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Immunohistochemical Localization and Ca^{2+}-Dependent Release of *Mytilus* Inhibitory Peptides in the ABRM of *Mytilus edulis*

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ABSTRACT—Immunohistochemical localization of *Mytilus* inhibitory peptides (MIPs) in the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* was investigated by using the anti-MIP polyclonal antibody. The antibody was shown to recognize the seven members of the MIP family that had been previously isolated from the ABRM extract. The MIP-like immunoreactivity was found abundantly in neuronal fiber-like structures in the ABRM and in the connective tissue sheath covering it. The immunoreactive fibers in both areas were rich in varicosities. In addition, it was demonstrated that the MIP-like immunoreactivity was released from the neuromuscular preparation to the bathing solution in response to the repetitive electrical pulses applied to the pedal ganglion. The release was Ca^{2+} dependent. These findings suggest that the seven MIP-family peptides, originally isolated from the muscle extract, are the inhibitory neuropeptides involved in physiological regulation of the ABRM contraction.

INTRODUCTION

A numerous number of neuropeptides have been isolated from various animal tissues in the last two decades. Evidently many more peptides than we imagined before are involved in physiological regulation of various systems. The anterior byssus retractor muscle (ABRM) of the bivalve mollusc, *Mytilus edulis*, seems to offer a good example of this multiple peptidergic regulation. Previous work has shown that the muscle is regulated by the excitatory neurotransmitter, acetylcholine (ACh), the relaxing transmitter, 5-hydroxytryptamine (5-HT), and several other modulators like dopamine and octopamine (for reviews, Muneoka and Twarog, 1983; Muneoka *et al.*, 1990). In addition to these substances, many peptides have been suggested to be involved in muscle contraction. The first neuropeptides isolated from the pedal ganglia of *Mytilus* were catch-relaxing peptide (CARP) which has a potent relaxing effect on catch tension of the ABRM (Hirata *et al.*, 1987) and two *Mytilus* inhibitory peptides (MIPs) which inhibit electrically induced, ACh-induced, and FMRFamide-induced contractions (Hirata *et al.*, 1988, 1989). Subsequently, from the acetone extract of the ABRM, thirteen peptides (including CARP and the two MIPs) which show excitatory, inhibitory, and relaxing effects on the muscle contractions have been isolated and sequenced (Fujisawa *et al.*, 1991, 1992, 1993a, b). Among them are as many as seven members of the MIP family (Table 1). They all possess the conserved C-terminal structure, -Pro-Xaa-Phe-Val (or Ile)-amide, and show similar inhibitory effects on the muscle contractions. Since they were purified from the muscle extract, they have been suggested to be peripherally acting neuropeptides. However, no direct evidence supporting their physiological involvement has been obtained so far.

It is of great interest to know the physiological significance of as many as seven homologous peptides present in the same muscle. Why are so many homologous peptides required in controlling a simple target? To clarify the mechanism by which a single target is controlled by multiple family peptides, various simple systems should be established as experimental models. Study on physiological roles that each member plays will then be possible. In the present study, an attempt was made to establish that the seven, or possibly more, MIP-family peptides are the neuropeptides involved in regulation of the ABRM contraction. First, neuronal localization of the MIP-like immunoreactivity is demonstrated using the polyclonal antibody which recognizes all the seven MIPs. Then the Ca^{2+}-dependent release of the MIP-like substances in response to neural stimulation is also described.

MATERIALS AND METHODS

Animals

Mytilus edulis (6-7 cm in shell length) was collected from the Hiroshima Bay and kept in the aerated artificial seawater (ASW) at 10-15°C. Specimens within a week of collection were used in all experiments.

Antibody preparation

The anti-MIP antiserum, named WM1, was raised by a commercial source (Immunobiological Laboratories, Gunma, Japan). Briefly, rabbits were immunized with synthetic MIP_1 (GSPMFVamide) conjugated to keyhole limpet haemocyanin (KLH) by glutaraldehyde. After seven injections of the immunogen, the serum was collected

Table 1. Seven members of the MIP family in the ABRM of *Mytilus*. Amino acid residues are shown by one letter abbreviations. MIP_1 and MIP_2 are found in both the ABRM and the pedal ganglion

