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Peptidergic innervation of the vasoconstrictor muscle of the abdominal aorta in *Aplysia kurodai*

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Summary

The arterial system of the marine mollusc *Aplysia* consists of three major arteries. One of them, the abdominal aorta, has a sphincter (the vasoconstrictor muscle) at the base of the artery. Contraction of this muscle reduces the blood flow into the abdominal aorta, thereby, playing a role in the regulation of the blood distribution in *Aplysia*. Here, we show the contractility of the vasoconstrictor muscle is modulated by three types of endogenous peptides, *Aplysia mytilus* inhibitory peptide-related peptides (AMRP), enterin and NdWFamide. Immunohistochemistry showed that putative neuronal processes containing the three peptides exist in the vasoconstrictor muscle. Enterin inhibited the muscle contraction elicited by the nerve stimulation or the application of a putative excitatory transmitter, acetylcholine (ACh). Enterin hyperpolarized the resting potential of the muscle and decreased the amplitude of the excitatory junction potential (EJP). AMRP also inhibited the nerve-evoked contraction although its action on the ACh-induced contraction was variable. AMRP also reduced the size of EJP, but had no effect on the resting potential of the muscle. NdWFamide enhanced the nerve-evoked contraction but not the ACh-induced contraction. NdWFamide augmented EJP without affecting the resting potential of the muscle. These results suggest that AMRP, enterin and NdWFamide are endogenous modulators of the contractile activity of the vasoconstrictor muscle, and that the peptidergic innervations of this muscle contribute to fine tuning of the blood distribution in *Aplysia*.

Key words: cardiovascular system, peptide, mollusc, *Aplysia*.

Introduction

The function of the circulatory system of animals is to exchange gases, transport nutrients and excrete wasted materials, to maintain an appropriate ambiance around the tissues. In animals with closed circulatory systems (e.g. vertebrates), the heart pumps blood into arteries connected to a network of capillaries in the tissues. In response to physiological demands, changes in blood vessel diameter modulate blood flow into the capillaries and, thus, modify the distribution of blood among the tissues (Musch et al., 1987; Norton et al., 1990; Rowell, 1993). In the open circulatory system of many invertebrates, the blood (hemolymph) carried by the vessels flows directly into tissue spaces, and eventually returns through sinuses to the heart. Some invertebrates (e.g. crustaceans and molluscs) have relatively well developed arterial systems, and the blood distribution is regulated by changing the arterial blood flow.

The blood distribution of crustaceans is regulated by neuronal as well as neurohormonal control of the cardioarterial valves located at the base of each artery (Kihara and Kuwasawa, 1984; Kuramoto and Ebara, 1984; Kuramoto et al., 1992; F.-Tsukamoto and Kuwasawa, 2003). Regulation of the blood flow by the valves is presumably a mechanism underlying the physiological modification of the blood distribution observed in a decapode crustacean *in vivo* (Airriess and McMahon, 1994; De Wachter and McMahon, 1996; McGaw and McMahon, 1996). A functionally similar mechanism is observed in a marine mollusc, *Aplysia*. There are three major arteries in *Aplysia* and one of them, the abdominal aorta, carries blood from heart to hepatopancreas and ovotestis. The abdominal aorta has a sphincter (the vasoconstrictor muscle) at the base of the artery (Mayeri et al., 1974). The activity of the vasoconstrictor muscle influences the blood distribution because the contraction of this muscle prevents the blood flow into the abdominal aorta and enhances the flow into the other two arteries (Mayeri et al., 1974; Koch and Koester, 1982; Koch et al., 1984). Activity of the vasoconstrictor muscle is relevant to feeding (Koch and Koester, 1982; Koch et al., 1984) and respiratory pumping (Koester et al., 1974; Byrne and Koester, 1978; Kandel 1979), and is known to be modulated by some peptides (Alevizos et al., 1989, 1991).

Recently, three different types of peptides (AMRP, enterin, NdWFamide) were identified in *Aplysia* (Morishita et al.,

1997; Fujisawa et al., 1999; Furukawa et al., 2001). Immunohistochemical experiments showed that the peptide-containing neuronal processes exist in the cardiovascular system including the abdominal aorta (Fujisawa et al., 1999; Sasaki et al., 2002b; Morishita et al., 2003a). Although the immunohistochemical results imply a regulatory role for these peptides in the abdominal aorta, physiological actions of the peptides on the contractile activity of the vasoconstrictor muscle are not yet established. The aim of the present study is to examine the actions of AMRP, enterin and NdWFamide in the vasoconstrictor muscle, and to delineate possible physiological roles of the peptides in the cardiovascular regulation of *Aplysia*.

Materials and methods

Animals

Aplysia kurodai Baba (80–200 g) were collected at the Hiroshima Bay and on the seashore in Shimane. Some animals were kindly provided by M. Kurokawa. Animals were maintained in an aquarium containing artificial seawater (Yashima, Japan) at 15°C and fed seaweed.

Immunohistochemistry

Animals were anesthetized by intra-abdominal injection of 0.33 mol l^{-1} $MgCl_2$. The abdominal aorta together with the abdominal ganglion was excised from the anesthetized animal and was bathed in a chamber containing the artificial sea water (ASW). ASW had the following composition (mmol l^{-1}): NaCl 460, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55, Tris-HCl 10, pH 7.8. The abdominal aorta was fixed by 4% paraformaldehyde for 20–24 h at 4°C or for 2–3 h at room temperature. Whole mount immunocytochemistry was carried out essentially as described previously (Fujisawa et al., 1999). The anti-enterin and anti-AMRP antibodies were kindly provided by F. S. Vilim. The preparation was viewed with a fluorescence microscope (Nikon, Tokyo, Japan) and photographed. The films were scanned by a film scanner (Coolscan III, Nikon) and printed using Photoshop (v5, Adobe Systems, San Jose, CA, USA).

Physiological recordings

The abdominal aorta was excised from the animal with the innervation from the pericardial nerve or the spermathecal nerve left intact. To record contractions of the vasoconstrictor muscle, the abdominal aorta was cut open longitudinally. One end of the vasoconstrictor muscle was pinned to the bottom of a recording chamber (0.8 ml volume) and the other end was connected to a force-transducer (Type 45196A, NEC San-ei Instrument Ltd, Tokyo, Japan) by a cotton thread. The signal from the transducer was monitored on a chart recorder (FBR-251A, TOA Electronics Ltd, Tokyo, Japan) and also digitized using a 12-bit AD converter (ADXM-AT10, Canopus, Kobe, Japan). The digitized data were stored on the hard disk of a personal computer (IBM, Tokyo, Japan). The data analysis and the compilation of figures were done using Origin (v6, Microcal Software Inc., Northampton, MA, USA). During the experiment, the recording chamber was continuously perfused with ASW (3-4 ml min^{-1}). All the peptides and drugs were applied by perfusing the bath with the peptide and/or drug containing ASW. In some experiments, we monitored the activity of the vasoconstrictor muscle by measuring the intra-arterial pressure change as described (Liebeswar et al., 1975). Briefly, one end of a three-way perfusion tube was inserted into the distal portion of aorta. The other two ends of the three-way tube were connected to a pressure transducer (DT4812J, Nippon Becton Dickinson Company, Ltd, Tokyo, Japan) and a peristalic pump, respectively. The abdominal artery was continuously perfused with ASW by the pump (4 ml min^{-1}). In this way, the contraction of the vasoconstrictor muscle was monitored as an increase in the inner pressure of the perfusion line. In most experiments, the contraction of the vasoconstrictor muscle was evoked by electrical stimulations of the pericardial nerve with a suction electrode. The pericardial nerve was stimulated by a train of the electrical pulse (1 ms, 1.0–3.0 V, 10 Hz) for 0.1–2.0 s every 20 s. In some experiments, the contraction was induced by bath application of acetylcholine (ACh) for 20–40 s.

Membrane potential of the vasoconstrictor muscle was measured using a conventional microelectrode method as described (Sasaki et al., 2002b). To restrict the movement of the vasoconstrictor muscle, a small piece of nylon mesh (approximately 100 μm between the grid) was pinned over the aorta, and the muscle fiber was penetrated through the mesh with a sharp microelectrode filled with a solution containing 3 mol l^{-1} CH_3COOK and 0.1 mol l^{-1} KCl (resistance 40–60 MΩ). The preparations were grounded directly using an Ag/AgCl electrode. Excitatory junction potentials (EJPs) were elicited by the pericardial nerve stimulation. The membrane potentials were amplified by the Duo 773 electrometer (World Precision Instruments, Sarasota, FL, USA), and were stored as described above. The results are expressed as means ± S.E.M. All the experiments were performed at room temperature (20–23°C). To assess the statistical difference between the control and the experimental groups, an *F*-test was conducted first to determine the equality of the variances between the two groups. When the variance was not considered to be equal, the difference between the groups was tested by a Mann-Witney *U*-test. Otherwise, a Student's *t*-test was used. The results were considered significant if $P<0.05$.

