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MASS SPECTROMETRIC SURVEY OF PEPTIDES IN CEPHALOPODS WITH AN EMPHASIS ON THE FMRFamide-RELATED PEPTIDES

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Summary

A matrix-assisted laser desorption/ionization (MALDI) mass spectrometric (MS) survey of the major peptides in the stellar, fin and pallial nerves and the posterior chromatophore lobe of the cephalopods *Sepia officinalis*, *Loligo opalescens* and *Dosidicus gigas* has been performed. Although a large number of putative peptides are distinct among the three species, several molecular masses are conserved. In addition to peptides, characterization of the lipid content of the nerves is reported, and these lipid peaks account for many of the lower molecular masses observed. One conserved set of peaks corresponds to the FMRFamide-related peptides (FRPs). The *Loligo opalescens* FMRFa gene has been sequenced. It encodes a 331 amino acid residue prohormone that is processed into 14 FRPs, which are both predicted by the nucleotide sequence and confirmed by MALDI MS. The FRPs predicted by this gene (FMRFa, FLRFa/FIRFa and ALSGDAFLRFa) are observed in all three species, indicating that members of this peptide family are highly conserved across cephalopods.

Key words: FMRFamide, neuropeptide, cephalopod, *Loligo opalescens*, *Dosidicus gigas*, *Sepia officinalis*, mass spectrometry, MALDI.

Introduction

Neuropeptides play important roles in the function of many neuronal networks. While much is known about many molluscan neuropeptides, few studies have characterized the peptides in the cephalopods, the molluscs with the most complex central nervous systems. This lack of information on the neuropeptides and hormones present in cephalopods hinders our understanding of many aspects of the function of neurons, even in simple networks, and of their global control of behavior.

Neuropeptides and neurohormones can be fairly well conserved across orders and, in many cases, across different phyla. Thus, one method of determining neuroactive peptides is to search for peptides in well-conserved peptide families (Chiu and Strumwasser, 1981; Nambu and Scheller, 1986; Nagle et al., 1988; Li et al., 1999a), and another strategy is to monitor peptides transported from the central nervous system to the periphery (Li et al., 1998; Lloyd, 1988). In this paper, we make accurate determinations of the molecular masses of easily ionized compounds in the stellar, fin and pallial nerves of cephalopods using mass spectrometric techniques well suited to the study of peptides. Specifically, we use matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry to generate a list of masses representing putative neuropeptides. These putative neuropeptides represent peptides transported to the central nervous system from the stellate ganglion and periphery, or *vice versa*, and thus may be involved in the control of chromatophore or mantle musculature. Two well-known cephalopod models have been used in this study, *Sepia officinalis* and *Loligo opalescens*. The unusual 1998 El Niño weather patterns allowed the Humboldt (jumbo) squid *Dosidicus gigas* to be collected off the coast of Monterey, California, enabling this species to be studied as well.

Advances in mass spectrometry allow peptides to be assayed directly from tissues using several new ionization techniques. In the present study, short sections of an isolated nerve, homogenized nerves or dissected neurons were subjected to direct MALDI MS analysis with high mass accuracy (Garden et al., 1998; Li et al., 1998). In contrast to many conventional biochemical and molecular biological approaches, MALDI MS does not require preselection of the peptides of interest and can provide a mass accuracy of 1 part in 10 000. In addition, its high sensitivity, compatibility with crude mixtures, minimal sample clean-up requirements and ease of use have made MALDI MS a method of choice for the direct peptide profiling of biological tissues (van Veelen et al., 1993; Jiménez et al., 1994; Li et al., 1994, 2000; Garden et al., 1996; Chiu and Zare, 1998).