Peptide	Sequence	References
MIP_1	GSPMFVamide	Hirata *et al.* 1988; Fujisawa *et al.* 1991
MIP_2	GAPMFVamide	Hirata *et al.* 1988; Fujisawa *et al.* 1991
MIP_3	DSPLFVamide	Fujisawa *et al.* 1991
MIP_4	YAPRFVamide	Fujisawa *et al.* 1991
MIP_5	ASHIPRFVamide	Fujisawa *et al.* 1991
MIP_6	RAPLFIamide	Fujisawa *et al.* 1993b
MIP_7	RSPMFVamide	Fujisawa *et al.* 1993b

and tested for the antibody titer in the enzyme-linked immunosorbent assay (ELISA). Since the obtained serum showed a considerably high level of immunoreactivity against the carrier protein, KLH, the anti-MIP antibody (IgG) was purified as follows. First, the IgG fraction was obtained by 33% saturated ammonium sulfate precipitation. The precipitate was dialyzed against 10 mM sodium-phosphate buffered saline (PBS, pH7.5) containing 0.02% sodium azide, and then incubated with the KLH-Sepharose gel at 4°C overnight. The KLH-Sepharose was prepared by coupling KLH to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Tokyo, Japan) following the manufacturer's instruction. The fraction which was not adsorbed to the KLH-Sepharose was collected by filtration of the gel suspension on a glass filter. By this treatment, the anti-KLH immunoreactivity was decreased to a negligible level in ELISA, while the anti-MIP immunoreactivity was not significantly reduced (data not shown). This purified antibody was used in the following ELISAs and immunohistochemistry.

Competitive ELISA for MIP-family peptides

To quantify the specificity of the antibody against different MIP-family peptides, a competitive ELISA was developed. First, the synthetic peptide, CGSPMFVamide ([Cys^0]-MIP_1), was coupled via the N-terminal Cys to bovine thyroglobulin by the bifunctional crosslinker, 3-maleimidobenzoyl-*N*-hydroxysuccinimide. The peptide-carrier conjugate thus prepared was confirmed to act as an effective immunosorbent in preliminary experiments. Using this conjugate, experimental conditions were optimized as follows. The conjugate (5 μg/ml) diluted in 100 μl/well of 50 mM bicarbonate buffer (pH9.6) was bound to polystyrene 96-well microtiter plates (SUMILON H plate, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at 37°C for 2 hr. Plates were then incubated with 150 μl/well of 10 mM PBS (pH7.5) containing 1% BSA at 37°C for 1 hr to prevent nonspecific binding. After three washes with 200 μl/well of PBS containing 0.1 % Tween-20, competitor peptides (0.01 pmol-10 nmol/well) and the anti-MIP antibody (1:4000) diluted in 100 μl of PBS containing 0.1% BSA were added to each well. Plates were incubated at 4°C overnight and then rinsed as described above. Biotinylated anti-rabbit IgG antibody (1:500 in 0.1% BSA/PBS) was added in 100 μl/well and incubated at 37°C for 1 hr. Plates were washed three times and incubated with 100 μl/well of alkaline-phosphatase-labelled streptavidin (1:1000 in 0.1% BSA/PBS) at 37°C for 1 hr. Finally, the substrate, *p*-nitrophenylphosphate, dissolved in 10 mM diethanolamine buffer (pH9.5) containing 0.5 mM $MgCl_2$, was added in 100 μl/well after four rinses. Plates were incubated at room temperature with rotation until OD_{405} of control wells (without competitors) attained to 1-2. Within this range, the relation between OD_{405} and the amount of reaction products was confirmed to be linear. The competitor peptides used in the assay were the seven MIP-family members, MRYFVamide (an inhibitory peptide with -Phe-Val-amide at the C terminus), CARP and FMRFamide, all of which were known to be present in the ABRM (Fujisawa *et al.*, 1991, 1992, 1993a, b). All assays were made in duplicate.

Immunohistochemistry

Whole-mount preparations of the intact ABRM and of the split muscle bundles were made in this study. The animals from which the visceral organs had been removed were fixed in 4% paraformaldehyde in ASW at 4°C overnight. A pair of the ABRM was excised from the shell after fixation, and the tissues left around the muscle were carefully removed. The pedal ganglion and the sheath covering the muscle were left intact in the whole muscle preparations. For the split preparations, the muscle was torn into several bundles in order to expose nerves running between the muscle fibers. The fixed tissues were washed with 1% Triton X-100 in 10 mM PBS (T-PBS, pH7.5) at 4°C for 8 hr. Non-specific binding was blocked by incubating the tissues in 5% normal goat serum in T-PBS at 4°C overnight. Then the preparations were incubated with the primary antibody (1:200 in T-PBS containing 1% BSA) at 4°C for 2-3 days. After washing at 4°C overnight, the TRITC-labelled anti-rabbit IgG antibody (goat) was applied at a dilution of 1:500 in T-PBS containing 1% BSA. Following the incubation (3-4 hr) and washing (5-8 hr), the tissues were mounted in PBS: glycerol (1:6) containing 0.5% *n*-propyl gallate, and examined under a fluorescent microscope with G filters (Labophot-2, Nikon).