Peptides and chemicals

ACh, 4-aminopyridine (4-AP) and hexamethonium were purchased from Sigma (St Louis, USA) and were dissolved in ASW just before use. APSFGHSFVamide (ENpa), GSPRFFamide, and NdWFamide were synthesized with an automated solid-phase peptide synthesizer (PSSM8, Shimadzu, Kyoto, Japan) and purified by reversed-phase high-performance liquid chromatography. Although enterins and AMRPs are families of multiple related peptides, we used ENpa (enterin) and GSPRFFamide (AMRP) throughout this study because previous studies in the anterior aorta showed little difference in potency among different enterins or AMRPs

(Sasaki et al., 2002a,b). Peptide was dissolved in distilled water to make a concentrated stock solution (10^{-2} mol l^{-1}). The stock solution was stored at –20°C and diluted appropriately just before use.

Results

Peptidergic innervation of the vasoconstrictor muscle in the abdominal aorta

The vasoconstrictor muscle of the abdominal aorta in *A. kurodai* constitutes a dense circular muscle band that is located at about a few millimeters from the origin of the aorta. We first confirmed the innervation of the vasoconstrictor muscle by monitoring the contraction elicited by the electrical stimulation of the nerves arising from the abdominal ganglion because it is known that the abdominal ganglion contains motoneurons that innervate the vasoconstrictor muscle in *A. californica* (Mayeri et al., 1974). We found two pathways to the vasoconstrictor muscle: the pericardial nerve and the spermathecal nerve (Fig. 1A). The branches of both nerves could be followed without staining under a stereomicroscope. The pericardial nerve is divided into two branches, and one of them innervates the vasoconstrictor muscle as described in *A. californica* (Mayeri et al., 1974). Conversely, the spermathecal nerve diverges many times after leaving the ganglion, and one of the branches was found to enter the vasoconstrictor muscle.

We next verified distribution of the AMRP-, enterin- and NdWFamide-immunoreactivity in the vasoconstrictor muscle. Fig. 1A shows an example of the distribution of AMRP-immunoreactivity in the vicinity of the abdominal ganglion and three main arteries. All three arteries were densely innervated by AMRP-immunoreactive fibers. In the abdominal aorta and gastroesophageal artery, all the AMRP-immunoreactive fibers originated from the pericardial nerve and the spermathecal nerve. Main immunoreactive nerve trunks on the abdominal aorta were oriented in the longitudinal direction, and many

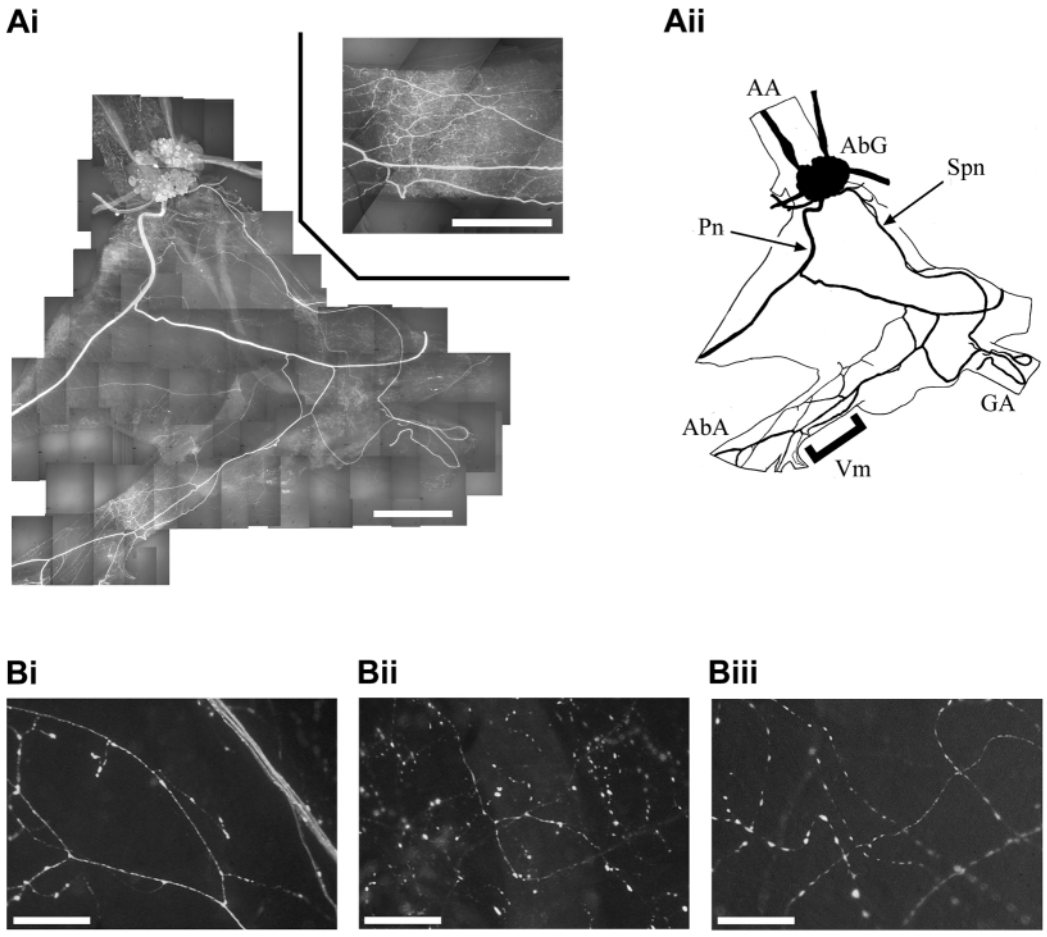

Fig. 1. Innervation pattern of the vasoconstrictor muscle viewed by immunohistochemistry. (Ai) AMRP-immunoreactivity in the vicinity of the abdominal ganglion and three major arteries. The preparation is placed ventral side up except for the abdominal ganglion (the ganglion is rotated 180° to see the dorsal surface). The abdominal aorta is mainly innervated by the branches of the pericardial and spermathecal nerves (see Aii). Scale bar is 2 mm. Inset shows a higher magnification of a part of the abdominal aorta including the vasoconstrictor muscle. Note the extensive ramification of the immunopositive fibers. Scale bar in the inset is 1 mm. (Aii) Diagram of the preparation shown in Ai. AA, anterior aorta; AbA, abdominal aorta; AbG, abdominal ganglion; GA, gastroesophageal artery; Pn, pericardial nerve; Spn, spermathecal nerve; Vm, a region containing the vasoconstrictor muscle. (B) Immunopositive fibers and varicosities in the vasoconstrictor muscle. Scale bars in all figures are 100 μm. (Bi) AMRP-immunoreactivity. (Bii) Enterin-immunoreactivity. (Biii) NdWFamide-immunoreactivity.