One of the most ubiquitous neuropeptides is FMRFamide (FMRFa). First characterized by Price and Greenberg (1977), it has been shown to be present in the central nervous system and periphery of organisms as diverse as cnidarians and

vertebrates (Price and Greenberg, 1989, 1994). In molluscs, FMRFa appears to be widely involved in the control of muscle contraction, and several studies in cephalopods have indictated the involvement of FMRFa-related peptides (FRPs). For example, in the European cuttlefish *Sepia officinalis*, FRPs have been identified on the basis of immunoreactivity in the optic gland (Le Gall et al., 1988) and appear to act on chromatophore muscles (Loi et al., 1996; Loi and Tublitz, 1997). In addition, FRPs have been isolated from the octopus *Octopus vulgaris* (Martin and Voigt, 1987) and from the squid *Loligo pealei* (Chin et al., 1994). Several physiological functions for FRPs such as chromatophore expansion in *Sepia officinalis* (Loi et al., 1996), potentiation of transmission at the giant synapse in *Loligo pealei* (Cottrell et al., 1992) and modulation of Ca^{2+} channels in *Loligo forbesii* (Chrachri et al., 2000) have been reported. More recently, it has been demonstrated that FMRFa and related peptides are involved in the regulation of egg laying in *Sepia officinalis* (Henry et al., 1999). In addition, a *Loligo pealei* FMRFa receptor has been characterized (Chin et al., 1994). In the present study, we have placed particular emphasis on confirming the presence and identity of FRPs because FMRFa is the best-characterized peptide in cephalopods. Multiple mass spectral peaks correspond to the molecular masses of putative FRPs so that we also report the characterization of an FMRFa gene in *Loligo opalescens* and compare this with the known *Sepia officinalis* FMRFa gene and an incompletely characterized *Loligo pealei* FMRFa gene (Chin et al., 1994).

Materials and methods

Animals

Sepia officinalis were obtained from the National Resource Center for Cephalopods (Galveston, TX, USA). *Dosidicus gigas* and *Loligo opalescens* were collected off Monterey, California, USA, by commercial fisherman and kept onboard in holding tanks until transferred to the Hopkins Marine Station. Animals were maintained in seawater tanks at either 14 or 18 °C.

Molecular biology

Loligo opalescens stellate ganglion (SG) and giant fiber lobe (GFL) cDNA libraries (8-Uni-Zap XR; Rosenthal and Gilly, 1993; Rosenthal et al., 1997) were used as templates for the polymerase chain reaction (PCR). Briefly, degenerate primers (Gibco degenerate primer code: GAYAARMGITTYYTIMG-ITTYGG; CATRAAYTTYTTYTC and the antisense primer AGGRTCACKACCRAAICKCAT, GibcoBRL, Rockville, MD, USA) were used for the initial PCR. Each 25 µl reaction mixture contained 2.5 µmol l^{-1} dNTPs, 0.5 µl of stock *Taq* polymerase, 2 mmol l^{-1} $MgCl_2$ and PCR buffer diluted to a final (1×) strength, all from Perkin Elmer (Norwalk, CT, USA). In addition, primer was added to give a final concentration of 10 µmol l^{-1}, and 1 µl of cDNA library and 0.5 µl of mineral oil were added to each tube. The PCR reaction was initiated by exposure to 94 °C for 600 s, followed by 30 cycles, each cycle consisting of denaturation (94 °C for 30 s), elongation (72 °C for 120 s) and annealing (55 °C for 60 s). The PCR products were assessed using agarose gels and visualized with ethidium bromide and ultraviolet excitation. Appropriately sized bands were manually removed and then subcloned using TOPO-TA PCR-II (Invitrogen, Carlsbad, CA, USA). Insert-bearing clones were identified, and the corresponding plasmids were purified with QIAquick DNA purification kits (Qiagen, Valencia, CA, USA) and sequenced using the Stanford PAN sequencing facility. On the basis of the sequencing results, additional primers were generated and the process was repeated; in this way, the entire coding region of the FMRFa gene was sequenced. The results presented in this paper represent the consensus sequence from multiple PCR experiments from the GFL and SG cDNA libraries. No differences in sequences were obtained between the two cDNA libraries (GFL and SG), so these libraries were used interchangeably.

Sample preparation for mass spectrometric analysis

The animal was decapitated, and the appropriate ganglia or nerve dissected. Extracellular salts were removed by replacing the sea water with the MALDI matrix solution, 10 mg ml^{-1} 2,5-dihydroxybenzoic acid (DHB) (ICN Pharmaceuticals, Costa Mesa, CA, USA) in deionized water (Garden et al., 1996). A small section of the nerve was placed onto a MALDI sample plate, followed by deposition of 0.5 µl of matrix solution. For the posterior chromatophore lobe (PCL), small numbers of isolated cells were placed on individual spots. The plates were dried at ambient temperature and stored at −20 °C until used for mass spectrometric analysis.