Release of the MIP-like immunoreactivity

The MIP-like immunoreactive substances released in response to neural stimulation both in the presence and absence of Ca^{2+} were measured by a competitive ELISA. A pair of the ABRM with the intact pedal ganglion was excised from the animal and placed horizontally in the experimental chamber (0.6 ml). The preparation was washed thoroughly with normal or Ca^{2+}-free ASW before collecting samples. In control experiments, the preparation was kept in 0.6 ml of normal or Ca^{2+}-free ASW with continuous aeration. After 1 hr, the ASW was collected. Then another 0.6 ml of normal or Ca^{2+}-free ASW was added to the chamber for test experiments. The condition of electrical stimulation was similar to that of Satchell and Twarog (1978). The repetitive electrical pulses (50 V, 2 msec, 10 Hz, 100 pulses) were applied to the pedal ganglion via a suction electrode at 90 sec intervals for 1 hr with aeration. Then the ASW was obtained. All the samples were acidified by adding 1/100 volume of trifluoroacetic acid (TFA) and kept frozen until use. For extraction of peptidic substances, the samples were applied onto reversed-phase cartridges (Sep-pak C18, Waters), and the adsorbed material was eluted with 60% acetonitrile/0.1% TFA. The eluate was dried up, reconstituted in chilled 0.1% BSA/PBS, and applied to the competitive ELISA using the anti-MIP antibody. The material collected from a pair of the ABRM was divided into two portions, each of which was added to a well. The conditions of the ELISA were basically the same as described above, but the lower concentration of the immobilized antigen (2 μg/ml) was employed. MIP_1 (0.01 pmol-1 nmol/well) was always included in each plate as a standard, and the MIP_1 equivalent for each sample was calculated from the standard curve.

Solutions and reagents

The composition of normal ASW was as follows; 445 mM NaCl,

55 mM $MgCl_2$, 10 mM KCl, 10 mM $CaCl_2$ and 10 mM Tris-HCl (pH7.8). In Ca^{2+}-free ASW, 10 mM $CaCl_2$ was replaced by osmotically equivalent $MgCl_2$. MIP_1, MIP_2 and CGSPMFVamide were synthesized with a solid-phase peptide synthesizer (PPSQ-10, Shimadzu, Kyoto, Japan). MIP_3, MIP_4 and CARP were custom-made at Peptide Institute (Osaka, Japan). MIP_5 was synthesized by Mr. M. Ohtani in our laboratory. MIP_6, MIP_7 and MRYFVamide were made by Drs. T. Nose and W. Miki (Marine Biotechnology Institute, Co. Ltd., Shizuoka, Japan). FMRFamide was purchased from Sigma (Missouri, U.S.A.). The biotinylated anti-rabbit IgG (H + L) antibody and the streptavidin alkaline phosphatase were purchased from Vector Laboratories (California, U.S.A). The TRITC-labelled anti-rabbit IgG antibody was from Biomedical Technologies, Inc. (Massachusetts, U.S.A.). BSA, KLH and bovine thyroglobulin were obtained from Sigma, and 3-maleimidobenzoyl-*N*-hydroxysuccinimide from Boehringer-Mannheim. Other chemicals were purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan).

Table 2. IC_{50} values of seven MIPs and other peptides in the competitive ELISA. Each value means the concentration yielding 50% displacement of the control binding which is obtained in Fig. 1

Peptide competitor	IC_{50} (pmol/well)
MIP_1	3.1
MIP_2	1.7
MIP_3	3.1
MIP_4	26
MIP_5	5.5
MIP_6	53
MIP_7	3.7
MRYFVamide	1,600
CARP	>10,000
FMRFamide	>10,000

RESULTS

Specificity of the antibody

Specificity of the purified anti-MIP antibody against different MIPs and unrelated peptides was quantitatively shown by the competitive ELISA. The binding of the antibody to the immobilized MIP_1-thyroglobulin conjugate was effectively displaced by increasing concentrations of the seven MIPs (Fig. 1). The IC_{50} values (concentrations yielding 50% displacement) for the seven MIPs ranged from one to 53 pmol/well (Table 2). On the other hand, the antibody showed only a weak cross-reactivity against MRYFVamide, which shared the C-terminal Phe-Val-amide with MIP-family peptides. The IC_{50} value for MRYFVamide was about two order of magnitude higher than that of MIPs (Table 2). Furthermore, no cross-reactivity was observed against CARP and FMRFamide. It was therefore confirmed that the antibody specifically recognized various MIP-family peptides in the ABRM.

