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Relaxing Action of Trp-Nle-Arg-Phe-NH_2 on the Anterior Byssus Retractor Muscle of *Mytilus*

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ABSTRACT

In the anterior byssus retractor muscle (ABRM) of *Mytilus*, low concentrations of the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) relax ACh-induced catch tension, whereas high concentrations cause contraction. In the present studies the actions of a FMRFamide-analog Trp-Nle-Arg-Phe-NH_2 (W-Nle-RFamide) on the ABRM were investigated in order to compare them with those of FMRFamide.

The actions of W-Nle-RFamide were qualitatively similar to those of FMRFamide, but catch-relaxing activity of the former peptide was found to be 10-30 times more potent than that of the latter.

Cytochemical studies showed that FMRFamide-like immunoreactivity was localized to nerve branches in the ABRM and also to neurons in the pedal ganglion.

A peptide which structurally resembles FMRFamide may be involved in the mechanism of the control of relaxation of catch tension in the ABRM of *Mytilus*.

In the anterior byssus retractor muscle (ABRM) of *Mytilus*, the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) discovered in the ganglia of the clam *Macrocallista nimbosa*[14] causes a relaxation or a contraction depending on its concentration. At 10^{-8}-10^{-7} M, FMRFamide relaxes catch tension induced by acetylcholine (ACh)[8], but at concentrations higher than 10^{-7} M, the peptide elicits a contraction[3,12].

Muneoka and Saitoh[9] have examined the structure-activity relations of FMRFamide for relaxation and contraction of the ABRM and have found that the relations for these two responses are different. That is, modifications of N-terminal of FMRFamide — extension with Tyr residue or Tyr-Gly-Gly, N-terminal acetylation and substitution of Tyr or D-Phe for Phe^1 — do not have a large effect on contractile potency, while the same modifications are all deleterious to relaxing activity.

In these studies of the structure-activity relations on the ABRM, however, the activity of Trp-Nle-Arg-Phe-NH_2 (W-Nle-RFamide) has not been investigated. It has been shown in various molluscan muscles that Nle can be substituted for Met^2 of FMRFamide[5,13]. In the ABRM, in fact, not only relaxing but also contractile activity of F-Nle-RFamide is comparable to that of FMRFamide[9]. It can be suspected, therefore, that the actions of W-Nle-RFamide on the ABRM may be comparable to those of WMRFamide in which Trp is substituted for Phe^1 of FMRFamide. Thus, it can be expected that relaxing activity of W-Nle-RFamide would be less potent than that of FMRFamide, though contractile activities of both of the peptides would be comparable.

In the present studies, we examined the actions of W-Nle-RFamide on the ABRM. As ex-

pected, contractile activity of the peptide was found to be comparable to that of FMRFamide, but contrary to our expectation, relaxing activity of the peptide was found to be 10-30 times more potent than FMRFamide. These results and others obtained in the present experiments led to the speculation that *Mytilus* may have a FMRFamide-like peptide involved in the mechanism of the control of relaxation of catch tension in the ABRM.

MATERIALS AND METHODS

Animals

Mytilus edulis L. was collected from Hiroshima Bay. The animals were stored in aerated artificial seawater (ASW) at temperatures between 18 and 23°C and were used for experimentation within 5 days of collection.

Tension recording

The method of recording of tension changes in the ABRM was essentially the same as that described by Muneoka and Twarog[11]. The ABRM was dissected out with its origin at the byssus organ and its insertion on the shell intact. The muscle was teased into bundles 0.7—1.0 mm in diameter, using a stainless needle. A small disk of shell on which the muscle is inserted was tied with a cotton thread to the experimental chamber. The byssus root was connected by a cotton thread to a force-displacement transducer. After mounting the preparation in the experimental chamber, repetitive electrical stimulation (15 V, 3 msec, 10 Hz) was apllied for 5 sec at 10 min intervals until the muscle relaxed completely and responded to the stimulation with a uniform phasic contraction. Only then were the experiments started. Drugs were applied to the muscle by replacing the bath solution.