Mass spectrometry

MALDI mass spectrometric analyses were performed using Voyager Elite and Voyager DE STR (PE Biosystems, Framingham, MA, USA) time-of-flight mass spectrometers equipped with delayed ion extraction. A pulsed nitrogen laser (337 nm) was used as the desorption/ionization source, and positive-ion mass spectra were acquired using both linear and reflectron mode with 20 kV acceleration potential. Each representative mass spectrum shown is the average of 128–256 laser pulses. Mass calibration was performed externally using bovine insulin (Sigma, St Louis, MO, USA) and synthetic *Aplysia* α-bag cell peptide (BCP) (American Peptide Co., Sunnyvale, CA, USA).

Results

Direct MALDI MS analysis of different nerve sections from three species was performed to determine the accurate molecular masses of the peptides present in the nerves. Fig. 1 shows representative MALDI mass spectra obtained from various nerves from *Loligo opalescen* (*N*=80 distinct samples with an average of three spectra per sample), *Sepia officinalis* (*N*=60) and *Dosidicus gigas* (*N*=60), giving a total of approximately 600 spectra. Table 1 lists the predominant masses observed. Essentially all peaks in these spectra

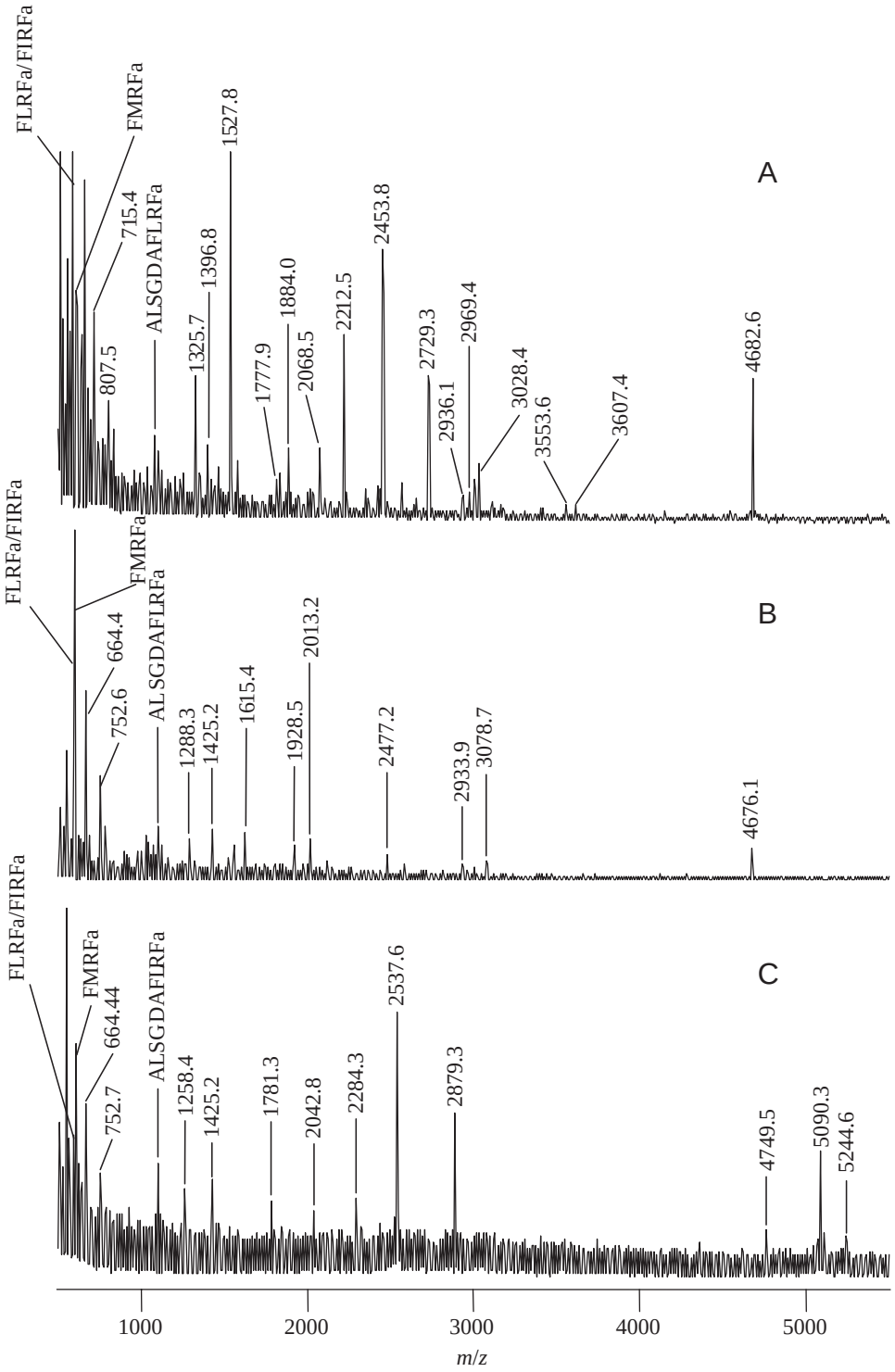

Fig. 1. Representative mass spectra from three species of cephalopod (A) *Loligo opalescens* stellar nerve, (B) *Sepia officinalis* pallial nerve and (C) *Dosidicus gigas* posterior chromatophore lobe. *m*, mass; *z*, charge.

correspond to the protonated molecular masses of peptides, with the exception of a cluster of peaks distributed over *m*/*z* 600–800, where *m* is mass and *z* is charge. This cluster probably corresponds to phospholipids associated with cellular membranes. Presumably because of the great microheterogeneity of the nerves, many individual mass spectra do not show the full complement of peptides that are observed with multiple samples. Hence, the physical location yielding a particular peptide peak has not been designated in Table 1. Fig. 2A shows a representative MALDI mass spectrum of the lower mass range (*m*/*z* 700–850) from a *Loligo opalescens* stellar nerve sample, which contains both glial Schwann cells and neuronal processes, and includes the mass range of many of the expected phospholipids.