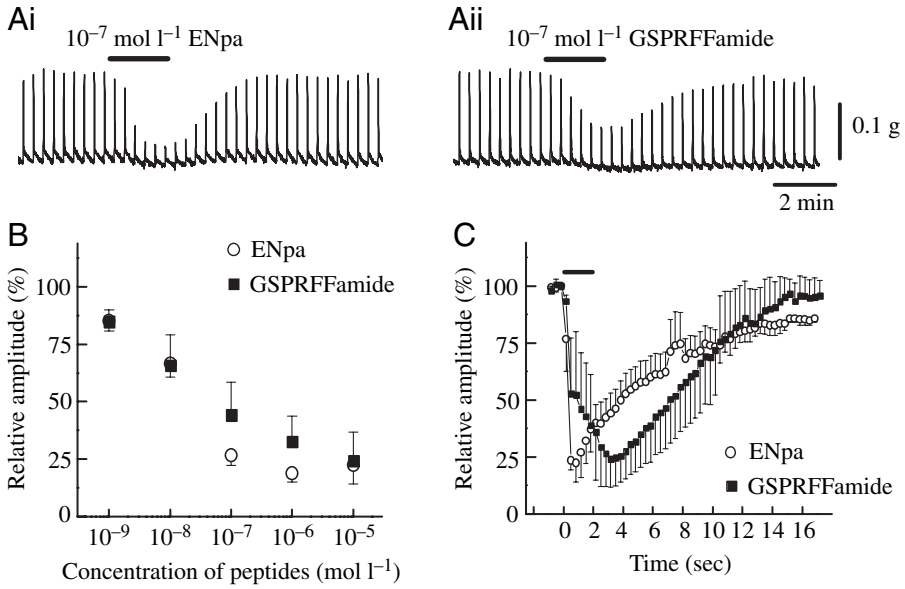

Fig. 2. ENpa and GSPRFFamide depress the contraction of the vasoconstrictor muscle evoked by the stimulation of the pericardial nerve. (A) Effects of 10^{-7} mol l^{-1} ENpa (Ai) and 10^{-7} mol l^{-1} GSPRFFamide (Aii) on the contraction evoked by a train of the electrical pulse (1 ms, 1.6 V, 10 Hz) for 1 s. The stimulus train was applied every 20 s. Peptides were applied for 2 min as indicated by bars. Ai and Aii are from the same preparation. (B) Concentration–response relationships of the effects of ENpa and GSPRFFamide. Amplitude of the most depressed contraction after the peptide application is normalized to that of the control contraction and the mean ± S.E. was plotted against the concentration of the peptides (N=3~6). (C) Time-dependent effects of the peptide actions. Amplitude of the muscle contraction was normalized to the control contraction just before the peptide application and plotted against the time (N=3). 10^{-5} mol l^{-1} of ENpa or GSPRFFamide was applied for 2 min as indicated.

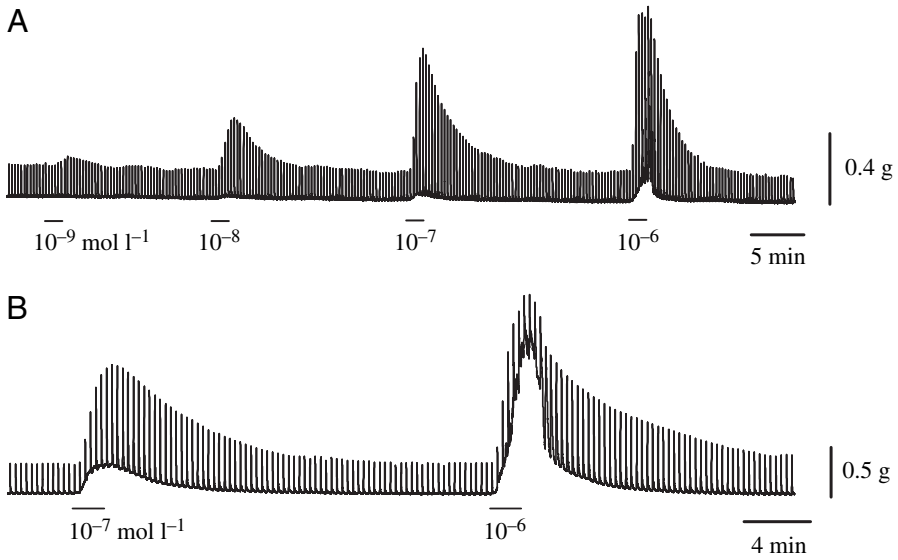

Fig. 3. NdWFamide enhances the nerve-evoked contractions of the vasoconstrictor muscle. (A) and (B) are from the different preparations. The contraction of the muscle was evoked by the nerve stimuli as described in Fig. 2. Duration of each stimulus train in A and B were 1 s and 0.4 s, respectively. Note a tonic contraction was also induced by NdWFamide at higher concentrations.

small immunoreactive fibers surrounded the vasoconstrictor muscle (Fig. 1A). No peripheral immunoreactive cell bodies were found in the abdominal aorta. The distribution pattern of the enterin- or NdWFamide-immunoreactive fibers in the abdominal aorta was almost the same to that of the AMRP-immunoreactivity (Fig. 1B). Quantitatively, however, the enterin- or NdWFamide-immunoreactivity seemed to be less than that of AMRP. These immunohistochemical analyses suggest that AMRP, enterin and NdWFamide may play roles in the regulation of the vasoconstrictor muscle of the abdominal aorta.

Action of the peptides on the contractility of the vasoconstrictor muscle

To clarify the function of the peptides, their actions on the nerve-evoked contraction of the vasoconstrictor muscle were examined. The stimulus trains applied to the pericardial nerve (see Materials and methods) reliably induced contractions of the vasoconstrictor muscle, which permitted testing the action of the peptides unequivocally. Although the data were not presented in this paper, similar results were obtained on the contractions elicited by the spermathecal nerve stimulation.

Fig. 2 shows the effects of 10^{-7} mol l^{-1} ENpa and GSPRFFamide on the nerve-evoked contractions of the vasoconstrictor muscle. The peptide was applied for 2 min because a longer application did not affect the contraction further in preliminary experiments. In our perfusion system, it took about 25 s to replace the bath solution completely (estimated by the dye perfusion). Amplitude of the contractions was reversibly inhibited by application of either peptide. Both peptides sometimes relaxed the basal tonus of the vasoconstrictor muscle slightly. To quantify the concentration–response relationship of the peptide action, the relative amplitude of the most depressed contraction after the peptide application was calculated and plotted against the concentration of the peptide (Fig. 2B). Threshold concentrations of both peptides were less than 10^{-9} mol l^{-1}, and

their overall dose–response relationships were almost identical. To compare the time course for the onset and the recovery of the inhibitory actions, each contraction elicited every 20 s was normalized and plotted as a function of time. The inhibitory actions of ENpa and GSPRFFamide had a similar time course up to the concentration of 10^{-7} mol l^{-1} or less. The differences in the time course, however, became apparent at the concentration of more than 10^{-6} mol l^{-1} (Fig. 2C). The inhibitory action of 10^{-5} mol l^{-1} ENpa became to its peak within a minute, and started to decline even in the presence of the peptide. By a sharp contrast, the inhibitory action of GSPRFFamide grew slowly, and its maximum effect was always seen during washing out the peptide. The different time course at higher concentration of the peptides suggests that the mechanisms for the inhibitory actions of ENpa and GSPRFFamide are not identical.

In contrast to ENpa and GSPRFFamide, NdWFamide enhanced the amplitude of the nerve-evoked contractions of the vasoconstrictor muscle (Fig. 3). The amplitude of the nerve-evoked contraction could be enhanced to more than 500% of the control contraction. NdWFamide also evoked a tonic contraction of the muscle although the amplitude of the tonic contraction was quite different among preparations. When the tonic contraction was large enough, the potentiation of the nerve-evoked contraction was masked (Fig. 3B). Although the threshold concentration for the potentiating action of NdWFamide was less than 10^{-9} mol l^{-1} in all the tested preparations, it was difficult to evaluate the potentiating action by the amplitude of the nerve-evoked contraction in most preparations because of the apparently mixed actions of NdWFamide. In those preparations which showed relatively small tonic contractions, the potentiating action of the nerve-evoked contraction reached a plateau at more than 10^{-6} mol l^{-1}. In general, the action on the nerve-evoked contraction was long lasting compared with the action on the basal tonus and persistent after washing out the peptide (see also Fig. 12).

