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Review

FMRFamide and related peptides in the phylum mollusca

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ABSTRACT

FMRFamide is one of the well-known peptides studied within the phylum Mollusca. It was first isolated from the clam *Macrocallista nimbosa* during the end of the 1960s. Since then, a number of reports related to FMRFamide have been published from different experimental approaches, revealing that it and its related peptides (FaRPs) are implicated in a variety of physiological processes. As this year is the 30th anniversary since its discovery, this review focuses on diverse findings related to both FMRFamide and FaRPs in the phylum Mollusca.

Contents

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1. Introduction

This year is the 30th anniversary of the isolation from the Sunray Venus clam *Macrocallista nimbosa* of a substance with an important activity in mollusk cardiac muscle. This substance, designated peak C by Frontali et al. [29], was further characterized and named FMRFamide peptide (Phe-Met-Arg-Phe-NH_2) by Price and Greenberg based on its amino acid sequence [61]. This tetrapeptide amide is only found in Mollusca since FMRFamide itself has not been identified in other phyla. However, other FMRFamide-related peptides (FaRPs) are present in both invertebrate and vertebrate phyla such as Nematoda, Annelida, Arthropoda, and Chordata, and these peptides are also known as RFamide or RF-NH_2 peptides.

This review covers the last three decades of FaRPs research in mollusks. It is now clear that much has been achieved regarding this peptide family, and there is much more work to be done in the future. We have divided the presentation of the findings in five sections, corresponding to the molluskan Classes in which the peptides have been found, and following a chronological order based on the first discovery within each Class. We have focused on the isolation, characterization, localization, as well as the activity of this class of peptides. We have not dealt in detail with the structure of the genes that code for FMRFamide and FaRPs, and the differential splicing and distribution of the corresponding mRNAs, which are included in the recent, elegant review by Di Cosmo and Di Cristo [24].

2. Discovery of FMRFamide

By the end of 1960s, the existence of some neurohumors from several specimens of mollusks was well known, including acetylcholine, serotonin, and dopamine. However, there was a component named peak C seen by chromatography of ganglion extracts that had cardioexcitatory activity. It was not until further characterization by Price and Greenberg using different separation techniques such as gel filtration and ion-exchange chromatography, and sequencing by dansyl Edman degradation after peptidase digestion that the full characterization of this neurohormone was completed [61], revealing a tetrapeptide with 3 standard amino acids and Phenylalanine at the 1st and 4th positions (Phe-Met-Arg-Phe-NH_2), named FMRFamide peptide.

The first attempts to assay the activity of this peptide were to apply either natural or synthetic FMRFamide on an isolated ventricle of clam *Mercenaria mercenaria* and on the radular protractor muscle of *Busycon contrarium*. Both preparations showed a response with a threshold about 10^{-8} and 10^{-9} M, respectively. Since then, a great number of FMRFamide-related peptides have been found throughout the animal kingdom, where they are involved in many behaviors [for reviews, see 24, 59].

3. Localization of FMRFamide in mollusks

Since the discovery of FMRFamide peptide as a potential neuropeptide in both invertebrate and vertebrate species, considerable work has been done to locate the structures or tissues where FMRFamide or FaRPs are present. It should be noted that the antisera used for these studies are not completely selective and do not necessarily distinguish between FMRFamide and certain FaRPs. Despite this, tests for FaRPs by radioimmunoassays (RIA) and immunohistochemistry (IH) have been reported in almost all major animal phyla, and FMRFamide is apparently limited to mollusks. Besides the discovery of FMRFamide in ganglion extracts from the clam *M. nimbosa*, this peptide is also found in every major Class of mollusks including other Bivalvia, Gastropoda, Cephalopoda, Polyplacophora, and Scaphopoda. For amino acid sequences of the FaRPs mentioned in the different molluskan Classes see Table 1.