While most masses in Table 1 do not correspond to the molecular masses of known peptides, masses corresponding to

Table 1. *Major mass spectral peaks observed in three cephalopod species*

Protonated molecular mass, *m/z*	*Sepia officinalis*	*Loligo opalescens*	*Dosidicus gigas*	Protonated molecular mass, *m/z*	*Sepia officinalis*	*Loligo opalescens*	*Dosidicus gigas*
428.29	x	x	x	1884.0		x	
508.70	x	x	x	2013.2	x		x
522.81	x	x		2042.8			x
542.26	x	x	x	2068.5	x	x	
564.32	x		x	2212.5		x	
581.33	x	x	x	2241.0	x		
599.29	x	x	x	2285.2			x
664.44	x	x	x	2326.2			x
752.71	x		x	2453.8		x	
781.48 (putative lipid)	x			2477.2			x
807.50 (putative lipid)	x	x	x	2537.6			x
887.47	x			2604.4			x
900.67	x			2646.3	x		
975.58	x	x		2729.3		x	
980.11	x			2834.6	x		
1028.6			x	2879.3			x
1037.6	x	x		2930.5	x		
1045.5	x	x	x	2936.1		x	x
1053.6		x		2969.4	x	x	
1079.3		x		3005.5		x	
1095.6	x	x	x	3028.4		x	
1244.6	x			3553.6		x	
1288.3	x			3607.4		x	x
1325.7		x		4009.8	x		
1334.7		x		4676.1	x		
1349.8		x		4682.6		x	
1396.8		x	x	4749.5			x
1425.2	x			5090.3			x
1527.8		x	x	5244.6			x
1560.3	x		x	5441.9		x	
1736.5		x	x	5455.6		x	
1818.4		x	x	8569.6		x	x

*Masses labeled in bold type indicates FMRFamide-related peptides; *m*, mass; *z*, charge.
A cross indicates the presence of a spectral peak.

FMRFa and FLRFa are detected. Two approaches are possible to confirm that these peaks are the FRPs, MS-based sequencing (Li et al., 1999b) and molecular biology. Specifically, we have previously demonstrated that accurate molecular mass measurements provided by MALDI MS combined with gene (prohormone) sequencing allows the confirmation of the expression of specific genes and the identification of peptide processing pathways (Garden et al., 1998; Li et al., 1998).