Immunohistochemistry

The MIP-like immunoreactivity was found abundantly in neuronal fiber-like structures both in the intact and the split muscle preparations. In the intact ABRM, the muscle bundles, the pedal ganglion and arising nerves were enveloped by the connective tissue sheath, throughout which the immunoreactive fibers were distributed (Fig. 2). Branching of the fibers occurred frequently, and therefore many fine fibers were observed netlike in every part of the muscle (Fig. 2A-C). In addition, highly immunoreactive varicosities were present abundantly on the fine fibers (Fig. 2D-F), while few varicosities on thicker parts. Furthermore, the immunoreactive fibers running along the muscle fibers were demonstrated both on the surface (Fig. 3A, B) and inside the muscle bundles (Fig. 3C), although the number of fibers was less than that found in the sheath. Immunoreactive fibers in these areas appeared to be fine and run almost parallel with muscle fibers with little branching. Varicosities with intense staining were observed

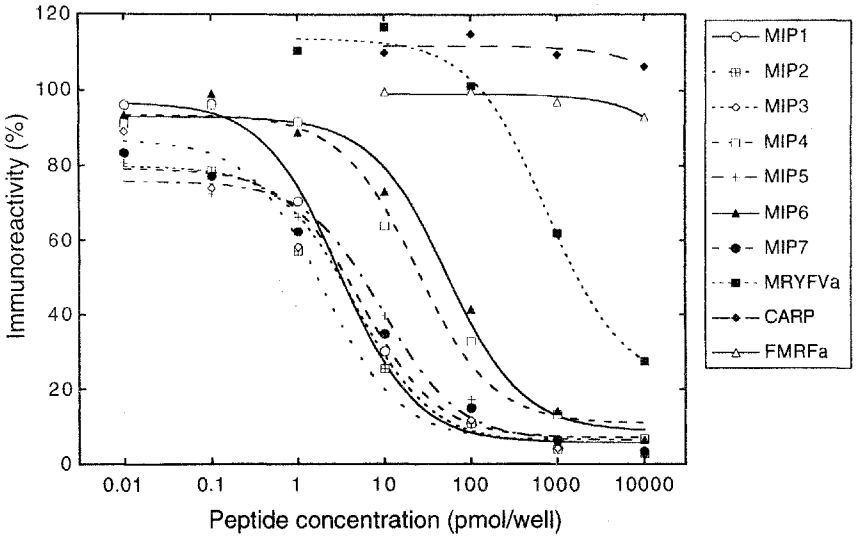

Fig. 1. Displacement curves for MIPs and unrelated peptides in the competitive ELISA. Immunoreactivity is shown in per cent of control reaction (in the absence of competitors). Each value is the mean of three different assays.