The experiments were carried out at room temperature (18—23°C).

Cytology

The ABRM and pedal ganglion were isolated and fixed for 12 hr with 4% paraformaldehyde in ASW. They were then dehydrated in an alcohol series. The dehydrated materials were treated with xylene and embedded in paraplast. Serial sections were cut at 10 μm. The sections were placed on gelatin-coated slide-glasses and used for immunocytochemical staining and haematoxylin-eosin staining.

The avidin-biotin-peroxidase complex method[16] using Vectastain Kit purchased from Vector Labs. was adopted for immunocytochemistry. The staining procedure consisted of the following incubation steps: after deparaffinized in xylene, the sections were incubated for 20 min in normal goat serum (1/50 in PBS), washed for 10 min with PBS, incubated for 12 hr in FMRFamide antiserum (1/4000 in PBS) purchased from Peninsula Labs., washed for 10 min with PBS, incubated for 30 min in biotinylated goat anti-rabbit IgG (1/200 in PBS), and washed for 10 min with PBS. They were then incubated for 30 min in the complex of avidin and biotinylated horseradish peroxidase (1/100 in PBS), washed for 10 min with PBS and exposed for 10 min to 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 (in 0.05 M Tris-HCl buffer). The sections were dehydrated again and mounted.

The specificity of the immunocytochemical localization was established by examining the sections incubated with antiserum preadsorbed with FMRFamide (10 nmoles/ml diluted antiserum).

Haematoxylin-eosin staining was carried out by a conventional method.

Solutions and drugs

The physiological saline was ASW of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCl; pH 7.6. In some experiments, 540 mM KCl containing 5 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (KCl-EGTA solution) was used to obtain a denervated preparation[6]. The solution was adjusted to pH 7.6 with NaOH.

Drugs used were as follows: acetylcholine bromide (Sigma), DL-α-methyldopa (Nakarai Chemicals, Ltd.), Phe-Met-Arg-Phe-NH_2 (Peninsula Labs.) and Trp-Nle-Arg-Phe-NH_2 (Cambridge Research Biochemicals, Ltd.).

RESULTS

High concentrations of W-Nle-RFamide, as well as FMRFamide, caused contraction in the ABRM in a dose-dependent manner. The threshold concentration of W-Nle-RFamide for contraction was found to be about 10^{-7} M (Fig. 1A) as in the case of FMRFamide[12]. That is, contractile activities of both of the peptides were

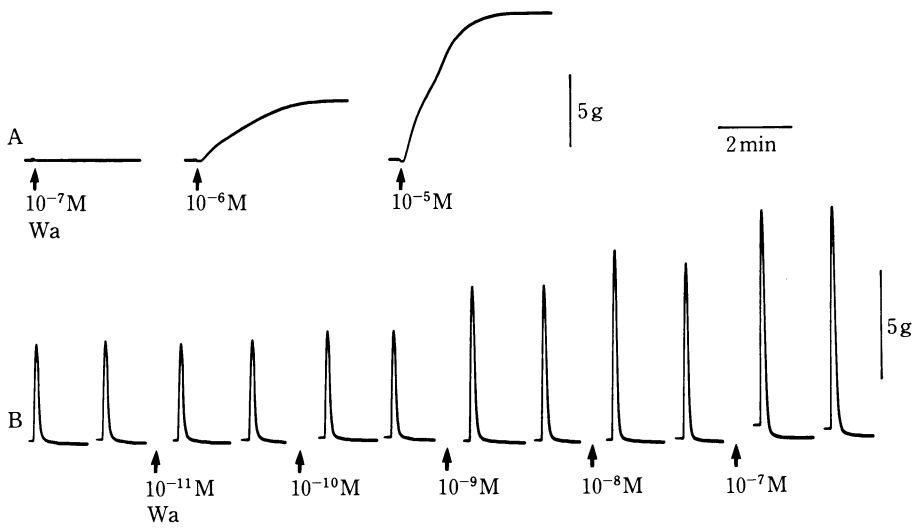

Fig. 1. Contractile (A) and contraction-potentiating (B) actions of W-Nle-RFamide (Wa). In A, W-Nle-RFamide was applied for 4 min at 15 min intervals. After the peptide was washed out, the muscle was stimulated with repetitive electrical pulses to relax it. In B, repetitive electrical stimulation was applied at 10 min intervals to produce phasic contractions. Each dose of the peptide was applied 8 min prior to the first stimulation in it.