The detection of putative FRPs in *Loligo opalescens* led us to attempt to characterize the FRP gene. Initial primers were generated on the basis of the published incomplete FMRFa gene from *Loligo pealei* (Chin et al., 1994) and consensus with other published FRP sequences. The initial primers were selected for the fairly conserved FRP amino acid sequences DKRFLRFG and EKKFM, and an antisense primer for MRFGRDP. Both the GFL and SG *Loligo opalescens* libraries were screened using PCR. On the basis of these initial sequencing results, additional exact primers were generated and further PCR and sequencing was performed. In this way, the entire open reading frame was determined and sequenced in triplicate. The consensus nucleotide sequence is shown in Fig. 3. The open reading frame consists of 331 amino acid residues. The presumed signal sequence, predicted using the Signal 1P prediction algorithm (Nielsen et al., 1997), is shown in blue. The open reading frame predicts one copy each of FIRFa, FLRFa and the decapeptide ALSGDAFLRFa and 11 copies of FMRFa. Each predicted peptide sequence is flanked with either monobasic (K or R) or dibasic (KR or KK) sites. The dibasic and monobasic sites are proteolytic processing sites for cleavage of the prohormone into the active peptides, with the peptides between the FRPs being acidic ‘linker’ peptides. In addition, one tetrabasic putative furin cleavage site exists at locations 129–132, after the FLRFa and the first FMRFa. All FRPs end in Gly, which can be enzymatically

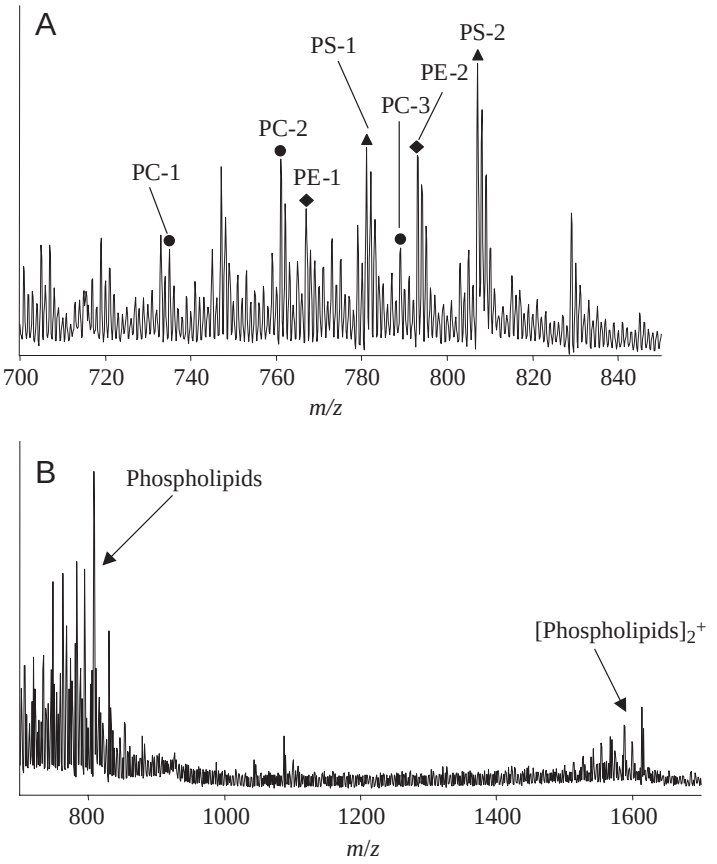

Fig. 2. Representative MALDI mass spectra of phospholipids in the *Loligo opalescens* distal stellar nerve. (A) Spectrum in the *m*/*z* 700–850 range, with peaks labeled as three classes of phospholipids. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Putative assignments are as follows: PC-1 with fatty acid moieties of 16:0 on both carbons; PC-2 with 16:0 on the C-1 and 18:1 on the C-2 positions; PC-3 with 16:0 on the C-1 and 20:1 on the C-2 positions; PE-1 with fatty acid moieties of 18:0 and 20:5 on the C-1 and C-2 positions; PE-2 with fatty acid moieties of 18:0 and 22:6 on two carbons; PS-1 with 16:0 and 20:5 fatty acids on C-1 and C-2; and PS-2 with 16:0 and 22:6 fatty acids on C-1 and C-2. (B) Mass spectrum of phospholipids in the range *m*/*z* 700–1700, showing the detection of dimer peaks. *m*, mass; *z*, charge.

processed into the biologically active amidated FRPs using peptidyl-glycine alpha-amidating mono-oxygenase (Eipper et al., 1992).