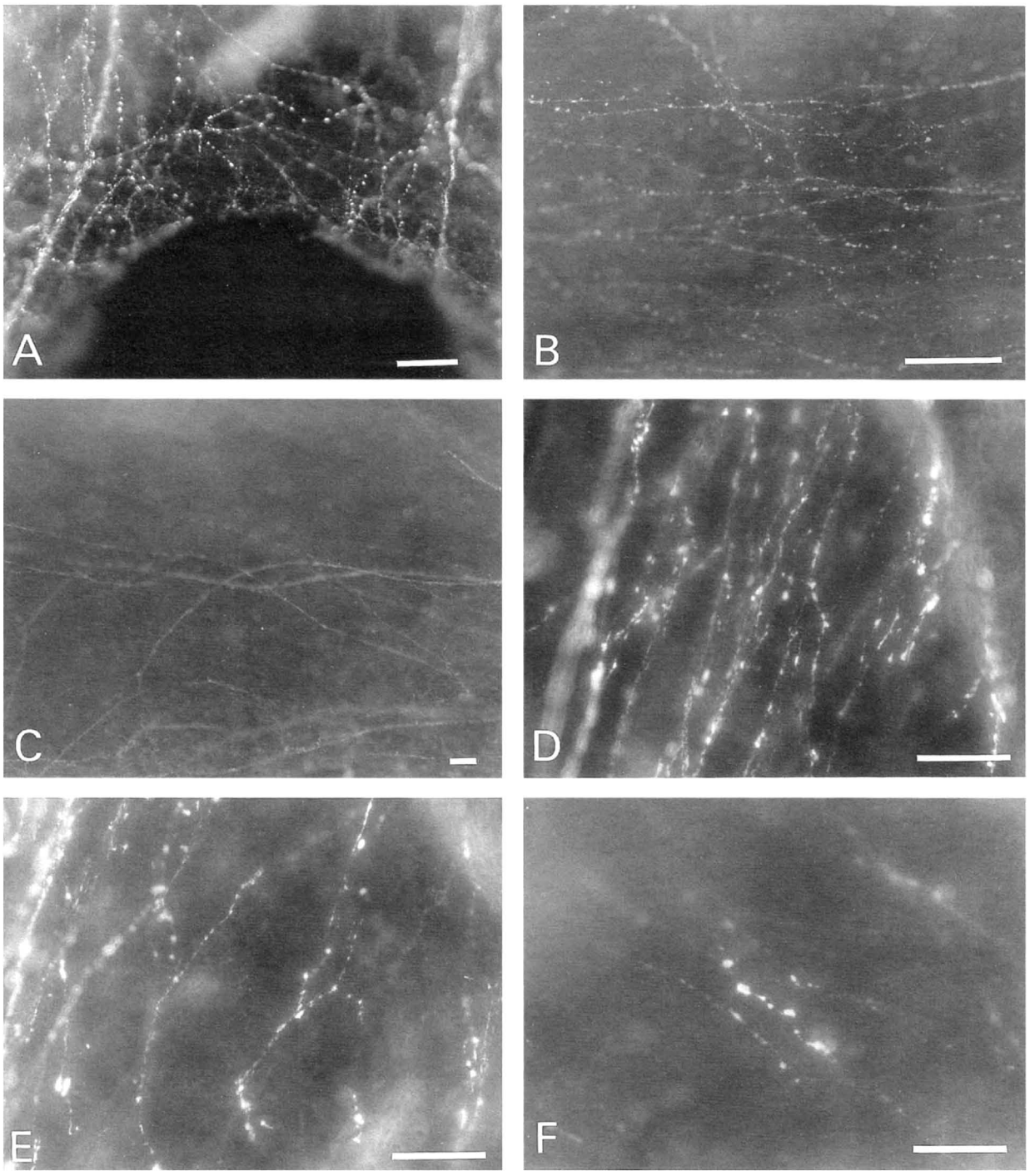

Fig. 2. A-F The MIP-like immunoreactive fibers in the connective tissue sheath surrounding the ABRM. A Posterior part of the muscle. B Middle part. C Anterior part. D-F Immunoreactive varicosities on fine fibers in the posterior part. Scale, 20 μm.

as in the sheath (Fig. 3D).

Specificity of the immunostaining was confirmed by adsorption of the antibody (1:200) with 10^{-3} M MIP_1 at 4°C overnight. By this treatment, the immunoreactivity was eliminated (data not shown).

Ca^{2+}-dependent release of the MIP-like immunoreactivity

The amount of the MIP-like immunoreactive material released from the pedal ganglion-ABRM preparation was measured by the competitive ELISA. The detection limit for MIP_1 was as low as ~ 0.1 pmol/well when a lower concentration (2 μg/ml) of the peptide-carrier conjugate was employed. The results are summarized in Fig. 4. In the presence of 10 mM Ca^{2+} (normal ASW), the MIP-like immunoreactive material was shown to be released in response to the electrical stimulation applied to the ganglion. The total amount released in response