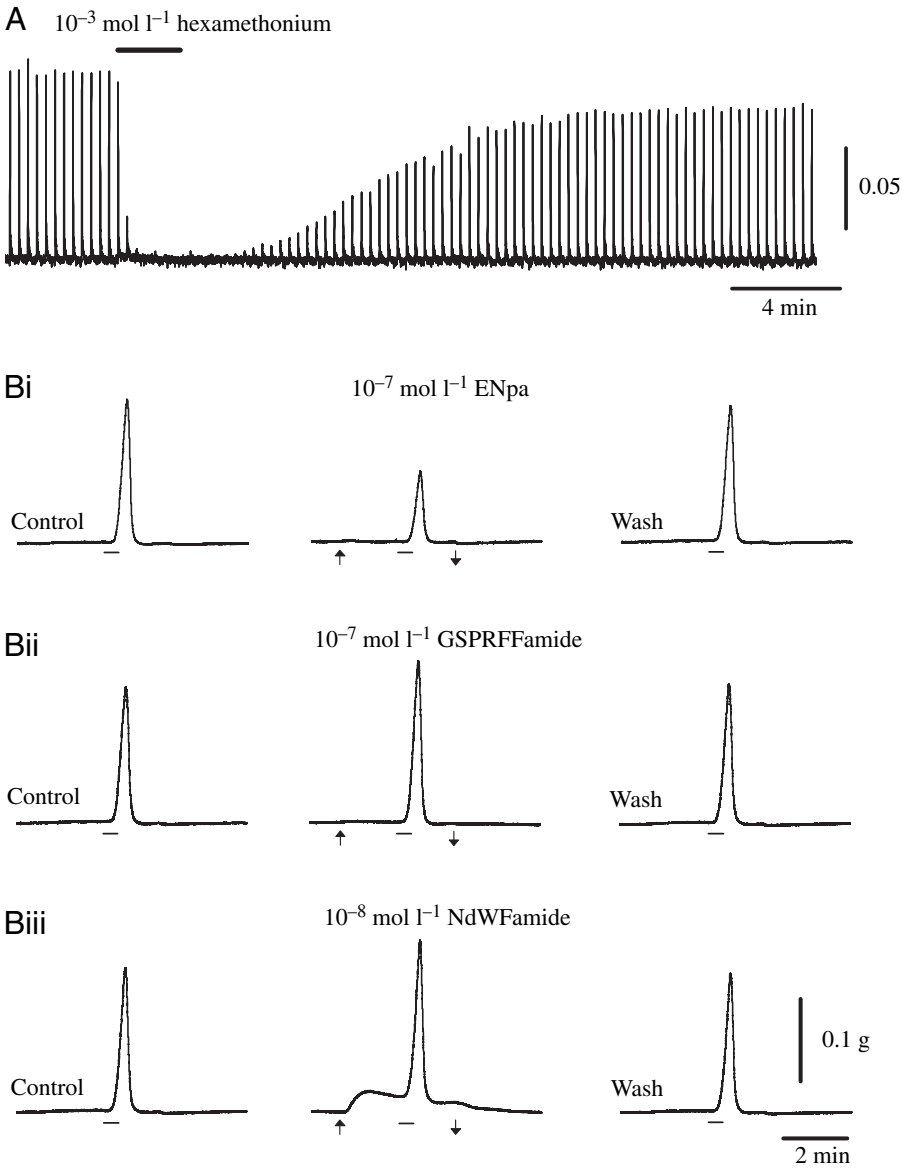

Fig. 4. Action of ENpa, GSPRFFamide or NdWFamide on the ACh-induced contraction of the vasoconstrictor muscle. (A) Blockade of the nerve-evoked contraction by 10^{-3} mol l^{-1} hexamethonium. Each contraction was evoked by the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.5 s every 20 s. (B) Effect of ENpa (Bi), GSPRFFamide (Bii) or NdWFamide (Biii) on the ACh-induced contraction. The contraction was evoked by perfusion of 10^{-6} mol l^{-1} ACh for 40 s as indicated by bars. Peptide was applied between arrows.

Action of the peptides on the ACh-induced contraction of the vasoconstrictor muscle

In *A. californica,* the identified vasoconstrictor motoneurons are suggested to be cholinergic because their actions are blocked by a cholinergic antagonist, hexamethonium (Liebeswar et al., 1975). The nerve-evoked contraction of the vasoconstrictor muscle in the present preparation was also blocked reversibly by 10^{-3} mol l^{-1} hexamethonium (Fig. 4A; N=4), suggesting that the excitatory innervation of the muscle in *A. kurodai* is also mainly cholinergic. To determine whether the modulatory actions of the peptides are mediated postsynaptically, we next examined actions of the peptides on the ACh-induced contraction. ACh was applied by a brief perfusion (<40 s) every 20–25 min to avoid a desensitization of the ACh response. In Fig. 4B, 10^{-6} mol l^{-1} ACh was

perfused for 40 s to evoke each contraction. In the presence of 10^{-7} mol l^{-1} ENpa, the ACh-induced contraction was depressed to 40% of the control (Fig. 4Bi). In the same preparation, 10^{-7} mol l^{-1} GSPRFFamide did not inhibit the ACh-induced contraction but rather enhanced it slightly (Fig. 4Bii). Effect of GSPRFFamide was quite variable depending on the preparations, and either modest inhibition or some potentiation of the ACh-induced contraction was observed (see Fig. 5). 10^{-8} mol l^{-1} NdWFamide did not affect the ACh-induced contraction (Fig. 4Biii).

Effects of each peptide on the nerve-evoked contraction and the ACh-induced contraction are compared in Fig. 5. ENpa always inhibited the contractions evoked by both methods although the inhibitory potencies were somewhat variable among the preparations (Fig. 5A). Mean ± S.E. of the relative contractions evoked by the nerve stimulation and the ACh application were 23.0±4.2% (*N*=13) and 51.7±8.5% (*N*=12), respectively. The different potencies of ENpa examined in the two conditions are statistically significant ($P<0.05$, Mann-Witney *U*-test), suggesting that the inhibitory action of ENpa is produced by both presynaptic and postsynaptic mechanisms. In some preparations, ENpa reduced the amplitude of the nerve-evoked contraction to 7.1±4.3% of the control although the ACh-induced contraction was rarely inhibited (93.9±1.2%, *N*=3).

Similarly, GSPRFFamide always decreased the nerve-evoked contraction (Fig. 5B). The mean inhibitory action (33.7±7.6% of the control, *N*=11) was comparable to that of ENpa. By contrast, its effect on the ACh-induced contraction was extremely variable as noted above. In the presence of GSPRFFamide, the relative contractions of seven preparations were between 70.2 and 156.9% of the control (109.6±11.9%, *N*=7). Such variability indicates the complexity of the AMRP action in this system. Nevertheless, the differences between the mean effects of GSPRFFamide in two conditions are statistically significant, suggesting that the inhibitory action of GSPRFFamide is mainly presynaptic.

NdWFamide enhanced the nerve-evoked contraction considerably (430.4±45.1% of the control, *N*=10; Fig. 5C), but had essentially no effect on the ACh-induced contraction (110.3±3.2%, *N*=4). These results clearly suggest that potentiation of the contractility of the vasoconstrictor muscle by NdWFamide is due to the enhancement of the excitatory transmitter release.

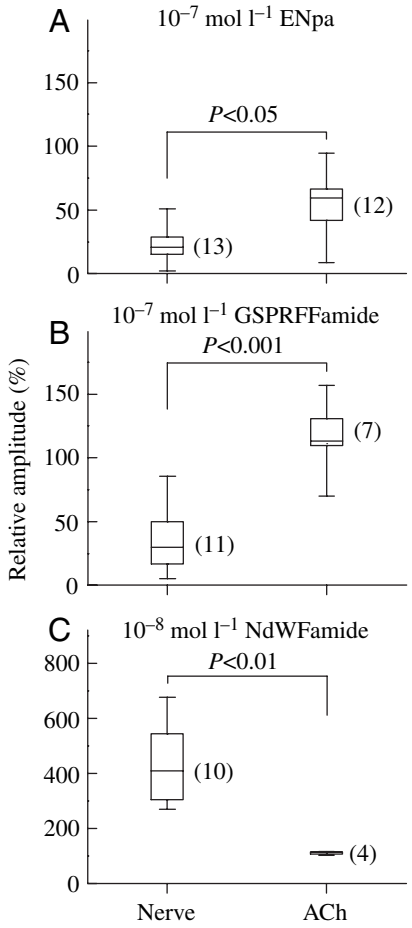

Fig. 5. Comparison of the effects of the peptides on the nerve-evoked and the ACh-induced contractions. (A) 10^{-7} mol l^{-1} ENpa, (B) 10^{-7} mol l^{-1} GSPRFFamide, (C) 10^{-8} mol l^{-1} NdWFamide. Upper, middle and lower lines of the box plots show 75th percentile, median and 25th percentile, respectively. Upper and lower bars show the largest and smallest values. Numbers of preparations are shown in parentheses. Statistical significance was assessed either by Mann-Witney *U*-test (A,C) or Student's *t*-test (B).