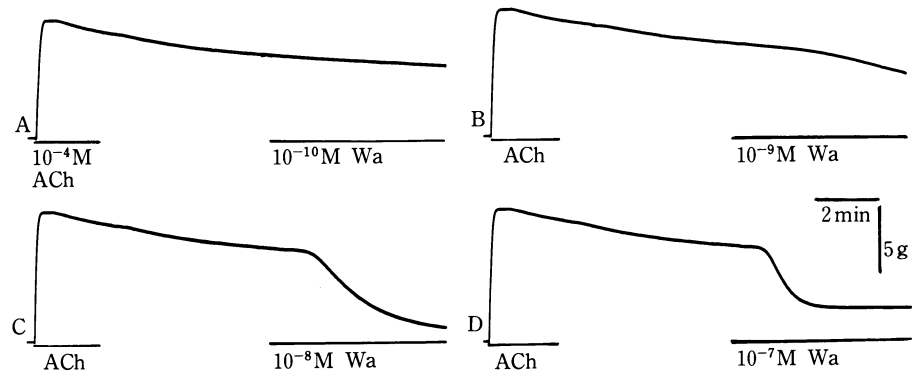

Fig. 2. Relaxing action of low concentrations of W-Nle-RFamide (Wa) on ACh-induced catch tension. ACh was applied at 30 min intervals. After each record, the peptide was washed out and the muscle was stimulated with repetitive electrical pulses to relax it completely.

comparable. In contrast, potentiating activity of W-Nle-RFamide on phasic contraction in response to repetitive electrical pulses (15 V, 3 msec, 10 Hz, for 5 sec) of stimulation was about 10 times more potent than that of FMRFamide[8]. The threshold concentration of W-Nle-RFamide for the potentiation was about 10^{-10} M (Fig. 1B).

At low concentrations, W-Nle-RFamide not only potentiated phasic contraction but also relaxed catch tension induced by ACh (Fig. 2). The threshold concentration for relaxation was found to be between 10^{-10} and 10^{-9} M in most of the muscles, but in some muscles it was about 10^{-10} M. That is, the relaxing activity of W-Nle-RFamide was 10-30 times more potent than that of FMRFamide[8]. At 10^{-8} M, W-Nle-RFamide completely or almost completely relaxed catch tension (Fig. 2C), but at 10^{-7} M the peptide did not completely relax catch, though the rate of relaxation at the initial phase was greater than that of relaxation induced by 10^{-8} M (Fig. 2D). Thus, it seems that although 10^{-7} M W-Nle-RFamide cannot evoke contraction in most of

