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Relaxing and Inhibitory Actions of Pedal Ganglion Extracts on the Anterior Byssus Retractor Muscle of *Mytilus*

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ABSTRACT

Pedal ganglion homogenates from the bivalve, *Mytilus edulis*, were subjected to gel filtration and the biological activities of the extracts were assayed on the ABRM of the mussel. The extracts resolved into a catch-relaxing peak and a contraction-inhibiting peak of activities.

Relaxation of ACh-induced catch tension in the ABRM by catch-relaxing peak was not affected by pretreatment of the muscle with 10^{-5} M FMRFamide, suggesting that the active principle of the peak is not FMRFamide. The relaxation was blocked by 5×10^{-4} M mersalyl, which suggests that the active substance is neither dopamine nor octopamine. The relaxation was markedly depressed after the muscle had been denervated by treating it with KCl-EGTA solution, suggesting that the substance is not serotonin and that it relaxes the catch tension acting on intramuscular relaxing nerve elements. The relaxing activity of the peak was destroyed by incubating it with a protease, subtilisin. Thus, the active substance in the peak seems to be a peptide which acts presynaptically to increase the release of relaxing transmitter serotonin.

The contraction-inhibiting peak also lost its activity when incubated with subtilisin, suggesting that the inhibitory substance in the peak is also a peptide. The substance inhibited not only phasic contraction by repetitive electrical stimulation but also ACh contraction and FMRFamide contraction, which suggests that it acts directly on muscle fibres to inhibit the contractions.

It has been shown that low concentrations of the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) and some of its analogs, such as Trp-Nle-Arg-Phe-NH_2, can relax catch tension induced by acetylcholine (ACh) in the anterior byssus retractor muscle (ABRM) of *Mytilus*[3,4,7]. It has also been demonstrated that crustacean red pigment concentrating hormone pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 (RPCH), whose analog has been suggested to be present in mollusc[1], can inhibit phasic contraction of the ABRM in response to repetitive electrical pulses of stimulation[6]. These facts lead to the speculation that the nervous system of *Mytilus* might have a FMRFamide-like catch-relaxing peptide and a RPCH-like contraction-inhibiting peptide.

In the present study, we assayed the biological activities of *Mytilus* pedal ganglion extracts on the ABRM of the mussel and found that the extracts resolved into a catch-relaxing peak and a contraction-inhibiting peak of activities.

The results obtained from pharmacological studies on the actions of the peaks suggest that both the catch-relaxing and contraction-inhibiting substances in the peaks are peptides. However, the results also suggest that the catch-relaxing substance is not FMRFamide and that, further,

the contraction-inhibiting substance is not RPCH.

MATERIALS AND METHODS

Animals

Mytilus edulis L. 5—7 cm in length were collected from Hiroshima Bay and stored in the laboratory as described previously[7]. The pedal ganglia were isolated from the animals within 10 hr of collection. For recording tension changes in the ABRM, the animals were used within 5 days of collection.

Pedal ganglion extracts

The pedal ganglia from 1000 mussels were excised, immediately frozen on dry ice, and stored at −20°C. The frozen ganglia were steeped in acetone (30 ml) and homogenized with Polytron. The homogenates were centrifuged at 3000 g for 15 min. The pellet was re-extracted with 80% acetone (10 ml). The two acetone supernatants were pooled and evaporated to dryness. The dried material was taken up in 2 ml of 0.1 N hydrochloric acid and the fluid was again centrifuged at 10000 g for 10 min. The supernatant was forced through a disposable C-18 cartridge (Waters Sep-Pak). The retained material was eluted with methanol (3 ml) and the effluent was evaporated to dryness. The residue was taken up in 0.1 M acetic acid (0.5 ml), applied to a column (2.6 × 40 cm) of Sephadex G-15, and eluted with the same solvent. Fractions of 60 drops, or about 4 ml, were collected and lyophilized. Each lyophilized material was taken up in 1 ml of distilled water and stored frozen at −20°C.

Bioassay

Small muscle bundle of the ABRM was dissected and mounted in an experimental chamber (10 ml), and tension changes in the muscle were recorded. The methods of dissection, stimulating and tension recording from the muscle have been described previously[7].

The frozen fractions were thawed before the bioassay experiments. Forty microliters of each fraction was removed, diluted in 10 ml of artificial seawater (ASW) and assayed on the ABRM.