Action of the peptides on the resting and excitatory junction potentials of the vasoconstrictor muscle

We next examined whether the resting potential of the

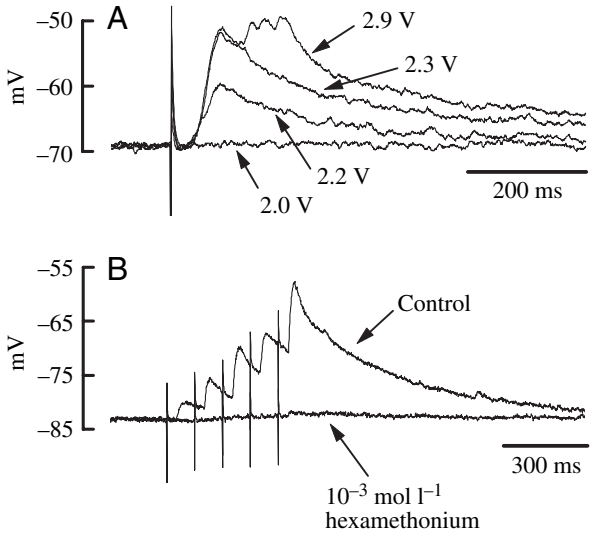

Fig. 6. Single vasoconstrictor muscle fiber is innervated by several excitatory axons. (A) Relationships between stimulus intensities and EJPs. The pericardial nerve was stimulated by a 1 ms pulse of various intensities as indicated. Four records are superimposed. Fast vertical deflections are stimulus artifacts. Note that the increase in stimulus intensity not only changes the peak amplitude of EJP, but also the shape of EJP. (B) Effect of 10^{-3} mol l^{-1} hexamethonium on EJPs evoked by the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.5 s.

arterial muscle and the excitatory junction potentials (EJPs) evoked by the nerve stimulation were affected by the peptides. In ASW, the resting potential of the vasoconstrictor muscle was –77.4±1.2 mV (*N*=37). We often observed spontaneous EJPs but not the inhibitory junction potentials (IJPs, not shown). Also, the nerve stimulation did not evoke IJPs in the present experiments. Fig. 6A shows the EJP in the vasoconstrictor muscle elicited by a single brief stimulation of the pericardial nerve. In this preparation, a threshold stimulus intensity to evoke an EJP was between 2.0 and 2.1 V. The threshold was different among preparations, ranging from 1.0 to 2.2 V. In the preparation shown in Fig. 6, the peak amplitude of the EJP evoked by 2.3 V became twice of that evoked by 2.2 V. Further increase in the stimulus intensity did not increase the size of the EJP. When the intensity was increased to 2.9 V, multiple EJPs having longer latencies were also observed in addition to the initial EJP. These results suggest that a single vasoconstrictor muscle fiber is innervated by multiple excitatory axons. Multiple excitatory innervation and the lack of IJPs are also described in *A. californica* (Mayeri et al., 1974). Repetitive nerve stimulations produced a summation of EJPs, and in some cases, a marked facilitation of EJPs was observed (Fig. 6B). EJPs were reversibly abolished by hexamethonium (Fig. 6B; *N*=4), supporting the above mentioned hypothesis that ACh is the main transmitter released from the excitatory nerve terminals following the stimulation of the pericardial nerve.

To quantify the effects of the peptides on EJP, stimulation intensity was adjusted before each experiment so that a single nerve stimulation evokes an EJP with a single peak. Because the nerve stimulation sometimes failed to evoke EJP and the size of a single EJP was often quite small, summated EJPs in response to several stimuli were examined to see the effects of peptides in most cases.

Fig. 7 illustrates the effect of ENpa on EJP. Summating EJPs were elicited by three consecutive stimuli of the pericardial nerve. Application of 10^{-7} mol l^{-1} ENpa reduced the amplitude of each EJP as well as the summated EJPs. In control, the peak depolarization of the summated EJPs was 17.7±0.7 mV whereas it became 10.1±1.3 mV in the presence of 10^{-7} mol l^{-1} ENpa (Fig. 7C, *N*=15). ENpa slightly hyperpolarized the resting potential of some muscle fibers (3.3±0.4 mV, 7 out of 15).

Fig. 8 shows the effect of GSPRFFamide on EJP of the vasoconstrictor muscle. When 10^{-7} mol l^{-1} GSPRFFamide was applied, EJP was depressed markedly. The summated EJPs of 18.6±0.6 mV was depressed to 8.4±1.2 in the presence of GSPRFFamide (Fig. 8C, *N*=14). Unlike ENpa, however, GSPRFFamide did not hyperpolarize the resting potential in all the tested muscle fibers (*N*=14).

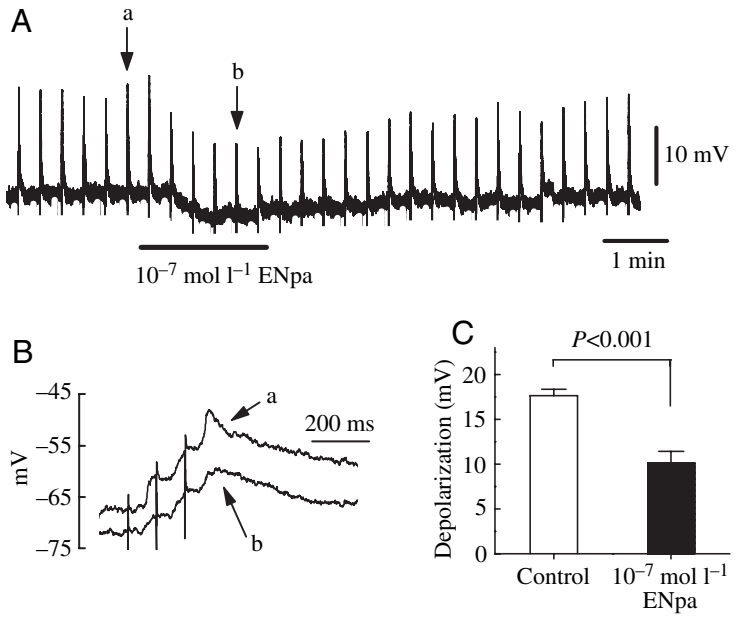

Fig. 7. ENpa decreases the amplitude of EJPs. (A) Effects of 10^{-7} mol l^{-1} ENpa. EJPs were evoked by the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.3 s every 20 s. Note ENpa also hyperpolarized the resting potential of the muscle fiber. (B) Comparison of EJPs before (a) and after (b) ENpa application. (C) Inhibitory action of ENpa on EJPs. Histogram shows mean ± S.E. of the maximum amplitude of summated EJPs before and after ENpa application (*N*=15). Statistical significance was assessed by Student's *t*-test.

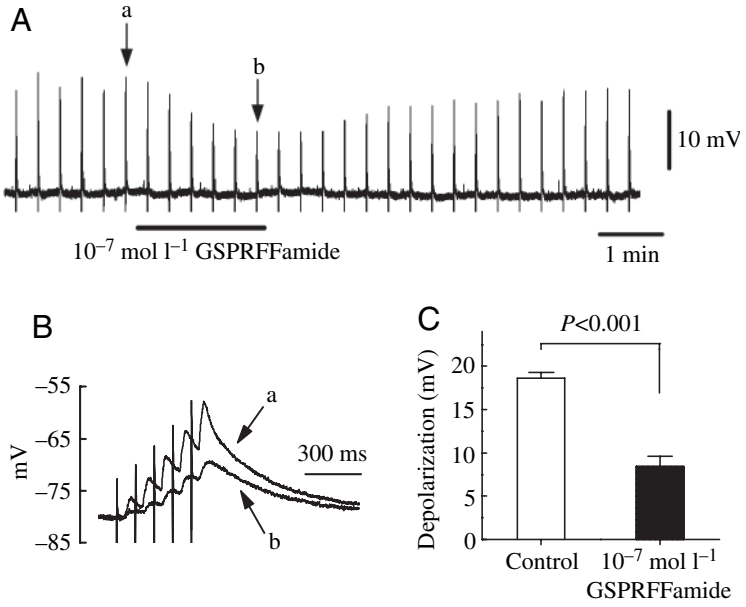

Fig. 8. GSPRFFamide decreases the amplitude of EJPs. (A) Effects of 10^{-7} mol l^{-1} GSPRFFamide. EJPs were evoked by the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.5 s every 20 s. (B) Comparison of EJPs before (a) and after (b) GSPRFFamide application. (C) Inhibitory action of GSPRFFamide on EJPs. Histogram shows mean ± S.E. of the maximum amplitude of summated EJPs before and after GSPRFFamide application (*N*=14). Statistical significance was assessed by Student's *t*-test.

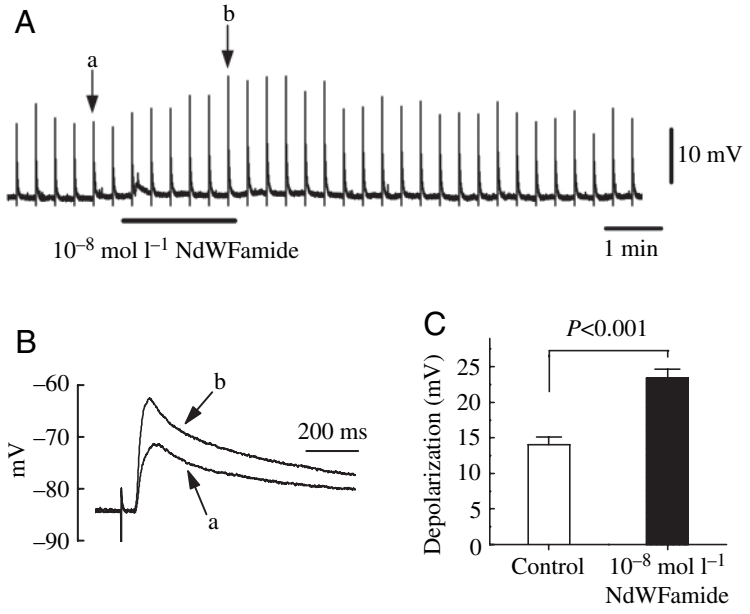

Fig. 9. NdWFamide increases the amplitude of EJP. (A) Effects of 10^{-8} mol l^{-1} NdWFamide on EJP. Each EJP was evoked by a single nerve stimulation (1 ms, 1.6 V). (B) Comparison of EJPs before (a) and after (b) NdWFamide application. (C) Excitatory action of NdWFamide on EJPs. Histogram shows mean ± S.E. of the maximum amplitude of summated EJPs before and after NdWFamide application (N=9). Statistical significance was assessed by Student's t-test.