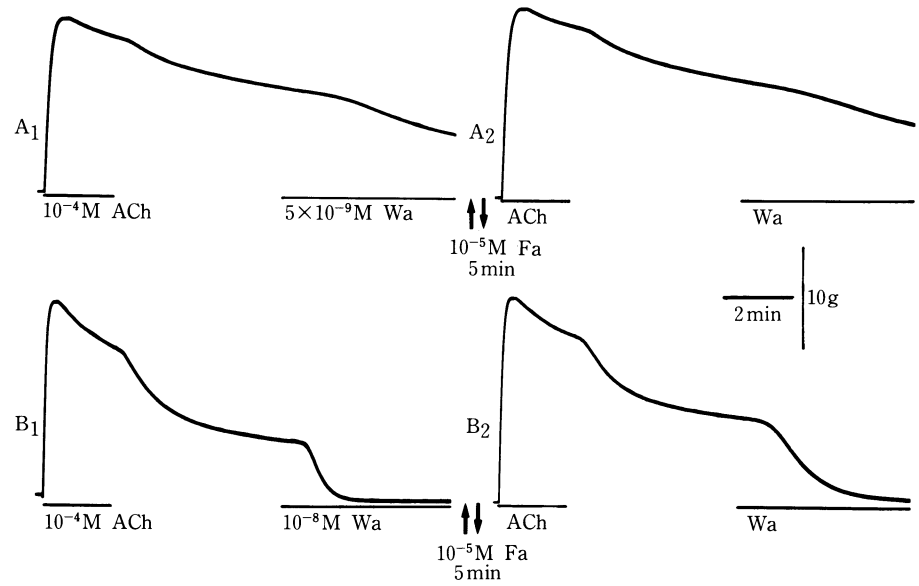

Fig. 3. After-effect of high concentration of FMRFamide (10^{-5} M Fa) on relaxations of ACh-induced catch tension by 5 × 10^{-9} M (A) and 10^{-8} M (B) of W-Nle-RFamide (Wa). After recording the control responses (A_1 and B_1), the muscles were washed and relaxed completely by stimulating with repetitive electrical pulses. Ten minutes after the stimulation, the muscles were exposed to FMRFamide for 5 min. Then, the muscles were again washed and relaxed by repetitive electrical stimulation. Twenty minutes after the last electrical stimulation, A_2 and B_2 were recorded, respectively.

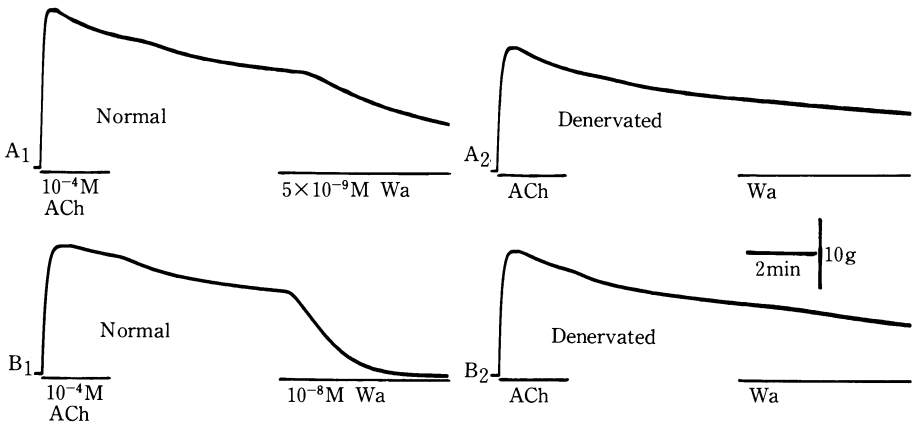

Fig. 4. Effect of denervation on relaxing responses to 5 × 10^{-9} M (A) and 10^{-8} M (B) W-Nle-RFamide (Wa). After recording the control relaxations of ACh-induced catch tension (A_1 and B_1), the muscles were washed and stimulated with repetitive electrical pulses to relax them completely. Then, they were denervated (for the denervation procedure, see text). After the denervation-treatment, the action of the low concentration of W-Nle-RFamide was again examined on ACh-induced catch tension (A_2 and B_2).

the muscles, the peptide at this concentration has two opposite effects; a relaxing effect on ACh-induced catch tension and an inhibitory effect on the relaxation by itself.

Muneoka and Matsuura[8] have shown that after the ABRM has been treated with concentrations of FMRFamide high enough to produce contraction, relaxation of ACh-induced catch tension by 10^{-7} M FMRFamide is markedly depressed for hours. In the present study, there-