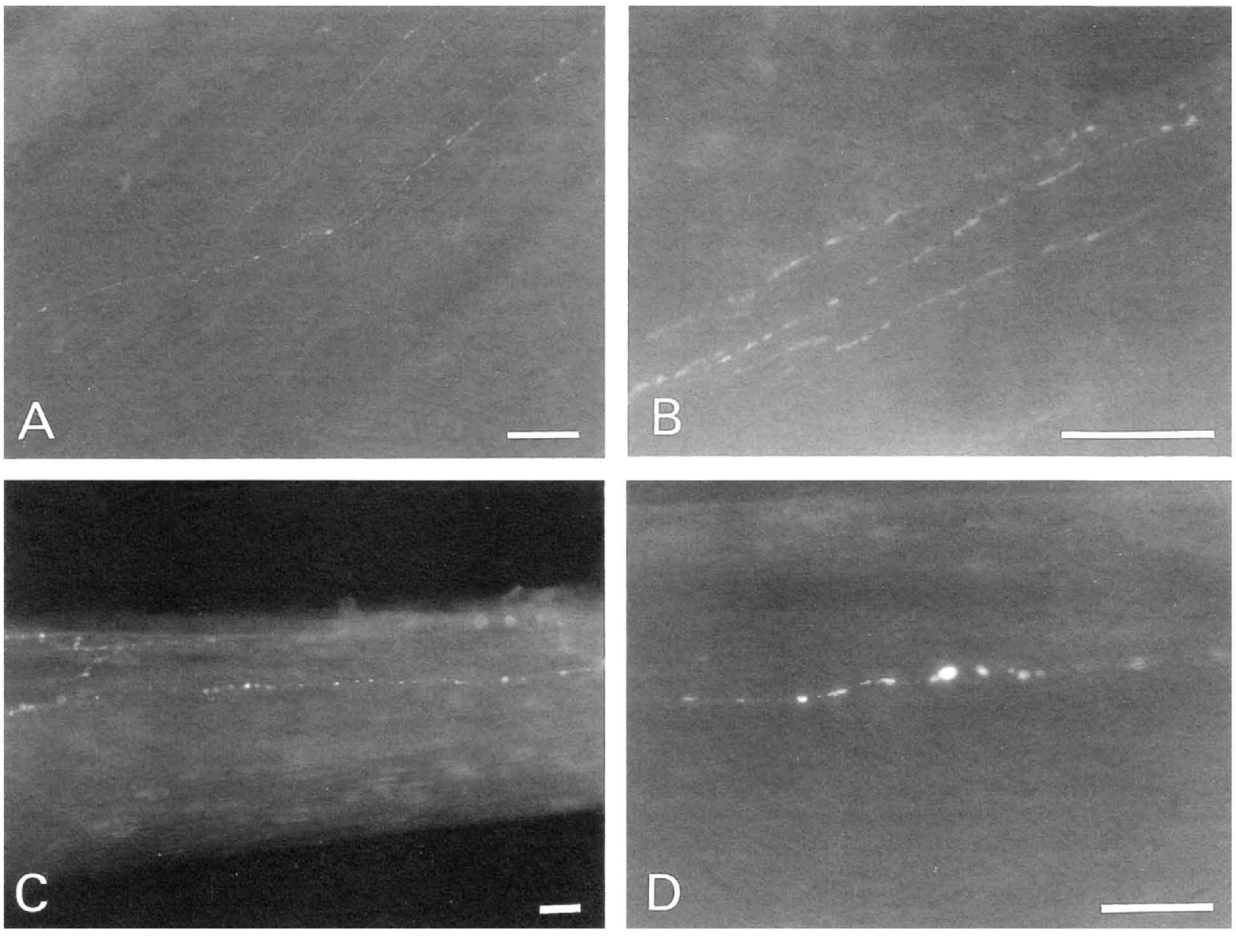

Fig. 3. A-D The MIP-like immunoreactive fibers running between muscle fibers. A Posterior surface of the intact muscle. B Middle surface of the intact muscle. C Fibers running inside the muscle bundle and exposed in the split preparation. D Varicosities on fibers in C. Scale, 10 μm.

to the stimulus was 9.24 ± 2.60 pmol MIP_1 equivalent/animal, while the basal release (1.21 ± 0.82 pmol/animal) was also observed even when the ganglion received no stimulation. In the absence of Ca^{2+}, however, no significant release of the MIP-like material was observed in response to the stimulation. The basal release was also blocked in the Ca^{2+}-free ASW. Contraction of the muscle could be observed only when it was stimulated in the normal ASW.

DISCUSSION

The present experiments were designed to demonstrate the neuronal localization and the Ca^{2+}-dependent release of the seven members of the MIP family, MIP_{1-7}, in the ABRM of *Mytilus*. These are prerequisite for establishing any substance as a physiological neurotransmitter/modulator. For this purpose, the polyclonal antibody was employed throughout the experiments to detect MIPs in the ABRM. The antibody had the advantage of specificity; it can recognize all of the seven MIPs without crossreacting to other peptide species as examined in the competitive ELISA. It cannot be ruled out that the antibody may recognize some other peptides that were not tested in this study, but it is considered to be sufficient for detecting the seven MIPs which are known to be contained in the muscle.

First, the immunoreactivity was localized in fiber-like structures, which could be morphologically identified as neuronal fibers. The results indicate that all the seven MIPs, first identified in the tissue extract, originate from peripheral neural elements in the ABRM. Some other unknown members of the MIP family might also be included in the immunoreactive fibers as described above, because it seemed possible that the antibody could recognize any MIP-family peptides with -Pro-Xaa-Phe-Val/Ile-amide at the C terminal region.

The putative MIP neurons running along the muscle fibers on the surface and inside the muscle bundles are supposed to make close contact with adjacent muscle cells. It has not been clarified whether they form synapses at the terminals, but, at least, intensely immunostained varicosities could be the potential sites of axonal release. On the other hand, numerous immunoreactive fibers found in the connective tissue sheath might diffuse peptides into broader space covering a whole muscle bundle. Since the antibody was not able to distinguish the seven (or more) isoforms, whether different sets of the peptides are colocalized in different fibers, or even in different terminals, is not clarified. But it is very likely that

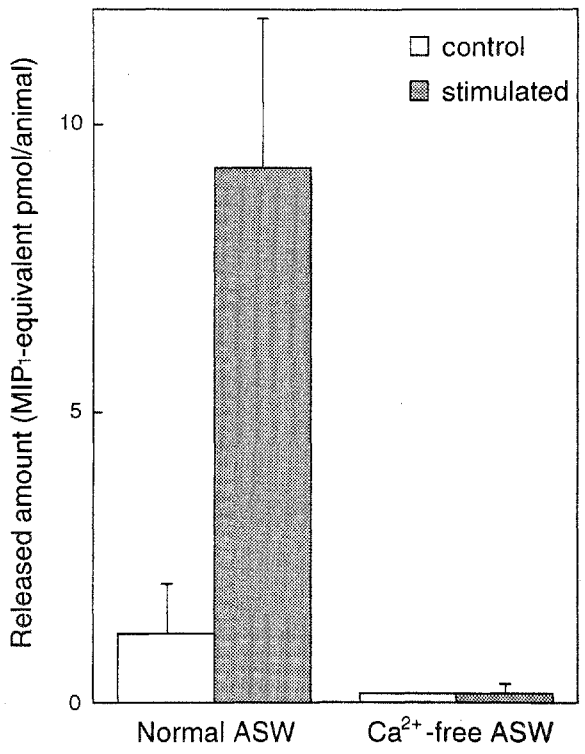

Fig. 4. Released amount of the MIP-like immunoreactivity in response to neural stimulation in the presence and absence of Ca^{2+}. The amount is expressed in pmol MIP_1 equivalent calculated from the standard curve made in each assay. Open columns show the basal release, and gray columns show the total release in response to the stimulation. Each value is expressed in mean ± SD of three preparations, with each of which a control and a subsequent test experiments was done.