The effect of NdWFamide on EJP is illustrated in Fig. 9. In these experiments, EJP was elicited by a single nerve stimulation. NdWFamide enhanced the amplitude of EJP reversibly but did not affect the resting potential. In the presence of 10^{-8} mol l^{-1} NdWFamide, the EJP (14.1±1.0 mV) was increased to 23.4±1.3 mV (Fig. 9C, N=9).

Action of enterin and AMRP in the presence of 4-AP

In the anterior aorta of *A. kurodai*, enterin hyperpolarizes the membrane potential of the arterial muscle through the activation of the 4-AP-sensitive K^+ channels, and the inhibitory action on the nerve-evoked contraction disappears in the presence of 4-AP (Sasaki et al., 2002b). AMRP also activates the K^+ channels that are highly sensitive to 4-AP in mechanosensory neurons of *A. californica* (McDearmid et al., 2002). To examine a possible involvement of the 4-AP sensitive K^+ channels in the inhibitory actions of ENpa and GSPRFFamide, the actions of the peptides were re-examined in the presence of 10^{-3} mol l^{-1} 4-AP that is enough to block the 4-AP sensitive K^+ channels (McDearmid et al., 2002; Sasaki et al., 2002b). The interval between the stimulus trains was prolonged to 25 min because the trains applied with shorter interval increased a basal tonus considerably in the presence of 10^{-3} mol l^{-1} 4-AP.

An example for the effect of 4-AP on the inhibitory action of ENpa is shown in Fig. 10A. In this series of experiments, the peptide actions in the absence or the presence of 4-AP were obtained in the same preparation to permit paired comparison. In the presence of 10^{-3} mol l^{-1} 4-AP, the basal tonus of the vasoconstrictor muscle was increased, and the nerve-evoked contraction was enhanced vigorously as described in the anterior aorta of *A. kurodai* (Sasaki et al., 2002b). In some preparations, spontaneous phasic contractions were

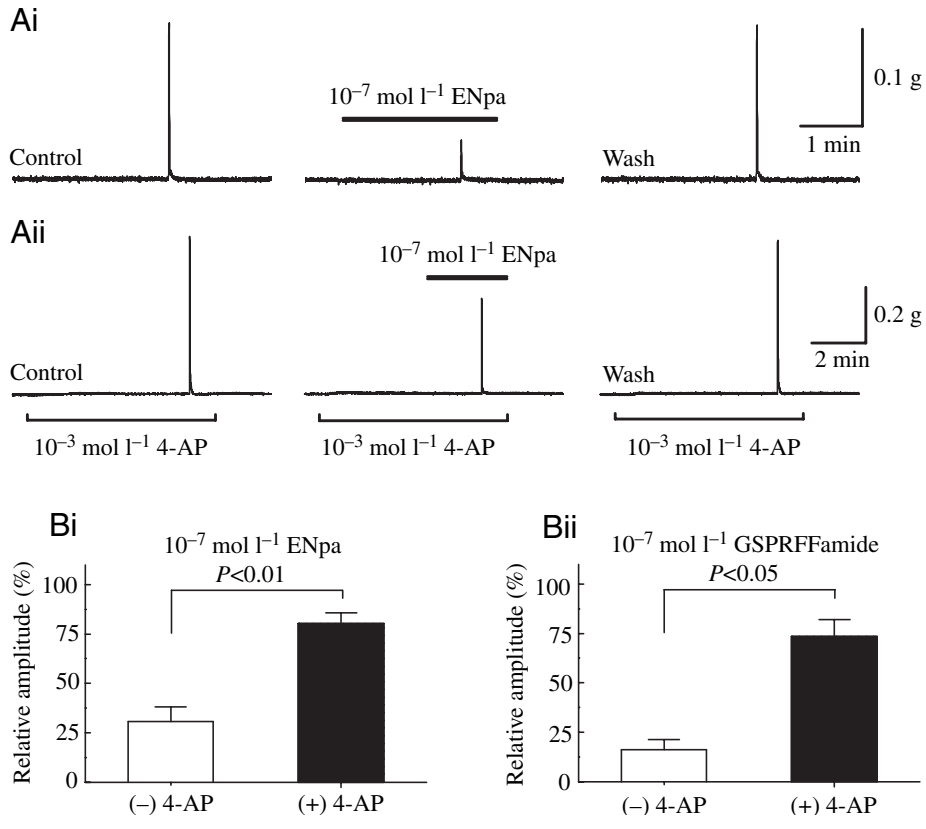

Fig. 10. 4-AP depresses the inhibitory actions of ENpa and GSPRFFamide on the nerve-evoked contraction. (A) Action of 10^{-7} mol l^{-1} ENpa on the contraction in the absence (Ai) or presence (Aii) of 10^{-3} mol l^{-1} 4-AP. The contraction was evoked by the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.4 s. (B) Inhibitory actions of 10^{-7} mol l^{-1} ENpa (Bi, N=5) and 10^{-7} mol l^{-1} GSPRFFamide (Bii, N=4) in the absence [(–)4-AP] or the presence [(+)4-AP] of 10^{-3} mol l^{-1} 4-AP. Statistical significance was assessed by Student's t-test.

also observed under this condition (Fig. 11A). The inhibitory action of ENpa was strongly depressed in the presence of 10^{-3} mol l^{-1} 4-AP. In the absence of 4-AP, the contraction was reduced to 30.7±7.4% of the control by 10^{-7} mol l^{-1} ENpa (Fig. 10Bi; *N*=5). However, in the presence of 10^{-3} mol l^{-1} 4-AP, the same concentration of ENpa reduced the contraction only to 80.4±5.4% of the control.

4-AP had similar effect on the inhibitory action of GSPRFFamide. In 10^{-7} mol l^{-1} GSPRFFamide, the contractions in the absence and the presence of 4-AP were depressed to 16.1±5.1% and 73.4±8.5% of the control, respectively (Fig. 10Bii; *N*=4). These results suggest that the inhibitory actions of the two peptides on the nerve-evoked contraction are at least partly mediated by the activation of the 4-AP sensitive K^+ channels.

Because ENpa inhibited the ACh-induced contraction consistently, we next examined the inhibitory action of ENpa on the ACh-induced contraction in the presence of 4-AP. In three out of four preparations, the ACh-induced contraction was markedly enhanced and prolonged in the presence of 4-AP (Fig. 11A). The amplitude of ACh-induced contraction was reduced to 43.9±12.7% of the control in the absence of 4-AP whereas it was 100.2±5.8% of the control in the presence of 4-AP (Fig. 11B, *N*=4). The result suggests that the inhibitory action of ENpa on the ACh-induced contraction was exclusively due to the activation of the 4-AP sensitive K^+ channels.