The experiments were carried out at room temperature (20—25°C).

Physiological saline and drugs

The physiological saline employed was ASW. Its composition has been described previously[7]. 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[2,3].

Drugs used were as follows: acetylcholine bromide (ACh, from Sigma), mersalyl acid (from Sigma), subtilisin (from Boehringer Mannheim Biochemicals) and Phe-Met-Arg-Phe-NH_2 (FMRFamide, from Peninsula Labs.).

RESULTS AND DISCUSSION

We first examined the relaxing activities of the extracts on catch tension in the ABRM. Catch tension was produced by a brief application (for 2 min) of ACh. Five minutes after washing out ACh, a fraction was applied for another 5 min to the muscle in catch state, and relaxation of the tension was recorded. After recording the relaxation, the muscle was washed with normal ASW and then stimulated with repetitive electrical pulses (15 V, 3 msec, 10 Hz, for 5 sec) to relax it completely. Fifteen minutes after the stimulation, the next experiment was started by the same procedure to examine the activity of other fractions. The amount of relaxation was expressed as a percentage as described previously[6].

As shown in Fig. 1A, a peak of catch-relaxing activity was observed. The maximum relaxation was induced by fraction No. 29. The degree of relaxation by fraction No. 29 varied from preparation to preparation (see Fig. $2C_1$ and Fig. $5B_2$). In some muscles, catch tension was almost fully relaxed during the 5 min application of the fraction.

We next examined the effects of the extracts on peak tension of phasic contraction in response to repetitive electrical pulses of stimulation. In these experiments, the electrical stimulation was applied at 15 min intervals. Each fraction was introduced 10 min prior to the stimulation. Soon after recording phasic contraction in a fraction, the muscle was washed with normal ASW. Peak tension of phasic contraction was expressed as a percentage of the control peak tension.

As shown in Fig. 1B, a peak of contraction-inhibiting activity was observed. The maximum inhibition was induced by fraction No. 36 (see also Fig. 2B). In addition to a contraction-inhibiting peak, a small contraction-potentiating peak was found at around fraction No. 29 (see

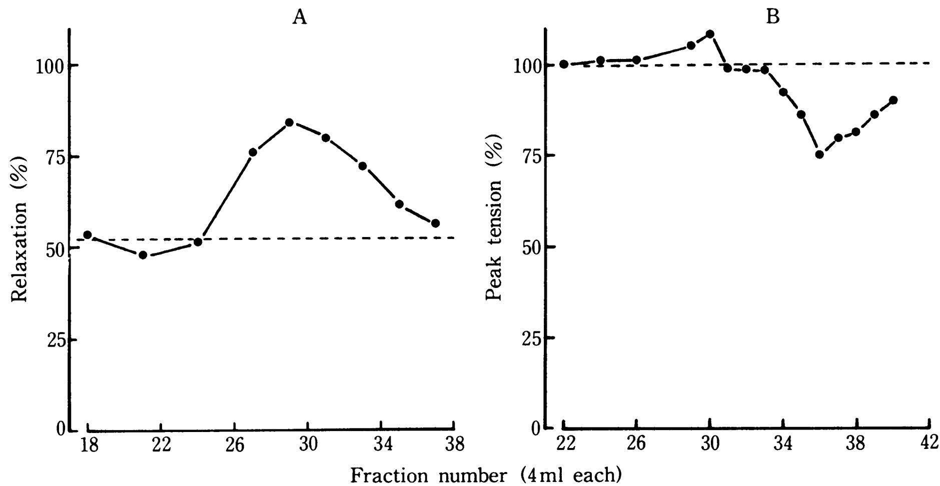

Fig. 1. Peaks of biological activities of the pedal ganglion extracts on the ABRM. A: catch-relaxing peak of activity. B: contraction-inhibiting peak of activity. Note that fraction No. 29 shows not only catch-relaxing activity but also weak contraction-potentiating activity. See also text.