multiple MIPs are derived from a single precursor, packed into the same vesicle and transported to the same terminals, as has been suggested for other molluscan neuropeptide isoforms such as FMRFamide-related peptides, myomodulins and buccalins (Linacre *et al.*, 1990; Saunders *et al.*, 1991, 1992; Miller *et al.*, 1993a, b). The discovery of three pentadecapeptides from the pulmonate, *Achatina fulica*, in which two MIP-family hexapeptides are linked by a endoproteolytic signal sequence, -Gly-Arg-Arg-, may support the above hypothesis (Ikeda *et al.*, 1992). Further study on colocalization and precursor organization of the MIP-family peptides is required.

Next, the MIP-like immunoreactivity was shown to be released from the ABRM-pedal ganglion preparation in response to repetitive electrical pulses applied to the ganglion. The release did not occur in the Ca^{2+}-free ASW. These results suggest that MIP neurons innervating the ABRM may, at least partly, originate from the pedal ganglion and that the peptides included in the neuronal fibers could be discharged when the neurons are excited with depolarizing stimuli. Since MIP_1 and MIP_2 were first isolated from the pedal ganglion, it is possible that at least the two are synthesized in the ganglionic somas and axonally transported by the putative MIP neurons. Requirement of extracellular Ca^{2+} may reflect the physiological mechanism of neurotransmitter release triggered by depolarization-dependent influx of Ca^{2+} into nerve terminals. In conclusion, the seven MIP-family peptides are located in neuronal fibers in the ABRM, and are probably released in response to neural excitation. Taken together, the present findings support the physiological roles of the seven MIP-family peptides as the neuropeptides regulating the ABRM contraction.

There are several other instances that multiple homologous members belonging to the same peptide family are involved in a single neuromuscular system. The best studied example is the myomodulin family in the accessary radula closer muscle of *Aplysia californica* and in the penis retractor muscle of *Lymnaea stagnalis*. In both cases, multiple peptide isoforms have been purified from the peripheral tissues (Cropper *et al.*, 1987, 1991; Březina *et al.*, 1995; Li *et al.*, 1994; Van Golen *et al.*, 1996) and shown to have overlapping yet distinct effects on muscle contractions (Březina *et al.*, 1995; Van Golen *et al.*, 1996). In case of MIPs in the ABRM, on the other hand, they all show similar inhibitory effects on electrically evoked and acetylcholine-evoked contractions although the potency of the inhibition differs from each other (Hirata *et al.*, 1989; Fujisawa *et al.*, 1991, 1993b). The dose-response relationship for the inhibitory effect differs from each other, but no qualitatively different effect has been observed so far. Therefore, it can be assumed that these seven isoforms play the same role in regulating the ABRM. Each of them could be replaced by another molecule. This hypothesis consequently implies that the structural variations of MIPs have little or no physiological significance and that each variant has been produced by the neutral molecular evolution, as has been suggested by the previous report (Fujisawa *et al.*, 1991). Further analysis on various parameters of the inhibitory effect of MIPs will be needed before concluding whether the above hypothesis can be generalized and applied to various systems that include multiple family peptides.

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REFERENCES

Březina V, Bank B, Cropper EC, Rosen S, Vilim FS, Kupfermann I, Weiss KR (1995) Nine members of the myomodulin family of peptide cotransmitters at the B16-ARC neuromuscular junction of *Aplysia*. J Neurophysiol 74: 54–72

Cropper EC, Tenenbaum R, Kolks MAG, Kupfermann I, Weiss KR (1987) Myomodulin: A bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. Prot Natl Acad Sci 84: 5483–5486

Cropper EC, Vilim FS, Alevizos A, Tenenbaum R, Kolks MAG, Rosen S, Kupfermann I, Weiss KR (1991) Structure, bioactivity, and cellular localization of myomodulin B: A novel *Aplysia* peptide. Peptides 12: 683–690

Fujisawa Y, Kubota I, Ikeda T, Minakata H, Muneoka Y (1991) A variety of *Mytilus* inhibitory peptides in the ABRM of *Mytilus edulis*: Isolation and characterization. Comp Biochem Physiol 100C: 525–531