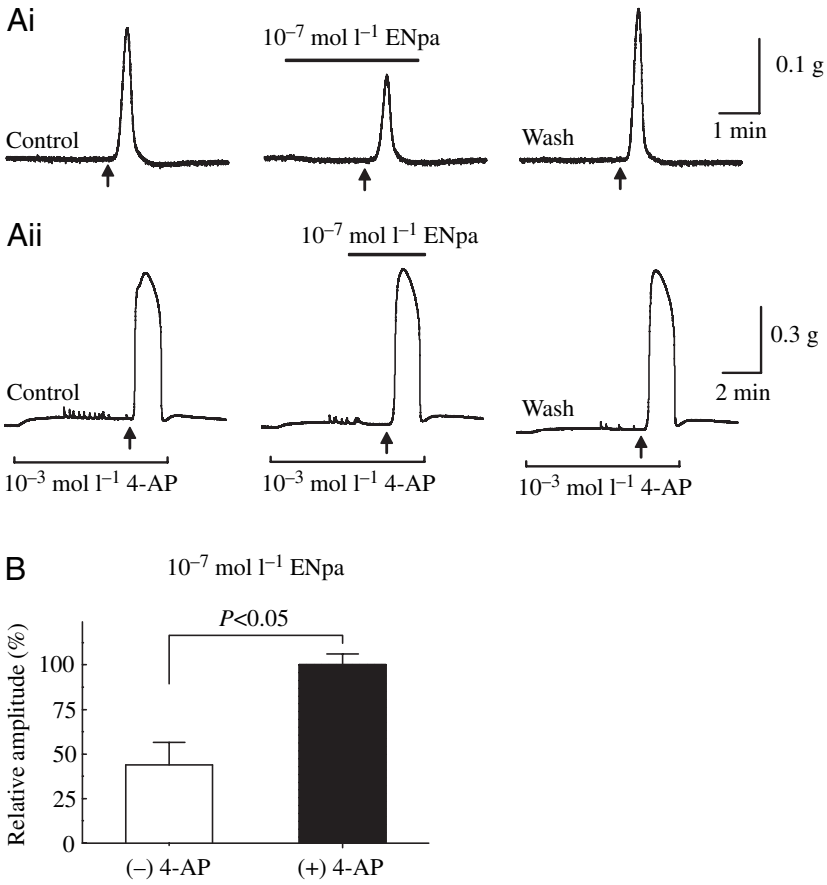

Fig. 11. 4-AP abolishes the inhibitory action of ENpa on the ACh-induced contraction. (A) Action of 10^{-7} mol l^{-1} ENpa on the contraction in the absence (Ai) or presence (Aii) of 10^{-3} mol l^{-1} 4-AP. 3×10^{-6} mol l^{-1} ACh was applied for 20 s at arrows. (B) Inhibitory action of ENpa in the absence [(–)4-AP] or the presence [(+)4-AP] of 10^{-3} mol l^{-1} 4-AP (*N*=4). Statistical significance was assessed by Student's *t*-test.

Modulation of the intra-arterial pressure by the peptides

In previous sections, we showed that ENpa, GSPRFFamide and NdWFamide had modulatory actions on the contractility of the vasoconstrictor muscle in the abdominal aorta. To determine whether the modulatory actions of the peptides on the muscle contraction affect the blood flow into the abdominal aorta effectively, we next examined the actions of the peptides on the intra-arterial pressure change (see Materials and methods). Following the stimulus trains applied to the pericardial nerve, reproducible pressure changes were evoked (Fig. 12), indicating that the constriction of the abdominal aorta was indeed brought up by the pericardial nerve stimulation. Bath application of ENpa or GSPRFFamide inhibited the evoked pressure change, and the concentration–response relationships of ENpa and GSPRFFamide were almost identical (Fig. 12A,B). The threshold concentration was less than 10^{-9} mol l^{-1}, and the nerve-evoked pressure change was almost completely blocked at the concentration of more than 10^{-7} mol l^{-1}. Bath application of NdWFamide potentiated the nerve-evoked pressure change with a threshold of $<10^{-10}$ mol l^{-1} (Fig. 12C). As expected from the result in Fig. 3, NdWFamide also increased the basal pressure at $>10^{-8}$ mol l^{-1} and the potentiation of the nerve-evoked pressure change was more persistent than the change of the basal pressure.

Discussion

In *Aplysia*, the vasoconstrictor muscle of the abdominal aorta is known to regulate the blood flow into the aorta and, thereby, controls the blood distribution. During the feeding behavior or the respiratory pumping, coordinated activation or inhibition of this muscle occurs associated with the behavioral movements (Koester et al., 1974; Byrne and Koester, 1978; Kandel, 1979; Koch and Koester, 1982; Koch et al., 1984). Modulation of the contractility of this muscle is, therefore, important and perhaps occurs in response to different behavioral demands. Previously, FMRFamide and R15 peptides are suggested to be involved in the regulation of the vasoconstrictor muscle (Alevizos et al., 1989, 1991).

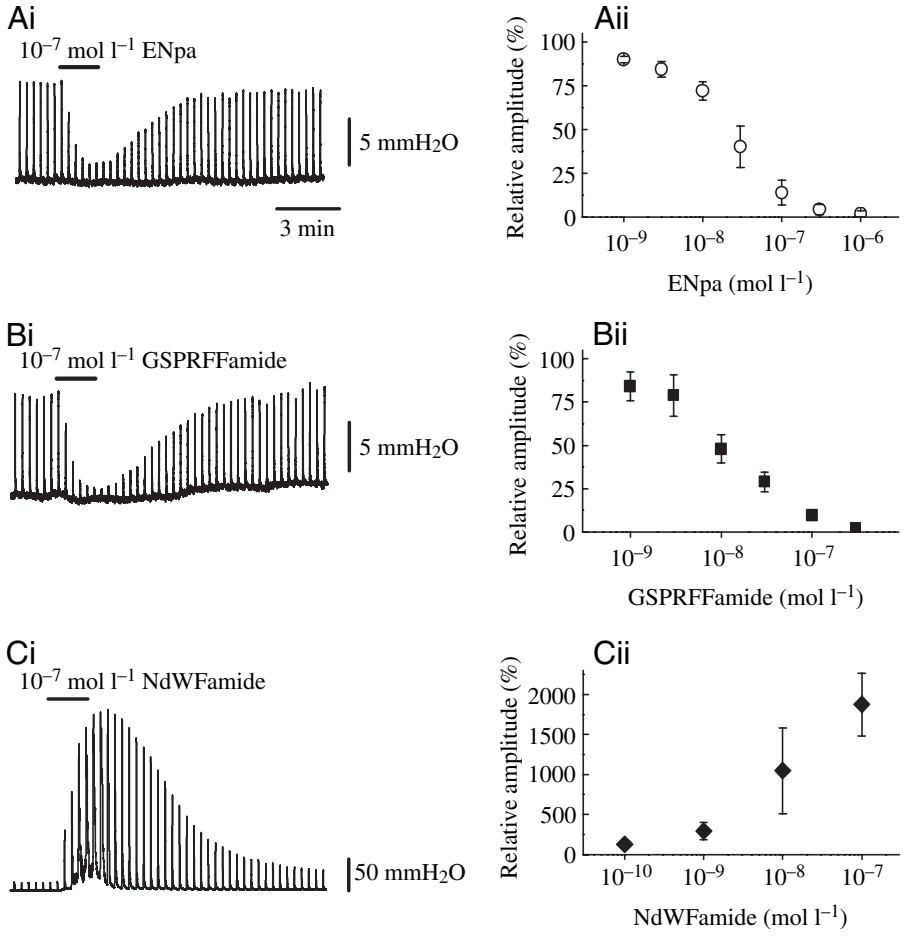

Fig. 12. Actions of the peptides on the vasoconstriction of the abdominal aorta. The vasoconstricion was monitored by an internal pressure change of the perfusion line as described in Materials and methods. Typical examples of the peptide actions (i) and the concentration–response relationships (ii) are shown. (A) Action of ENpa (*N*=4). (B) Action of GSPRFFamide (*N*=3). (C) Action of NdWFamide (*N*=3). The pressure change was evoked by stimulation of the pericardial nerve using the stimulus train (1 ms, 1.6 V, 10 Hz) for 0.9 s (A,B) or 0.5 s (C).

FMRFamide selectively inhibits the contraction of the vasoconstrictor muscle by the firing activity of the neuron LBvc (Alevizos et al., 1989), and R15 peptides acts synergistically with ACh on the vasoconstrictor muscle (Alevizos et al., 1991). In the present study, we focused on the three endogenous peptides (AMRP, enterin and NdWFamide) that have recently been suggested to be involved in the regulation of the cardiovascular system of *Aplysia* (Morishita et al., 1997; Fujisawa et al., 1999; Sasaki et al., 2002a,b) because immunohistochemistry revealed that AMRP-, enterin- and NdWFamide-immunopositive fibers exist in the vasoconstrictor muscle.

The main concern of the present study was whether AMRP, enterin or NdWFamide affects the contractile activity of the vasoconstrictor muscle. As described previously in *A. californica* (Liebeswar et al., 1975), a main excitatory innervation of the vasoconstrictor muscle of *A. kurodai* seems to be cholinergic because the nerve-evoked contraction as well as EJP are completely blocked by a cholinergic blocker, hexamethonium. Having confirmed the cholinergic excitatory innervation, we discuss the feature of the actions of the peptides one by one in the following sections. Our conclusion is summarized in Fig. 13.