On the basis of the FMRFa gene sequence, the peak at *m*/*z* 1095.6 can be assigned to the decapeptide ALSGDAFLRFa. Interestingly, this mass is observed in all three species. Although three putative N-linked glycosylation sites exist in the *Loligo opalescens* FMRFa gene (Fig. 3), neither these linker peptides nor any glycosylated forms of the peptides are detected. This may be because of the lower sensitivity of MALDI MS to glycosylated peptides (because of the heterogeneity of carbohydrate moieties), because of the degradation of the peptides or because these sites are not used.

Discussion

The mass profiles for each individual species are characteristic, with some conserved masses between the species. FMRFa is one of the most well-characterized neuropeptides in cephalopods, with authentic FMRFa having been isolated from *Sepia officinalis*, *Loligo pealei* and *Octopus vulgaris* (Loi et al., 1996; Martin and Voigt, 1987; Chin et al., 1994). Hence, it is not surprising that FMRFa and FLRFa/FIRFa were detected in all three species. Because of the detection of three predicted FRPs including FMRFa and FLRFa/FIRFa, which cannot be differentiated because of their identical molecular masses, and the decapeptide ALSGDAFLRFa, the FMRFa gene in *Loligo opalescens* was characterized and strongly supports these MS assignments.

Recently, Loi and Tublitz (1997) reported on a complete FMRFa gene from *Sepia officinalis*, which shows a surprisingly high degree of homology to the sequence reported here. For example, these two prohormone sequences share an overall identity of 92 %, with all amidated FRPs being 100 % conserved. Not surprisingly, the majority of the sequence differences between these two prohormones occur in the acidic 'linker' peptides. Two potential N-linked glycosylation sites (indicated with arrowheads in Fig. 3) are also conserved, with the *Loligo opalescens* gene containing one additional site. The extra glycosylation site is the last one marked in Fig. 3 starting with Asn225. Recently, a partial sequence for a *Loligo pealei* FRP gene was reported (72 amino acid residues) that contains one copy of FLRFa and four copies of FMRFa (Chin et al., 1994). This partial sequence is nearly identical to the *Loligo opalescens* gene presented here for residues 205–276. As shown in Table 2, there are eight differences in this region of the two *Loligo* sequences, only one of which alters a predicted FRP, the Met210 residue of FMRFa of *Loligo opalescens versus* the Leu19 residue in *Loligo pealei*. The sequence of the corresponding *Sepia officinalis* FRP is also included in Table 2; all predicted FRPs are identical to those of *Loligo opalescens*.

The FRPs found in *Loligo opalescens* are also identified in the *Sepia officinalis* nerve mass spectra when combined with

Fig. 3. Nucleotide sequence of *Loligo opalescens* FMRFa cDNA (1599 base pairs, numbered on the left). The predicted amino acid sequence is shown above the nucleotide sequence (331 amino acid residues, numbered on the right). The predicted signal sequence is indicated in blue, and the monobasic, dibasic and the tetrabasic putative furin cleavage sites are in red. There are 14 FMRFamide-related peptides (red acid residues) and three potential N-linked glycosylation sites (arrowheads above the amino acid residues). GenBank accession number AF303160.

the previous known precursor information (Loi and Tublitz, 1997). On the basis of accurate molecular mass measurements, the same series of FRPs is detected in the PCL nerves of *Dosidicus gigas*. Although the gene encoding these peptides has not been characterized in this species, the MS data suggest that the *Dosidicus gigas* FMRFa gene is highly homologous to those in *Sepia officinalis* and *Loligo opalescens*. The existence of the enzyme peptidyl-glycine alpha-amidating monooxygenase in all three species is inferred because the molecular masses measured by MALDI MS listed in Table 1 are consistent with the removal of Gly and the presence of the amidated FRPs.