Fujisawa Y, Ikeda T, Nomoto K, Yasuda-Kamatani Y, Minakata H, Kenny PTM, Kubota I, Muneoka Y. (1992) The FMRFamide-related decapeptide of *Mytilus* contains a D-amino acid residue. Comp Biochem Physiol 102C: 91–95

Fujisawa Y, Kubota I, Nomoto K, Minakata H, Yasuda-Kamatani Y, Ikeda T, Muneoka Y (1993a) A *Mytilus* peptide related to the small cardioactive peptides (SCPs): Structure determination and pharmacological characterization. Comp Biochem Physiol 104C: 469–475

Fujisawa Y, Takahashi T, Ikeda T, Muneoka Y, Kubota I, Minakata H, Nomoto K, Nose T, Miki W (1993b) Further identification of bioactive peptides in the anterior byssus retractor muscle of *Mytilus*: Two contractile and three inhibitory peptides. Comp Biochem Physiol 106C: 261–267

Hirata T, Kubota I, Takabatake I, Kawahara A, Shimamoto N, Muneoka Y (1987) Catch-relaxing peptide isolated from *Mytilus* pedal ganglia. Brain Res 422: 374–376

Hirata T, Kubota I, Iwasawa N, Takabatake I, Ikeda T, Muneoka Y (1988) Structure and actions of *Mytilus* inhibitory peptides. Biochem Biophys Res Commun 152: 1376–1382

Hirata T, Kubota I, Iwasawa N, Fujisawa Y, Muneoka Y, Kobayashi M (1989) Effects of *Mytilus* inhibitory peptides on mechanical responses of various molluscan muscles. Comp Biochem Physiol 93C: 381–388

Ikeda T, Yasuda-Kamatani Y, Minakata H, Kenny PTM, Nomoto K, Muneoka Y (1992) *Mytilus*-inhibitory peptide analogues isolated from the ganglia of a pulmonate mollusc, *Achatina fulica*. Comp Biochem Physiol 101C: 245–249

Li KW, Van Golen FA, Van Minnen J, Van Veelen PA, Van der Greef J, Geraerts WPM (1994) Structural identification, neuronal synthesis, and role in male copulation of myomodulin-A of *Lymnaea*: A study involving direct peptide profiling of nervous tissue by mass spectrometry. Mol Brain Res 25: 355–358

Linacre A, Kellet E, Saunders S, Bright K, Benjamin PR, Burke JF (1990) Cardioactive neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) and novel related peptides are encoded in multiple copies by a single gene in the snail *Lymnaea stagnalis*. J Neurosci 10: 412–419

Miller MW, Beushausen S, Cropper EC, Eisinger K, Stamm S, Vilim FS, Vitek A, Zajc A, Kupfermann I, Brosius J, Weiss KR (1993a) The buccalin-related neuropeptides: Isolation and characterization of an *Aplysia* cDNA clone encoding a family of peptide cotransmitters. J Neurosci 13: 3346–3357

Miller MW, Beushausen S, Vitek A, Stamm S, Kupfermann I, Brosius J, Weiss KR (1993b) The myomodulin-related neuropeptides: Characterization of a gene encoding a family of peptide cotransmitters in *Aplysia*. J Neurosci 13: 3358–3367

Muneoka Y and Twarog B (1983) Neuromuscular transmission and excitation-contraction coupling in molluscan muscle. In "The Mollusca" Ed by Saleuddin ASM and Wilbur KM, Academic Press, New York, Vol 4, pp35–76

Muneoka Y, Fujisawa Y, Fujimoto N, Ikeda T (1990) The regulation and pharmacology of muscles in *Mytilus*. In "Neurobiology of *Mytilus edulis*" Ed by Stefano GB, Manchester University Press, Manchester, pp209–245

Satchell DG and Twarog BM (1978) Identification of 5-hydroxytryptamine (serotonin) released from the anterior byssus retractor muscle of *Mytilus californianus* in response to nerve stimulation. Comp Biochem Physiol 59C: 81–85

Saunders SE, Bright K, Kellet E, Benjamin PR, Burke JF (1991) Neuropeptides Gly-Asp-Pro-Phe-Leu-Arg-Phe-amide (GDPFLRFamide) and Ser-Asp-Pro-Phe-Leu-Arg-Phe-amide (SDPFLRFamide) are encoded by an exon 3' to Phe-Met-Arg-Phe-NH_2 (FMRFamide) in the snail *Lymnaea stagnalis*. J Neurosci 11: 740–745

Saunders SE, Kellet E, Bright K, Benjamin PR, Burke JF (1992) Cell-specific alternative RNA splicing of an FMRFamide gene transcript in the brain. J Neurosci 12: 1033–1039

Van Golen FA, Li KW, Chen S, Jiménez CR, Geaerts WPM (1996) Various isoforms of myomodulin identified from the male copulatory organ of *Lymnaea* show overlapping yet distinct effects on the penis muscle. J Neurochem 66: 321–329

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