbreviations, see Fig. 1. Question marks indicate unidentified peaks. **B:** Mass spectrum of a single ventral CDC cell. The somata of six ventral CDCs randomly taken from the two clusters of the same CDC network as in Fig. 2A were individually examined and found to be very similar to each other. Also, the mass spectra of ventral CDCs somata were very similar to those of dorsal CDCs (cf. A). **C:** Mass spectrum of the COM. The spectrum shown is obtained from a piece of ~ 0.5 mm of the COM of the same CDC system studied in A and B. Six trials of the same COM were examined, all yielding similar spectra. The spectra obtained from the COM are similar to those obtained from the somata of both types of CDCs (see A and B). **D:** Mass spectrum of the COM of a different animal as shown in C. The spectrum illustrates that spectra of COMs obtained from different animals are highly similar.

the present technique or that they exist in a form different from that predicted by previous cDNA cloning studies.

Both the sensitivity of the MALDI-MS technique and its ability to predict novel and complex patterns of neuropep-

tide expression could convincingly be demonstrated in the identified neuron VD1. A typical example of a mass spectrum of a single VD1 (Fig. 3) shows the peaks representing α_1 , α_2 , and β peptides derived from two related precursors, pro-VD1/RPD2 A and B (Fig. 1), that are generated by alternative splicing from the single VD1/RPD2 gene (Bogerd et al., 1993). These peptides have been isolated and chemically characterized (Bogerd et al., 1993). As in the earlier study (Bogerd et al., 1993), the putative ϵ and δ peptides were not detected, suggesting that they are not processed as such. However, several other peaks were detected that represent putative peptides that were overlooked in the earlier study using conventional molecular biological and peptide chemical techniques. To identify the novel peptides, we returned to the conventional techniques, dissected 1,500 VD1 neurons, and isolated peptides by using reversed-phase HPLC. The fractions containing peptides with masses corresponding to some of the novel peptides determined by single-cell analysis were subjected to amino acid sequencing. These peptides have the same amino-terminal sequence as α_2 . However, in view of the fact that they have masses larger than α_2 peptides, which cannot be explained by previous studies, we tentatively conclude that (an)other gene(s) related to the prepro-VD1/RPD2 gene is (are) coexpressed in VD1, or that the VD1/RPD2 gene is alternatively spliced in a more complex way than hitherto thought. In addition, we identified a peptide (C.R.J., unpublished data) related to a small cardioactive peptide previously isolated from *Aplysia* (Morris et al., 1982), which indicates that yet another neuropeptide gene may be expressed in VD1. We are currently identifying the peptides of the remaining unknown peaks as well as their corresponding cDNAs.

The results obtained so far convince us of the advantage of the MALDI-MS technique for neuropeptide studies, such as tissue- and cell-specific processing of prohormones, temporal and spatial changes in neuropeptide expression, post-translational modifications, and peptide sorting and targeting to different intracellular sites. The present work represents the first direct mass spectrometric detection of high-molecular-weight compounds, viz., peptides, in single neurons and axon terminals in the neurohemal area. Although the peptides are in a physiological environment, nei-

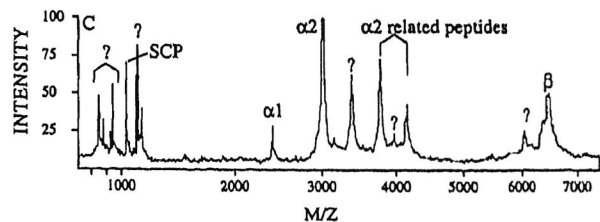


FIG. 3. Mass spectrum of a single VD1 neuron. The spectrum confirms the presence of α_1 , α_2 , and β peptides identified in earlier cDNA cloning and peptide studies (Bogerd et al., 1993). In addition, carboxy-terminally extended forms of α_2 peptides and *Lymnaea* small cardioactive peptide (SCP) are present. The peak at 6,400 Da was quite broad and is a mixture of several peptides (β peptides?). The masses of the identified peaks are α_1 (measured protonated mass, MH^+ , is 2,403 Da; the mass as calculated from previous studies is 2,404 Da), α_2 (2,998 vs. 2,998 Da), β (6,379 vs. 6,376 Da), and SCP (1,043 vs. 1,042 Da). The masses of the two extended α_2 peptides are 3,757 and 4,135 Da, respectively. Question marks indicate unidentified peaks.

ther sample pretreatment nor separation steps are necessary, which allows fast screening of many cells and minimizes artifacts inherent to the conventional techniques. Until now, only qualitative information has been extracted from the experimental data; however, quantitative analysis by mass spectrometry is underway (Tang et al., 1993). Moreover, postsource decay MALDI-MS enables (partial) sequencing of peptides at low concentrations. We predict that with the ongoing refinement of the technique also smaller neurons, including those of vertebrates and humans, will soon become accessible for direct MALDI-MS analysis.

Acknowledgment: We thank Mrs. T. Laan for typing the manuscript.

REFERENCES

- Bogerd J., Li K. W., Jiménez C. R., van der Schors R. C., Ebberink R. H. M., and Geraerts W. P. M. (1993) Processing, axonal transport and cardioregulatory functions of peptides derived from two related prohormones generated by alternative splicing of a single gene in identified neurons VD1 and RPD2 of *Lymnaea*. *Mol. Brain Res.* (in press).
- Fresenius Z. (1981) Special issue. *Z. Anal. Chem.* 308, 193–320.
- Geraerts W. P. M., Smit A. B., Li K. W., Vreugdenhil E., and van Heerikhuizen H. (1991) Neuropeptide gene families that control reproductive behaviour and growth in molluscs, in *Current Aspects of the Neurosciences, Vol. 3* (Osborne N. N., ed), pp. 244–304. Macmillan Press, London.
- Hökfelt T. (1991) Neuropeptides in perspective: the last ten years. *Neuron* 7, 867–879.
- Karas M., Bachmann D., Bahr U., and Hillenkamp F. (1987) Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Processes* 78, 53–68.
- Morris H. R., Panico M., Karplus A., Lloyd P. E., and Riniker B. (1982) Elucidation by FAB-MS of the structure of a new cardioactive peptide from *Aplysia*. *Nature* 300, 643–645.
- Tang K., Allman S. L., Jones R. B., and Chen C. H. (1993) Quantitative analysis of biopolymers by matrix-assisted laser desorption. *Anal. Chem.* 65, 2164–2166.
- van der Wilt G., van der Roest M., and Janse C. (1988) The role of two peptidergic giant neurons in modulation of respiratory behaviour in the pond snail *Lymnaea stagnalis*. *Symp. Biol. Hung.* 36, 377–385.
- van Veelen P., Jiménez C. R., Li K. W., Wildering W. C., Geraerts W. P. M., Tjaden U. R., and van der Greef J. (1993) Direct peptide profiling of single neurons by matrix-assisted laser desorption ionization mass spectrometry. *Org. Mass Spectrom.* (in press).