Table 1 – The FMRFamide-related peptides of mollusks

Peptide	Class/order	Reference
The tetrapeptides		
FMRF-amide	Bivalvia, Cephalopoda, Gastropoda, Polyplacophora	[44,48,68,76]
FLRF-amide	Gastropoda, Cephalopoda	[44,48,68]
FIRF-amide	Cephalopoda	[76]
The pentapeptides		
NFLRF-amide	Bivalvia	[58]
AFLRF-amide	Cephalopoda	[52]
TFLRF-amide	Cephalopoda	[52]
TIFRF-amide	Cephalopoda	[47]
EFLRI-amide	Gastropoda	[44,68]
pQFYRI-amide	Gastropoda	[44,68]
pQFYRF-amide	Gastropoda	[58]
The hexapeptides		
GNLFRF-amide	Cephalopoda	[47]
GSLFRF-amide	Cephalopoda	[47]
NSLFRF-amide	Cephalopoda	[47]
HDYMRF-amide	Gastropoda	[68]
SDPYLR-amide	Gastropoda	[68]
The heptapeptides		
GDPFLRF-amide	Gastropoda	[68]
SDPFLRF-amide	Gastropoda	[48,68]
NDPFLRF-amide	Gastropoda	[48]
SKPYMRF-amide	Gastropoda	[68]
NDPYLRF-amide	Gastropoda/ pulmonata	[48]
pQDPFLRF-amide	Gastropoda/ pulmonata	[48]
pQDPFLRI-amide	Gastropoda/ pulmonata	[48]
SEPYLRF-amide	Gastropoda/ pulmonata	[48]
The decapeptides		
ALSGDAFLRF-amide	Cephalopoda	[76]
ALAGDHFFRF-amide	Gastropoda	[47]
ALTNDHELRF-amide	Bivalvia	[47]
Other peptides		
ENNNGYIRF-amide	Gastropoda	[48]
GPMGWVPVFYRF-amide	Gastropoda	[50]
SYGWAFGDTTDEYLRF-amide	Gastropoda	[48]

The one-letter code is employed for standard amino acids. For post-translational modifications: pQ, pyroglutamic acid; -amide, amidated C-terminus.

4. FMRFamide and FaRPs in Bivalvia

As mentioned above, in 1977, FMRFamide was isolated and characterized from ganglion extracts of *M. nimbosa* by Price and Greenberg [60,61]. Four years later Nagle demonstrated, by RIA and electron micrographs, the presence of RIA-positive FMRFamide-like material in neurosecretory granules from ganglia of *M. nimbosa* [56]. These findings were the first support for the notion that FMRFamide was a neurosecretory product. Although the activity of FMRFamide was first demonstrated in the heart of *M. mercenaria,* little was known about the distribution of FMRFamide and RF-NH_2 peptides in this species. Deaton et al. [20] confirmed the presence of FMRFamide and FLRFamide in extracts of the bulbus arteriosus organ of *M. mercenaria* by RIA.

Stefano and Martin in 1983 studied the distribution of enkephalin-like peptides by immunoreactivity in the pedal ganglion of the bivalve *Mytilus edulis* [75]. Their findings showed a colocalization of enkephalin-like and FMRFamide immunoreactivity, particularly in intraganglionic regions. Using techniques of molecular biology Price and Greenberg isolated a decapeptide FaRP from the mussel *M. edulis* (ALAGDHFFRFamide) and also from *Geukensia demissa* [62]. The full-length precursor encoding this FaRP together with some other FaRPs including FMRFamide peptide was cloned from *M. edulis* by Pascal et al. [58].

Ram et al. [66] demonstrated positive immunoreactivity against FMRFamide in both the central and peripheral nervous system of the zebra mussel *Dreissena polymorpha*. RIA-positive material was plentiful in all ganglia and fibers in mantle and siphon regions.

FMRFamide has also been immunohistochemically localized in the cerebral, pleuropedal, and visceral ganglia of the sea scallop *Placopecten magellanicus* by Too and Croll [78].

5. FMRFamide and FaRPs in Gastropoda

Much more work related to FMRFamide has been done in gastropods than in other major Classes of mollusks. In 1981, after FMRFamide was identified in the bivalve *M. mercenaria*, Cottrell et al. found FMRFamide-like activity in ganglia of the pulmonate snail *Helix aspersa* [15]. Its existence, together with a novel hepta-FaRP (pQDPFLRFamide), was confirmed 4 years later by Price et al. [63]. By 1987 Lehman and Price had observed FaRP immunoreactivity in the ganglia, male reproductive organs, and digestive tract of *H. aspersa* [41], confirming previous results of Marchand and Dubois [51] and Cardot and Fellman [11]. Two more species of the genus *Helix* have been found to contain FMRFamide: it occurs in nerve fibers of the mid-intestinal gland of the snail, *H. lucorum,* and in ganglia of *H. pomatia* [43,67].