Table 2. *Comparison of a section of the FMRFa gene sequence from Cephalopods*

A	Pro Glu Lys Arg Phe Met Arg Phe Gly Lys Ser Asp Asp Lys Arg Phe	219
B	Glu Lys Arg Phe **Leu** Arg Phe Gly Lys Ser Glu Asp Lys Arg Phe	15
C	Pro Glu Lys Arg Phe Met Arg Phe Gly Lys Ser Asp Asp Lys **Lys** Phe	219
A	Met Arg Phe Gly Arg Asn Pro Ser Asp Ala Glu Asp Glu Leu Glu Glu	235
B	Met Arg Phe Gly Arg **Asp** Pro Ser Asp **Val** Glu Asp Glu Leu Glu Glu	31
C	Met Arg Phe Gly Arg Asn Pro **Gly** Asp Ala Glu Asp Glu Leu Glu Glu	235
A	Asp Lys Arg Phe Met Arg Phe Gly Arg Gly **Gly** Glu Asp Asp Glu Glu	251
B	Asp Lys Arg Phe Met Arg Phe Gly Arg Gly **Ala** Glu Asp Asp Glu Glu	47
C	Asp Lys Arg Phe Met Arg Phe Gly Arg Gly **Asp** Glu **Glu** Asp Glu Glu	251
A	Glu Ala Glu Lys Arg Phe Met Arg Phe Gly Arg Asp Pro Glu Lys Lys	267
B	Glu Ala Glu Lys Arg Phe Met Arg Phe Gly Arg Asp Pro Glu Lys Lys	63
C	Glu Ala Glu Lys Arg Phe Met Arg Phe Gly Arg Asp Pro Glu Lys Lys	267
A	Phe Met Arg Phe Gly Lys **Ser** Gly Glu **Asp** Lys Arg Phe Met Arg Phe	283
B	Phe Met Arg Phe Gly Lys **Arg Phe Met**	72
C	Phe Met Arg Phe Gly Lys **Asn** Gly Glu **Glu** Lys Arg Phe Met Arg Phe	283

A, *Loligo opalescens* FMRFa gene sequence from Fig. 3; B, partial *Loligo pealei* sequence from Chin et al. (1994); C, *Sepia officinalis* FMRFa gene sequence from Loi and Tublitz (1997).

Residues that differ among species are printed in bold type.

Underlined sequences are FMRFamide-related peptides.

The decapeptide ALSGDAFLRFa is identical in all three species. The extended forms of FRPs can exhibit different biological activities compared with the shorter forms (Henry et al., 1999; van Golen et al., 1995; Lange et al., 1995). While the opisthobranch and pulmonate molluscs do not appear to contain a decapeptide FRP, ALSGDAFLRFa is similar to the decapeptides isolated from the bivalve *Mytilus edulis* (ALAGDHFFRFa) and the prosobranch *Fusinus ferrugineus* (ALTNDHFLRFa) (Price and Greenberg, 1994), indicating the widespread distribution of such an extended decapeptide FRP throughout the Mollusca. Several FRPs have been isolated from *Octopus vulgaris* (Martin and Voigt, 1987), including AFLRFa, which has the same five C-terminal amino acid residues as the squid peptide ALSGDAFLRFa. This peptide was isolated biochemically, and it may therefore represent a proteolytically degraded form of a peptide related to the decapeptides from *Sepia officinalis*, *Loligo opalescens* and *Dosidicus gigas*.

The existence of the tetrabasic furin-processing site in this prohormone is intriguing as this indicates the possibility of packaging the N-terminal FRPs (FLRFa, FIRFa and ALSGDAFLRFa) into one set of vesicles and of the multiple copies of FMRFa into another set. Such differential packaging of gene products has been suggested for *Lymnaea stagnalis* FMRFa gene products (Santama et al., 1993; Favrel et al., 1998) and demonstrated for the distinct egg-laying hormone prohormone gene-products from *Aplysia californica* (Sossin et al., 1989) and *Lymnaea stagnalis* (Li et al., 1994). While using MS, we occasionally observed molecular masses corresponding to FLRFa/FIRFa/ALSGDAFLRFa peptides in distinct nerve samples compared with the FMRFa. As this may be due to sampling artifacts, this point needs further work to confirm such a differential packaging/distribution hypothesis.