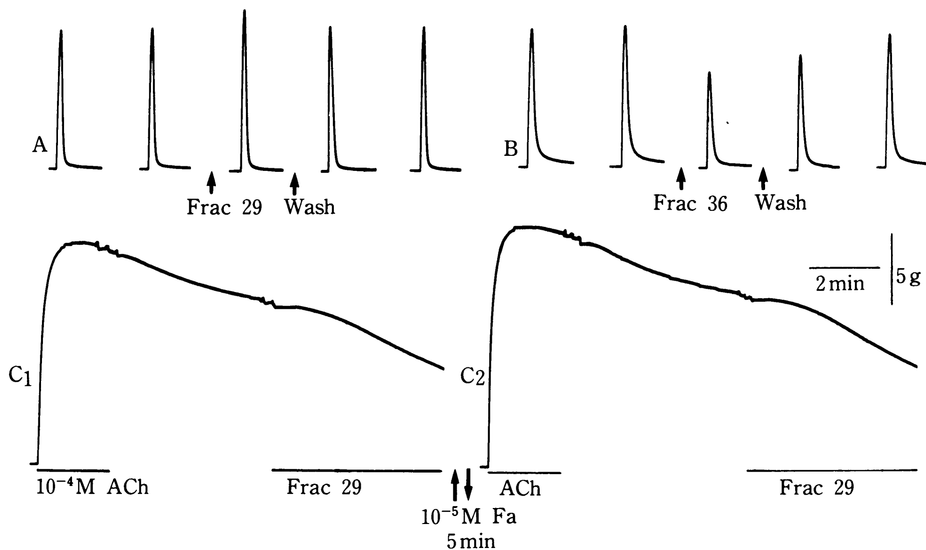

Fig. 2. Potentiating effect of fraction No. 29 (A) and inhibitory effect of fraction No. 36 (B) on phasic contraction, and relaxing effect of fraction No. 29 on catch tension (C). Phasic contraction was produced by stimulating the ABRM with repetitive electrical pulses (15 V, 3 msec, 10 Hz, for 5 sec) at 15 min intervals, and catch tension was induced by application of ACh for 2 min. A and B: fraction No. 29 or No. 36 was introduced 10 min prior to the stimulation and washed out soon after recording the contraction in the fraction. C: the muscle was treated for 5 min with 10^{-5} M FMRFamide during the 30 min interval between C_1 and C_2. A and C were obtained from the same muscle. Note that relaxing response to fraction No. 29 is not changed by FMRFamide treatment.

also Fig. 2A). This finding suggests that the catch-relaxing substance in fraction No. 29 may also have a contraction-potentiating action.

It has been shown that low concentrations of FMRFamide can relax ACh-induced catch tension in the ABRM and that they can also poten-

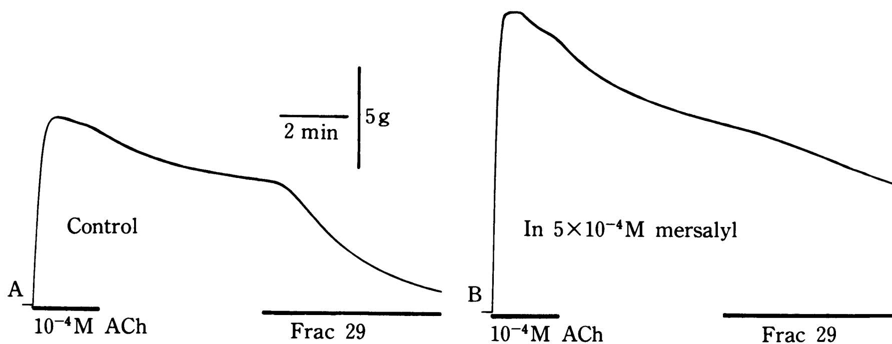

Fig. 3. Effect of 5 × 10^{-4} M mersalyl on relaxation of ACh-induced catch tension in response to fraction No. 29. A: control relaxation. B: block of relaxation in mersalyl. Mersalyl was introduced 10 min prior to ACh. Interval between responses is 30 min.

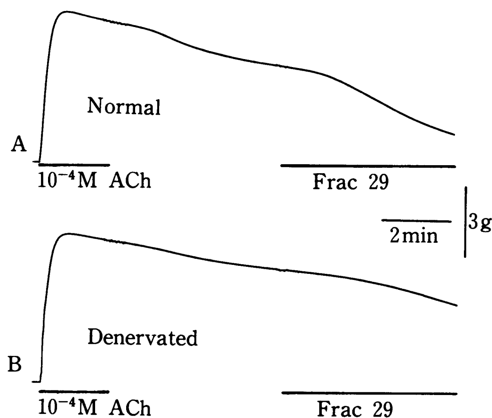

Fig. 4. Effect of treatment of the ABRM with KCl-EGTA solution on relaxing response to fraction No. 29. A: control relaxation of ACh-induced catch tension. B: relaxation after the treatment. Between A and B, the muscle was immersed in KCl-EGTA solution for 30 min and then returned to normal ASW for another 30 min, and test response was recorded.