Continuing with the description of work done in pulmonate snails, many studies have been done in the great pond snail *Lymnaea stagnalis.* Schot and Boer [70,71] showed immunoreactivity in nerve fibers of both the central and peripheral nervous system. Ebberink et al. [27] biochemically characterized an extract of the central nervous system and found that it contained 20% FMRFamide and 80% other FaRPs. Further characterization was done by Buckettet al. [7], who reported that heart motoneurons contain FaRPs. Skingsley et al. [73] identified FaRPs in cardiorespiratory interneurons by in situ hybridization and later using antibody against FaRPs. In 1987, Price et al. [64] showed evidence for the presence of an hepta-FaRP in the limpet *Siphonaria pectinata* using extracts of the whole animal. The FMRFamide locus in *L. stagnalis* is one of best characterized, and its organization, expression and physiological implications have been elegantly reviewed by Santama and Benjamin [68].

Aplysia is the second Genus of gastropods that has been extensively examined for the presence of FMRFamide. Found first in *Aplysia brasiliana* was the small cardioactive peptide (SCP) [54], which shares some sequence similarity with FMRFamide. Lehman et al. [42], using RIA and IH in parallel, clarified the occurrence of FMRFamide in the pleural, pedal, buccal cerebral, and abdominal ganglia of *A. brasiliana*, with the highest concentration in the pedal and pleural ganglia.

Schaefer et al. [69] also demonstrated immunoreactivity for FMRFamide-like peptides in many neurons throughout the sea-slug *Aplysia californica* nervous system, and they isolated and characterized a cDNA clone; the cDNA encodes a precursor for 19 copies of FMRFamide. In the same year, Brown et al. [6] detected positive immunocytochemical signals in large numbers of neurons in the abdominal ganglia. Using molecular biology techniques, DesGroseillers et al. [22] identified FaRPs in *A. californica*. In 1995 the presence of both FMRFamide and FaRPs in the heart of *A. californica* was investigated by IH localization, high performance liquid chromatography, and RIA. A higher density of FaRP-immunoreactivity was found in the atrium and atrioventricular as compared with the ventricle [33]. In general FMRFamide is widely distributed in the *Aplysia* nervous system.

The presence of FMRFamide or FaRPs has been reported in a number of other gastropod species, including: *Helisoma trivolis*, where FaRPs were detected in kidney, skin, mantle, hemolymph, ganglia, and salivary gland [8,49,55]; in *Helisoma duryi,* immunoreactive neurons were mostly in the nervous system, but also in kidney [37]; *Pomacea paludosa*, where FLRFamide represented between 10 and 20% of the total FMRFamide-like immunoreactivity [59]. In *Limax maximus*, FaRPs were identified in all ganglia of the central nervous system and also in peripheral buccal nerve roots [14]; in *Haliotis asinina* FaRPs were localized in the cerebral, pleuropedal, and visceral ganglia [57]; in *Achatina fulica*, FMRFamide immunoreactivity was found in cerebral ganglia and in the heart: in the atrium and the aortic end of the ventricle [30]; in *Melampus bidentatus*, FMRFamides were present in cerebral and visceral ganglia and almost all other parts of the body such as kidney, reproductive tract, hemolymph, and connective tissue [38]; in *Viviparus ater*, immunoreactivity against FaRPs was found in early embryonic development in the nervous system and extended to the digestive tract and endocrine cells [28].

In *Cepea nemoralis*, using paper chromatography as well as immunoreactivity, FMRFamide was detected in neurons of the stomach wall, nerves of the visceral ganglion, and the nerve from the buccal ganglia [67]; in *Phestilla sibogae*, FMRFamide was present in cerebropleural ganglia, pedal ganglia, peripheral nervous system, and early larva [17,18,19]. FaRPs were present in veliger stages on the second day after the eggs were laid. The immunoreactivity for FaRPs was limited to neuro-

pilar regions where the cerebropeural ganglion will develop; furthermore, on subsequent days FaRP-positive cells were detected in additional areas implicated in the development of other ganglia. Therefore, FaRPs are involved in the development of the central nervous system [17].