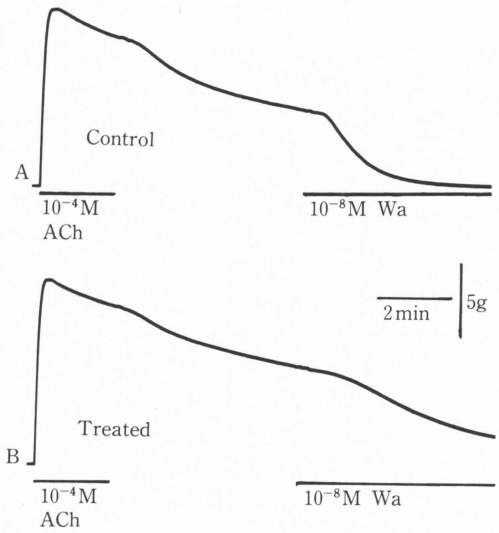

Fig. 5. Effect of treatment of the muscle with α-methyldopa on relaxing response to 10^{-8} M W-Nle-RFamide (Wa). After recording the control relaxation of ACh-induced catch tension (A), the muscle was treated with 10^{-3} M α-methyldopa (for the treatment procedure, see text). After the treatment, the action of W-Nle-RFamide was again examined (B).

fore, we examined whether relaxations of catch in response to low concentrations of W-Nle-RFamide are depressed or not by the treatment of the muscle with high concentration of FMRFamide.

As shown in Fig. 3, relaxations in response to low concentrations (5×10^{-9} M and 10^{-8} M) of W-Nle-RFamide were elicited even after the muscle had been treated for 5 min with 10^{-5} M FMRFamide, though the degree of the relaxing rate was found to be depressed slightly; i.e. the after-effect of 10^{-5} M FMRFamide on the relaxation in response to low concentrations of W-Nle-RFamide was slight.

Muneoka and Matsuura[8] have also shown that, in the denervated ABRM preparations, relaxing response to 10^{-7} M FMRFamide is not elicited, though contractile response to 10^{-5} M FMRFamide can be produced almost without any change. In the present study, therefore, we examined the effect of low concentrations of W-Nle-RFamide on ACh-induced catch tension in the denervated preparations. To obtain the denervated preparation, the ABRM was immersed for 30 min in KCl-EGTA solution after recording the control relaxation. Then, the muscle was immersed for another 30 min in normal ASW and the relaxing response to a low concentration of W-Nle-RFamide was examined.

As shown in Fig. 4, slight or little relaxing responses to low concentrations of W-Nle-RFamide were observed in the denervated preparations. On the contrary, contractile response to 10^{-5} M W-Nle-RFamide could be produced almost without any change in the denervated muscles. Thus, W-Nle-RFamide, as well as FMRFamide, seems to bring about relaxation by acting on the relaxing nerve terminals to increase release of relaxing-neurotransmitter serotonin[8].

Muneoka et al[10] have shown that treatment of the ABRM with α-methyldopa decreases the rate of relaxation of the phasic contraction in response to repetitive electrical pulses of stimulation, probably by inhibiting synthesis and thus decreasing the amount of relaxing-transmitter serotonin in the nerve terminals. In the present study, we found that the rate of relaxation of catch tension in response to 10^{-8} M W-Nle-RFamide was also decreased after the muscle had been treated with α-methyldopa (Fig. 5). In these experiments, control relaxation was first recorded, and then the muscle was immersed for 4 hr in 10^{-3} M α-methyldopa. During immersion in α-methyldopa, the muscle was stimulated every 5 min with repetitive electrical pulses (15 V, 3 msec, 10 Hz, for 5 sec)[10]. Alpha-methyldopa-containing solution in the chamber was exchanged every 30 min with a newly-prepared solution, because the drug was easily oxidized during immersion.

Cytochemical study showed that FMRFamide-like immunoreactivity was localized to a number of cell bodies in the pedal ganglion (Fig. 6B). The FMRFamide-like immunoreactivity was also found in the nerve branches in the ABRM (Fig. 6C).