Enterin is a family of nona/decapeptides identified in the nervous system of *Aplysia* (Furukawa et al., 2001). We previously showed that enterin is an inhibitory regulator of the anterior aorta in *A. kurodai* (Sasaki et al., 2002b). In the present study, enterin was found to inhibit the contractile activity of the vasoconstrictor muscle. We suggest that enterin inhibits the contraction of this muscle both presynaptic and postsynaptic mechanisms based on the following observations: (1) both the nerve-evoked and the ACh-induced contraction were depressed by enterin; (2) the potency of the inhibitory actions on the nerve-evoked and the ACh-induced contraction was not identical (the same potency would be expected if the inhibitory action is totally postsynaptic); and (3) the enterin hyperpolarized the resting potential of the muscle. The inhibitory mechanisms of enterin has been described in some detail in the anterior aorta of *Aplysia* (Sasaki et al., 2002b). In the anterior aorta, enterin activates the 4-AP sensitive K^+ channels, thereby hyperpolarizes the resting potential of the arterial muscle. The activation of the 4-AP sensitive K^+ channels is a main factor for the inhibitory action of enterin on the nerve-evoked contraction of the anterior aorta (Sasaki et al., 2002b). Because enterin also hyperpolarized the resting potential of some vasoconstrictor muscles, we examined whether the inhibitory action of enterin on this muscle was also mediated by the 4-AP sensitive K^+ channels. Although the action of enterin on the ACh-induced contraction was completely blocked by 4-AP, the inhibitory action on the

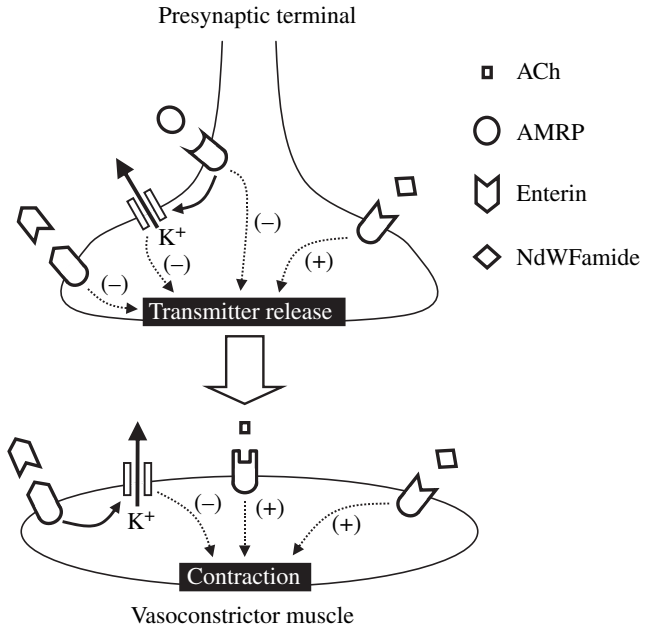

Fig. 13. A model of the peptide actions on the excitatory transmission in the vasoconstrictor muscle. AMRP acts mainly on the presynaptic receptors to inhibit the excitatory transmitter release. Part of this inhibition is assumed to be due to the activation of the 4-AP sensitive K^+ channels. Because 4-AP does not completely block the inhibitory action, other route to inhibit the transmitter release seems to exist. Enterin activates both pre and postsynaptic receptors. Activation of the postsynaptic receptors induces the opening of the 4-AP sensitive K^+ channels, and inhibits the contraction. Because 4-AP does not completely inhibit the action of enterin, the presynaptic inhibitory receptors are also assumed. NdWFamide also activates both pre and postsynaptic receptors. Activation of the postsynaptic receptors evokes the muscle contraction, and that of the presynaptic receptors enhances the transmitter release.

nerve-evoked contraction was not abolished by 4-AP. The result is consistent with the hypothesis that enterin acts at both pre- and postsynaptic sites to inhibit the nerve-evoked contraction of the vasoconstrictor muscle. A main postsynaptic action is probably the activation of the 4-AP sensitive K^+ channels, which should inhibit the nerve-evoked depolarization of the muscle membrane. Because the inhibition of the nerve-evoked contraction by enterin was still observed in the presence of 4-AP, we think that enterin may act on the presynaptic receptor to reduce the release of the excitatory transmitter.

AMRP belongs to the *Mytilus* inhibitory peptide family (Fujisawa et al., 1999) whose members have been isolated from various molluscs (Muneoka et al., 2000). The family members are shown to have inhibitory actions in many peripheral organs and in the central neurons of different species (Muneoka et al., 2000). In accord with the previous reports, AMRP inhibited the contraction of the vasoconstrictor muscle. The inhibitory action of AMRP in the vasoconstrictor muscle is different from that of enterin because AMRP decreases the amplitude of EJP without affecting the resting potential of the muscle and inhibitory action on the ACh-induced contraction was not always observed (see below). Thus, a main inhibitory action of AMRP in the vasoconstrictor muscle seems to be the reduction of the transmitter release from the excitatory nerve terminals. This hypothesis is consistent with the observations in molluscan neurons including *Aplysia* neurons that AMRP as well as other *Mytilus* inhibitory peptides activate 4-AP sensitive K^+ channels (Kiss et al., 1999; McDearmid et al., 2002). The activation of such K^+ channels, in principle, suppresses depolarization-dependent Ca^{2+} influx into the nerve terminals, thereby, reducing the transmitter release. In the present study, the inhibitory action of AMRP on the nerve-evoked contraction was indeed markedly depressed in the presence of 4-AP. Action of AMRP on the ACh-induced contraction was variable among the preparations. In some cases, no effect or modest inhibition was observed. In the other cases, potentiation of the contraction was observed. Such diverse effects are not easily explainable at present, and more experiments are required to clarify the phenomenon.

NdWFamide is originally purified from the heart extract of *A. kurodai* (Morishita et al., 1997), and identified recently from the extract of the central nervous systems of two other gastropod molluscs, *Euhadra congenita* and *Lymnaea stagnalis* (Morishita et al., 2003b). NdWFamide-immunopositive neurons are widespread in the central and peripheral nervous systems of *Aplysia*, and modulates the contractility of a variety of peripheral organs of this animal, suggesting that NdWFamide is ubiquitous signaling molecule in *Aplysia* (Morishita et al., 2003a). NdWFamide evoked a contraction of the vasoconstrictor muscle and potentiated the nerve-evoked contraction. Although NdWFamide is known to enhance the activity of L-type Ca^{2+} channels in *Aplysia* ventricular myocytes (Kanemaru et al., 2002), the upregulation of L-type Ca^{2+} channels by NdWFamide is not seemed to be related to the NdWFamide-induced contraction of the vasoconstrictor muscle because the resting potential of this muscle (well below –70 mV) is far from the activation range of L-type Ca^{2+} channels. We also did not observe meaningful depolarization of the muscle membrane following the application of 10^{-7} mol l^{-1} NdWFamide (data not shown). NdWFamide may, therefore, evoke the contraction by releasing stored Ca^{2+} inside the muscle without the membrane depolarization. NdWFamide-induced vasoconstriction may restrict the blood flow into the abdominal aorta tonically, unlinked to the activity of the vasoconstrictor motoneurons.

We previously proposed that NdWFamide may have presynaptic actions in the arteries because NdWFamide did not evoke longitudinal contractions of the abdominal aorta and the gastroesophageal artery in spite of their dense NdWFamide-immunoreactivity (Morishita et al., 2003a). In the present study, two pieces of evidence suggest that the potentiation of the nerve-evoked contraction of the vasoconstrictor muscle by NdWFamide is due to presynaptic modulation of the excitatory transmitter release: (1) NdWFamide enhances EJP without affecting the resting potential of the muscle; and (2) NdWFamide does not affect the ACh-induced contraction. A

plausible mechanism may be upregulation of the voltage-gated Ca^{2+} channels in the nerve terminals by NdWFamide as is shown for L-type Ca^{2+} channels in *Aplysia* ventricular myocytes (Kanemaru et al., 2002).

In the present study, three types of peptides (AMRP, enterin and NdWFamide) were shown to affect the contractile activity of the vasoconstrictor muscle in the abdominal aorta of *Aplysia*. The modulatory actions of the peptides were strong enough to affect the intra-arterial pressure of the aorta in the present *in vitro* preparation. Although the contraction of the vasoconstrictor muscle is basically determined by the activity of the motoneurons (Mayeri et al., 1974; Koch et al., 1984; Alevizos et al., 1989), the potency of the muscle contraction seems to be modulated by peptidergic innervations. The present results as well as others (Alevizos et al., 1989, 1991) indicate that some neuropeptides modulate the contractility of the vasoconstrictor muscle in the abdominal aorta of *Aplysia*. Peptidergic innervations of the vasoconstrictor muscle would enable the fine tuning of the vasoconstriction and the blood distribution in *Aplysia*.

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