In 2002, biochemical studies by Maillo et al. identified the first FaRP purified from any venom [50]. The peptide (GPMGWVPVFYRFamide) was named conorfamide-Sr1 (CNF-Sr1) because of its origin from a cone snail (*Conus spurius*), and the sequence of its two C-terminal residues (-RFamide). This peptide is one of the major components found in the venom duct of this predator snail. These authors discussed that the presence of CNF-Sr1 in the venom could be the result of the expression in the venom duct of a gene that could also be expressed, for endogenous purposes, in other tissues. However, in the venom, CNF-Sr1 might be involved in the envenomation of the prey, which is polychaete worms (Annelida), in the defense from predators, or in competitive interactions with other carnivorous snails. Given the high sequence identity of the four C-terminal residues of CNF-Sr1 (-FYRFamide) with FMRFamide, these authors compared the biological effects of these peptides in mice. Both CNF-Sr1 and FMRFamide elicited similar hyperactivity syndromes when injected intracranially (at doses higher than 100 pmol/g) in animals $>$ 16 days old, but they had no activity in mice 11–13 days of age. Recently, another 12-residue conorfamide was isolated from *C. spurius*. This peptide, conorfamide-Sr2 (CNF-Sr2), is the first FaRP in which glutamate residues have been found to be post-translationally modified to gamma-carboxyglutamate [1]. CNF-Sr2 has biological activity in marine and freshwater mollusks and in the mouse, which might be explained by its sequence similarity with FaRPs expressed in these organisms. In addition, CNF-Sr2 has sequence similarity with FaRPs from polychaetes that affect the longitudinal muscles of these organisms, which might be related to its biological function (for example, by hampering the movements of the prey).

6. FMRFamide and FaRPs in Cephalopoda

The tetrapeptide was discovered, in 1987, by Martin and Voigt in the neurosecretory system of vena cava of *Octopus vulgaris* [52] and by Le Gall et al., in 1988, in the CNS of *Sepia officinalis* [40].

Chin et al., in 1994, demonstrated clear evidence of FMRFamide and FaRPs present in the optic lobe of the squid *Loligo pealei* using RIA and radioligand receptor assays. The same authors reinforced their results by the detection of a gene encoding the FMRFamide precursor protein [12]. However, unidentified peaks of FMRFamide reactivity were also detected, and they bound to both the FMRFamide antiserum and the receptor [12]. Such peaks, which correspond to FaRP homologs, occur in all mollusks. FaRPs were also found in the optic lobe of *O. vulgaris* by Martin and Voigt [52].

Di Cosmo and Di Cristo [23] and Di Cristo et al. [25] demonstrated the presence of FMRFamide both in the CNS and PNS of *O. vulgaris* by immunohistochemistry. Moreover they isolated from the CNS of *O. vulgaris* an FMRFamide cDNA containing several FMRFamide-related peptides (FaRPs) showing a high degree of identity with the FaRPs encoded in the precursor of *S. officinalis*.

In the CNS the tetrapeptide is involved in the inhibition of the secretory activity of the optic gland which controls in turn the gonads maturation. FMRFamide is present in several lobes of *O. vulgaris* CNS, such as optic, subpedunculate and olfactory lobes, that integrate and relay the olfactory, chemical and visual inputs to the optic gland in order to modulate its secretory activity. Indeed, stimulation of retina isolated from the eyes of *O. vulgaris* demonstrated that this tetrapeptide, coupled with dopamine, induces an extreme adaptation of the retina to the light condition. This situation *de facto* inhibits sexual maturation. [23,26].

In the PNS Henry et al. [35] demonstrated, using a myotropic assay, the role exerted by FMRFamide and FaRPs in the peptidergic control of egg laying in *S. officinalis*. Di Cristo et al. [25] showed the presence in the fusiform ganglion of *O. vulgaris* of immunoreactive neurons and fibres that innervated the reproductive ducts of female and male. Both these findings support, or regulates secretory products such as mucus and mucilaginous substances from the oviducal gland and the seminal vesicle [25].