DISCUSSION

Up to the present, structure-activity relations of FMRFamide have been examined on seven responses in six molluscan muscles[5,9,13]. In all of these examinations, it has been shown that Nle can be substituted for Met[2]. In the ABRM of *Mytilus*, F-Nle-RFamide is almost equipotent

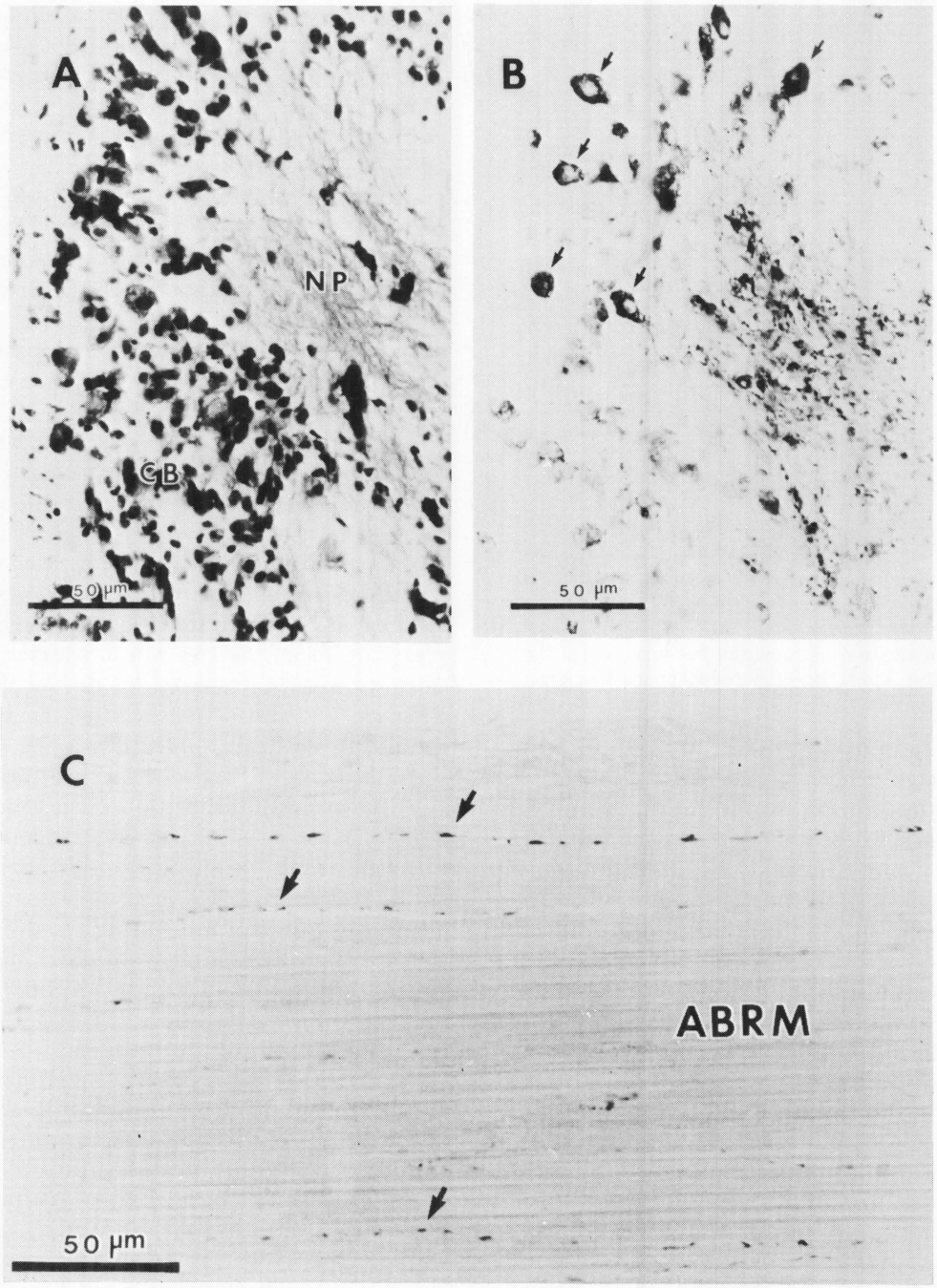

Fig. 6. Haematoxylin-eosin stained (A) and FMRFamide-antiserum stained (B) neurons in the pedal ganglion, and FMRFamide-antiserum stained nerve branches (C) in the ABRM. CB: cell body. NP: neuropile.

to FMRFamide in producing not only contraction but also relaxation[9]. From these facts it can be suspected that the effects of W-NleRFamide on the ABRM may be comparable to those of WMRFamide. It has also been shown that Phe[1] of FMRFamide can be replaced by

other amino acids for contraction-inducing activity on the ABRM[9]. Thus, we expected that contractile activity of W-Nle-RFamide would be comparable to that of FMRFamide. As shown in the present experiments, this is the case, both of the peptides being almost equipotent in producing contraction.