In addition to the FRPs, peaks at *m*/*z* 428.3, 508.7, 542.3, 664.4 and 807.5 are also present in all three species. Some other putative peptides are common in two species, whereas some peaks are unique for one species. For example, a mass spectral peak at *m*/*z* 1425 is present in both the pallial nerve from *Sepia officinalis* and the PCL from *Dosidicus gigas* but it is absent from *Loligo opalescens*. This may be an interesting peptide for further characterization. In all three species, there is a peak around *m*/*z* 4600–4700, with slightly different molecular masses. This may indicate a homologous peptide with a few amino acid substitutions or modifications. Because of the limitations of direct MS profiling and the need to work with larger number of animals to sequence a peptide of this molecular mass, further characterization was not attempted at this time.

Although most of the peaks in the mass region of *m*/*z* 1000–5000 can be attributed to peptides, a group of peaks ranging from *m*/*z* 500 to *m*/*z* 900 are often observed in cellular samples, especially in nerve tissues, and are generally attributed to phospholipids (Li et al., 1998; Garden and Sweedler, 2000). We attempted to determine whether the assignment of these low-molecular-mass peaks to lipids is correct by accurate comparisons with expected lipids in this model system. The squid giant axon has proved a useful preparation for examining the physiological and biochemical properties of axonal processes such as impulse propagation, axonal transport and their link to behavior (Baker, 1984; Preuss and Gilly, 2000; Neumeister et al., 2000), and an understanding of the role of lipids in the function of this nerve is of considerable importance. An essential step towards this goal is to examine the molecular composition of these lipid components of the squid giant axon. Accordingly, several studies have investigated lipid metabolism in various regions of squid giant nerve fibers and characterized lipid composition in different squid nerve tissues (Camejo et al., 1969; Tanaka et al., 1987; Yamaguchi et al., 1987). More recently, several reports describe the use of the MALDI MS technique for lipid

analysis (Harvey, 1995; Marto et al., 1995; Schiller et al., 1999), although the direct profiling of lipids from neuronal tissues has not been reported.

Many of the peaks in the mass region *m*/*z* 700–850 correspond to the calculated masses of the known phospholipids in *Loligo opalescens*. Among these peaks, seven match the calculated values within 10 p.p.m. and are labeled in the spectra with different symbols indicating three classes of phospholipid (Fig. 2A). By matching up many of these peaks, phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine with fatty acid moieties ranging from 16:0 to 22:6 appear to be the major lipid components in these nerve tissues, which is consistent with the findings of prior studies (Yamaguchi et al., 1987). On the basis of these assignments, it is interesting to note that phosphatidylethanolamine and phosphatidylserine are enriched with polyunsaturated fatty acids, mainly 20:5 and 22:6. Phosphatidylcholine, in contrast, consists of mostly saturated or mono-unsaturated fatty acids. For example, a spectral peak at *m*/*z* 792.554 is assigned to a phosphatidylethanolamine containing 18:0 and 22:6 fatty acid moieties, and the intense signal at *m*/*z* 807.504 can be attributed to a phosphatidylserine with fatty acids on two carbons being 16:0 and 22:6, respectively. However, because MS/MS analysis is required for unambiguous assignment, these assignments must be regarded as tentative. The lipids are present in such significant amounts that dimers of these lipid cluster peaks are observed (Fig. 2B).

Our assignment of the low-mass envelope of peaks to phospholipids remains a strong possibility on the basis of these analyses. Because of the ubiquitous presence of phospholipids in tissue samples, one has to exercise caution when interpreting the mass spectral peaks observed in the low-mass region. The ability of MALDI to characterize lipids directly from a nerve should aid future lipid compositional studies. The combination of MALDI MS with other conventional biochemical methods enables the rapid characterization of a wide variety of substances across species and thus provides a powerful tool for comparative biology.

In summary, we have demonstrated that MALDI MS molecular mass profiling allows a large number of putative peptides and phospholipids to be detected in several species of cephalopod. The detection of numerous FRPs in these species confirms the widespread and highly conserved structure of this peptide family. Furthermore, a new FMRFa gene has been characterized, and its expression in the tissue samples has been confirmed.

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