Unlike traditional RIA and IH analysis to find FMRFamide or FaRPs, Sweedler et al. [76] used techniques of mass spectrometry to analyze different nerve sections (stellate ganglion and giant fiber lobe) from three species of cephalopods: *S. officinalis, Loligo opalescens,* and *Dosicus gigas*. Their mass spectrometric profiling together with gene cloning confirmed the existence and expression of FaRPs in these species.

7. FMRFamide in Polyplacophora

Using immunocytochemical techniques, in 1994, Moroz et al. [53] demonstrated FMRFamide reactivity in neurons at the pedal region of the chiton *Lepidopleurus asellus*. Haszprunar et al. [34], looking at the sensory system called the ''ampullary system'' of the larval stages of different chiton species (*Chiton olivaceus, Lepidochitona* aff. *corrugata*, *Mopalia muscosa*) found positive immunoreactivity against FMRFamide in *M. muscosa*.

8. FMRFamide in Scaphopoda

Less work has been reported in this Class of mollusks; nevertheless Wanninger and Haszprunar [80], using confocal laser scanning microscopy, showed that FMRFamide is present in larval stages during the development of the nervous system of *Antalis entalis*.

9. Physiological implications of FMRFamide

FMRFamide and FaRPs are involved in numerous physiological processes that are not exclusive to mollusks; their roles extend to other phyla such as Nematoda, Arthropoda, and Chordata, among others. It is well known that these peptides act predominantly on excitable tissues such as cardiac muscle and nerve, as seen in the classic experiments done by Price and Greenberg in 1977 where application of FMRFamide

augments the contractile force in cardiac muscle and prolongs contraction in the radula protactor muscle of *M. mercenaria* and *B. contrarium*, respectively [61]. Since FMRFamide increases and extends the contractions of molluskan hearts, remedies arrhythmias and starts beating in inactive hearts, it was nicknamed ''the molluscan cardioexcitatory peptide'' [65]. In mammals, FMRFamide has been studied as a pain modulator based on its ability to elicit hyperalgesia when is injected intracerebroventricularly in rats [77,82].

Among the functions of FMRFamide and FaRPs in mollusks are the following: the modulation of sensory organs, reproduction, motility, osmoregulation, feeding, and neurogenesis. For example, in *P. sibogae*, Croll et al. [19] speculated that the expression of FaRPs in patches in rhinophore fibers (glomerulus structures) may be involved in local processing of distant stimuli for olfactory processes; in the same study FaRPs were also observed in oral tentacles, where their effects relate to relaxation and/or muscular contractibility. Based on these muscular modulation findings, Weiss et al. [81] examined the effect of FaRPs in isolated gill preparations of *A. californica*. An application of FMRFamide at the threshold level of 3×10^{-9} M elicited gill contractions and was 10-fold more potent than dopamine (EC_{50}, 10^{-7} M). This study confirms previous results obtained by Voshart and Lukowiak, where the application of FMRFamide increased the amplitude of the gill withdrawal reflex and prevented its habituation [79]. In *Dreiseena polymorpha*, FMRFamide caused contraction of siphon and mantle preparations at concentration similar to and even lower than acetylcholine. The mechanism of action of FMRFamide differs from that of acetycholine, since the contractile response was unaffected by the presence of the cholinergic blocker hexamethonium bromide (HxBr) [66]. In *A. californica*, biphasic, prolonged excitatory/inhibitory responses were seen by exogenous application of FMRFamide onto the siphon neurons. More surprising is the fact that the excitatory response was seasonally dependent. During the summer, the excitatory response showed an increase of incidence compared with the rest of the year; this result apparently was not temperature dependent [4,5].