In contrast to the contractile activity of W-Nle-RFamide, its catch-relaxing activity can be expected to be less potent than that of FMRFamide. This is because it has been shown in the study of the structure-activity relation of FMRFamide that substitution of Tyr or D-Phe for Phe[1], N-terminal extensions and N-terminal acetylation are all deleterious to relaxing activity[9]. These facts have led us to suppose that substitution of Trp for Phe[1] would also be deleterious to relaxing activity. Contrary to our expectation, however, we found in the present experiments that W-Nle-RFamide is not less potent but 10-30 times more potent than FMRFamide in relaxation-inducing activity.

It has been suggested in the ABRM that FMRFamide elicits a contraction by a direct action on the muscle fibres[8,12] and that it causes a relaxation through its action on elements of the relaxing nerves in the muscle[8]. W-Nle-RFamide seems to produce a contraction or a relaxation by a similar manner to that of FMRFamide. After the muscle has been denervated by treating with KCl-EGTA solution, only slight or little relaxation is elicited by W-Nle-RFamide, whereas contractile response to the peptide can be produced almost without any change. Relaxation of catch tension in response to W-Nle-RFamide is also depressed by treating the muscle with α-methyldopa, an inhibitor of aromatic-L-aminoacid decarboxylase. These facts suggest that W-Nle-RFamide produces contraction acting directly on the muscle fibres, while it brings bout relaxation by acting on the relaxing nerve terminals to increase release of relaxing-neurotransmitter serotonin.

Low concentrations of W-Nle-RFamide, as well as FMRFamide[8], potentiate phasic contraction in response to repetitive electrical stimulation. The potentiating activity of W-Nle-RFamide is about 10 times more potent than that of FMRFamide. W-Nle-RFamide, at least at low concentrations, may exhibit its potentiating action through the release of relaxing-neurotransmitter serotonin. It has been shown that relaxing-transmitter serotonin not only relaxes catch tension but also potentiates contraction in the ABRM[7,15,18].

After the ABRM has been treated with high doses of FMRFamide, relaxations of ACh-induced catch tension caused by low concentrations of FMRFamide are markedly inhibited for hours[8]. In contrast, relaxations of catch tension caused by low concentrations of W-Nle-RFamide are inhibited only slightly by the pretreatment with high dose of FMRFamide. From these facts, it can be suspected that if *Mytilus* has a neuropeptide which activates relaxing nerve terminals, the peptide might be FMRFamide-like one rather than FMRFamide itself. Hirata et al[4] have suggested that the pedal ganglion has a peptide which can relax catch tension probably by acting on the relaxing nerve terminals and that the peptide is not FMRFamide, though it might be FMRFamide-like one.

It has been known that at least most of the neurons controlling the ABRM come from the pedal ganglion through the cerebro-pedal connective[1,17,19,20]. The present immunocytochemical study has shown that FMRFamide-immunoreactive neurons are present in the pedal ganglion and that FMRFamide-immunoreactive nerve branches are present in the ABRM. Painter[12] has reported that immunoreactive FMRFamide is present in the ABRM. Since FMRFamide antiserum used in these studies can recognize not only FMRFamide but also other FMRFamide-like peptides, the observed immunoreactivities might due to FMRFamide-like peptide and might not due to FMRFamide.

From the foregoing, it seems likely that a FMRFamide-like peptide or both FMRFamide and FMRFamide-like peptide are involved in the regulation of function of the ABRM of *Mytilus*. If this is the case, it is then very important to determine the structure of the FMRFamide-like peptide. This is because FMRFamide-like peptides have been detected in many animals including mammalians and suspected to play various important roles in those animals[2], but only several of the peptides has been structurally elucidated.

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