tiate phasic contraction in response to repetitive electrical pulses of stimulation[3]. Therefore, it can be assumed that the relaxing substance in fraction No. 29 might be FMRFamide, but this was not the case. This is because, as shown in Fig. 2C, relaxation of ACh-induced catch tension by fraction No. 29 was not depressed after the muscle had been treated with high concentration (10^{-5} M) of FMRFamide. It has been shown that relaxation of catch tension in response to low concentrations of FMRFamide is markedly depressed after the muscle has been treated with high concentrations of FMRFamide[3,4].

It has been observed that relaxation of catch tension by serotonin can be blocked by 5 × 10^{-4} M mersalyl, whereas relaxation by dopamine or octopamine is not blocked[5,8]. As shown in Fig. 3, relaxation of catch tension by fraction No. 29 was blocked by 5 × 10^{-4} M mersalyl. Thus, it can be ruled out that the relaxing substance in the fraction is either dopamine or octopamine.

The foregoing results lead to the speculation that the relaxing substance in fraction No. 29 might be serotonin, but this possibility can also be ruled out. This is because, after the muscle had been denervated by treating it with KCl-EGTA solution, relaxation in response to the fraction was markedly depressed (Fig. 4). It has been demonstrated that relaxing response to serotonin is not depressed after the denervation treatment[2].

Contraction-inhibiting activity of fraction No. 36 was destroyed by incubating it with a protease, subtilisin (Fig. 5A). Catch-relaxing activity of fraction No. 29 was also destroyed by subtilisin (Fig. 5B). Thus, both of the inhibitory and relaxing substances in the fractions seem to be peptides.

Fraction No. 36 inhibited not only phasic contraction by repetitive electrical stimulation but also ACh contraction (Fig. 6) and FMRFamide contraction (Fig. 7). These findings suggest that the inhibitory substance in the fraction acts directly on the muscle fibres to inhibit the contractions.

The present experimental results suggest that

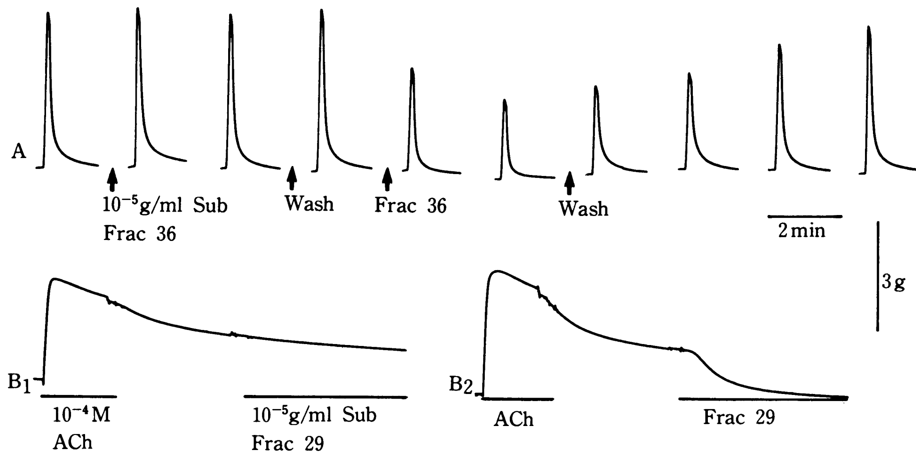

Fig.5. Loss of contraction-inhibiting activity of fraction No. 36 (A) and of catch-relaxing activity of fraction No. 29 (B) by incubating the fractions with a protease, subtilisin. Before dilution of the fractions with ASW, they were incubated with 10^{-5} g/ml subtilisin at 35°C for 30 min. The control fractions were kept at 35°C for 30 min without subtilisin. A: fraction No. 36 incubated with subtilisin was introduced 10 min before the second contraction (at the first arrow) and washed out soon after the third contraction (at the second arrow), and control fraction No. 36 was introduced 10 min before the fifth contraction (at the third arrow) and washed out soon after the sixth contraction (at the fourth arrow). B: after testing the effect of fraction No. 29 incubated with subtilisin on ACh-induced catch tension (B_1), the ABRM was washed with normal ASW and stimulated with repetitive electrical pulses to relax it, and then effect of control fraction No. 29 was examined (B_2). Interval between ACh responses is 30 min. The other procedures are the same as in Fig. 2.