Loi and Tublitz [47] presented evidence for actions of four endogenous FaRPs including FMRFamide. Their effects on the expansion of the brown chromatophores in *S. officinalis* occurred with different thresholds: FLRFamide (10^{-9} M) was the most potent followed by ALSGDAFLRFamide (10^{-8} M), FMRFamide, and FIRFamide (10^{-7} M). The direct physiological evidence of the action exerted by FMRFamide on the inhibition of screening pigment migration of the retina of *O. vulgaris* in combination with dopamine demonstrate the role played by this tetrapeptide on the control of the reproduction. Indirect evidence suggests that FMRFamide negatively modulates optic gland activity, thereby inhibiting sexual maturity [26]. Madrid et al. [49] exposed osmoregulatory tissues to hyposmotic and isosmotic conditions. Kidney and mantle tissues contained low levels of FaRPs (1.5–2.0 pmol/pg protein), while isosmotic conditioning increased the level of FaRPs in both tissues (to 3.0 and 5.5 pmol/pg protein, respectively). Taken together with autoradiographic studies, these data indicate the existence of two types of neurosecretory cells in the visceral and parietal ganglia whose axons terminate in close proximity to the smooth muscle of the kidney, and they support a role for FaRPs in osmoregulation since they are acting as diuretic and antidiuretic hormones [37,38].

Feeding behavior consists of a number of different rhythmic motor patterns, including biting, swallowing, and rejection, mediated by networks that can generate rhythmic outputs known as central pattern generators (CPG). Although this behavior seems to be a complicated process, it is one of the best understood in *Aplysia* [21,39]. Various techniques, principally electrophysiology and immunoreactivity, have helped to elucidate the role of FaRPs in the feeding behavior; in this regard, FMRFamide inhibits the closure of the radula muscle while its activation is carried out for the biogenic amine (5-hydroxytryptamine) [2,72,74].

10. FaRPs target ion channels

Several G-protein-coupled receptors have been identified as receptors for the mammalian and molluskan FaRPs [24]. However, another molecular target in mollusks is the FMRFamide-gated receptor/channel protein (FaNaCh). The first evidence for this FaNaCh was seen by Cottrel et al. [16] in single channel recordings of isolated neuron membranes of *Helix aspersa*. Application of FMRFamide elicited inward currents even in the presence of guanosine, which blocks the G-protein-coupled receptors. These data indicated the existence of a new, ligand-gated channel. Five years later, the FMRFamide-gated sodium channel (FaNaCh) gene was cloned by Lingueglia et al. [45]. It is now known that FaNaCh belongs to the amiloride-sensitive sodium channel and degenerin family ion channels (DEG/ENaC).

Heterologous expression in *Xenopus* oocytes of FaNaCh from different species of gastropods such as *H. aspersa, A. californica*, and *H. trivolvis* displays a fast depolarizing response to FMRFamide application via activation of a sodium conductance [4,16,36]. Similarly, FMRFamide-activated currents have been seen in native tissues such as C2 neurons of *Helix*, as well as in dopamine neurons and serotonin neurons in the pedal ganglia of *H. trivolvis* [31,32]. The implications of the fast depolarization evoked by FMRFamide are unclear, but its action on FaNaCh is comparable to the activation of other ligand-gated channels; therefore, FaNaCh may be involved in synaptic processes [46].

In *Aplysia*, FMRFamide modulates the probability of opening and closing of S-type K^+ channels in sensory neurons. This modulation is mediated in part by second messengers [3,9]. In the same type of neurons chloride currents are evoked by FMRFamide via the cGMP cascade [10].

FMRFamide can also regulate spontaneous and evoked excitatory post-synaptic currents as well as inhibitory post-synaptic currents (sIPSCs), depending the region, in the optic lobe of *S. officinalis* [13].

11. Conclusion

In this work, we review the findings regarding FMRFamide and FaRPs found in mollusks in the 30 years of research since FMRFamide was fully characterized by Price and Greenberg in 1977. As mentioned throughout the text, FaRPs participate in a

variety of physiological processes, including basic life functions, such as feeding, reproduction, development and osmoregulation. Many of the processes where FMRFamide and FaRPs operate are well known, but others need to be clarified, for example the presence of the FaRPs named conorfamides, from *Conus spurius*; these peptides, isolated from the venom duct may be useful as an offensive agent to ease in the capture of the prey (such as by interfering with the movements of a fleeing polychaete), as a defensive substance against predators, or as a chemical resource to overcome competitors; another possibility is that they are involved in self-regulatory mechanisms of contraction and relaxation of the proboscis, a structure used to locate and inject the prey.

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