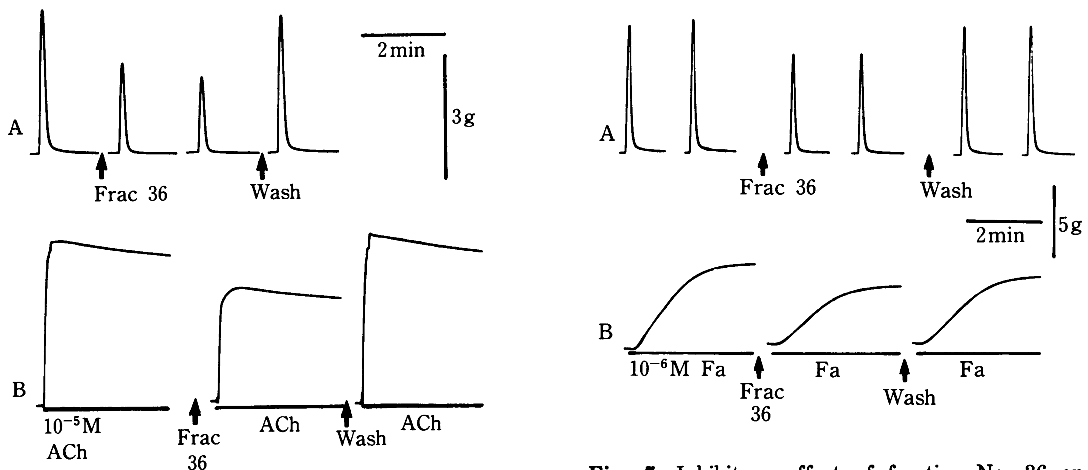

Fig. 6. Inhibitory effect of fraction No. 36 on phasic contraction in response to repetitive electrical pulses of stimulation (A) and on ACh contraction (B). After each record of contraction in response to ACh, which was applied for 3 min at 15 min intervals, the muscle was washed and stimulated with repetitive electrical pulses to relax it. A and B were obtained from the same muscle. The other procedures are the same as in Fig. 2.

Fig. 7. Inhibitory effect of fraction No. 36 on phasic contraction in response to repetitive electrical pulses of stimulation (A) and on FMRFamide (Fa) contraction. After each record of contraction in response to FMRFamide, which was applied for 3.5 min at 20 min intervals, the muscle was washed and stimulated with repetitive electrical pulses to relax it. A and B obtained from the same muscle. The other procedures are the same in Fig. 2.

a catch-relaxing peptide (CARP) and a contraction-inhibiting peptide (COIP) are present in the pedal ganglion of *Mytilus*.

Although CARP is probably not FMRFamide, the peptide, as well as FMRFamide and its some analogs[3,4,7], seems to bring about a relaxation of catch tension by acting on the relaxing nerve elements in the muscle. This is because relaxation of catch tension in response to fraction No. 29 is markedly depressed after the ABRM has been denervated by treating it with KCl-EGTA solution. CARP may increase release of relaxing neurotransmitter serotonin by acting on the relaxing nerve terminals in the muscle. Mersalyl block of relaxation of catch tension in response to fraction No. 29 may be a result of blocking action of the mercurial on released serotonin[8]. Thus, it is possible to suspect that CARP might be a novel FMRFamide-like peptide. Determination of the structure of CARP and examination of its action on the ABRM are required.

In contrast to CARP, COIP probably acts directly on the muscle fibres to inhibit contraction and thus, COIP is not RPCH. This is because RPCH inhibits phasic contraction by repetitive electrical stimulation but does not inhibit ACh contraction and FMRFamide contraction[6]. In addition, RPCH inhibits relaxing action of repetitive electrical stimulation, but COIP does not[6]. However, the possibility that COIP is a RPCH-like peptide cannot be ruled out.

Inhibitory peptide which acts physiologically on the ABRM is not yet known. In the present experiments, we could not suggest that COIP is a physiological inhibitory peptide in the ABRM, but this possibility remains to be confirmed. Therefore, determination of the structure of COIP and further studies of its action on the ABRM are also required.

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