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The enterins inhibit the contractile activity of the anterior aorta of *Aplysia kurodai*

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Summary

The anterior aorta is one of the largest blood vessels in the marine mollusc *Aplysia kurodai*. We examined the actions of recently identified neuropeptides, the enterins, on this blood vessel. Immunohistochemistry revealed that the enterin-immunopositive nerve fibers and varicosity-like structures are abundant in the aorta. When the enterins were applied to the aorta, the basal tonus of the arterial muscles was diminished. The enterins also decreased the contraction amplitude of the anterior aorta evoked either by the application of an *Aplysia* cardioactive peptide, NdWFamide, or by the stimulation of a nerve innervating the aorta (the vulvar nerve). We found that the enterins activate the 4-aminopyridine (4-AP)-sensitive K^+ channels, and thereby hyperpolarize the membrane potential of the aortic muscles. In the presence of 4-AP, the enterins failed to inhibit the muscle contraction evoked by the vulvar nerve stimulation, suggesting that the inhibition is mainly due to the activation of the 4-AP-sensitive K^+ channels. The inhibition of the NdWFamide-evoked contraction by the enterin was not, however, affected by 4-AP. These results suggest that the enterins are involved in inhibitory regulation of the contractile activity of the anterior aorta, and that the inhibition could be due to multiple mechanisms.

Key words: peptide, artery, smooth muscle, cardiovascular system, enterin, mollusc, *Aplysia kurodai*.

Introduction

Physiological control of the cardiovascular system is associated with various animal behaviors. During several aspects of the behaviors, the neuronal regulation of the vasculature system is required for tuning the flow volume of blood, which restricts or facilitates the blood flow into some body regions. In a marine mollusc *Aplysia californica*, the relationships between the behaviors and the cardiovascular regulation were studied in some detail. Presentation of food elicits arousal of the animal, which results in an increase in heart rate (Dieringer et al., 1978; Weiss et al., 1981). During the food-induced arousal, the blood pressure as well as the blood flow recorded from the anterior aorta that carries blood into the feeding apparatus is increased (Koch and Koester, 1982), whereas during egg-laying behavior, blood flow to the reproductive organ is considered to be enhanced and it is thought that the flow to the digestive organ is decreased (Ligman et al., 1985). These studies in *A. californica* clearly indicate a causal relationship between the type of cardiovascular regulation and the behavior.

The anterior aorta is one of the major arteries in the *Aplysia* cardiovascular system, and carries hemolymph to many parts of the body including the central nervous system (CNS), the buccal mass, the genital organs, the opaline gland and the anterior somatic tissues (Koester and Koch, 1987). Previous studies on *A. californica* and *A. kurodai* have provided evidence for regulation of the anterior aorta by several classical transmitters, including glutamate, serotonin, acetylcholine and glycine (Sawada et al., 1981a,b, 1984a,b,c). It is, however, highly likely that the neuropeptides are also involved in fine-tuning the function of blood vessels, as shown in other systems of *Aplysia* (Weiss et al., 1992). In fact, several immunohistochemical studies suggest that the nerve fibers containing different neuropeptides are distributed in the anterior aorta (Miller et al., 1991; Skelton and Koester, 1992; Giardino et al., 1996; Fujisawa et al., 1999; Morishita et al., 2001). Although these observations suggest numerous peptidergic innervations of the anterior aorta, the functional roles and mechanisms of such putative peptidergic innervations are poorly understood.

The enterins are multiple neuropeptides, recently identified in the gut and CNS of *A. californica* and *A. kurodai* (Furukawa et al., 2001). The enterins inhibit gut motility of *Aplysia*, and also reduce excitability of some identified *Aplysia* neurons that are involved in the feeding network. Because the distribution of the enterin-immunopositive neurons is widespread in the nervous system of *Aplysia*, it is suggested that the enterins have

actions in the control of several systems. In the present study, we examined the possibility that the enterins are involved in regulation of the arterial system of *Aplysia*. Immunohistochemical results showed that the enterin-immunopositive nerve fibers are abundant in the proximal region of the anterior aorta of *Aplysia*. Physiological as well as pharmacological experiments revealed that the enterins inhibit the contractility of the aortic muscles by at least two distinct mechanisms, and that the multiple enterins are almost equally potent. These results suggest that the enterins are functionally redundant multiple myoinhibitory peptides in the arterial system of *Aplysia*.

Materials and methods

Animals and their preparation

Aplysia kurodai Baba (80–300 g) were collected at the Hiroshima Bay and on the sea shore in Shimane during March to July (1999–2001). Some specimens were provided by the marine biological station of Tohoku University (Asamushi, Japan) during October 1999 to January 2000. Animals were maintained in an aquarium containing artificial seawater (Yashima, Japan) at 15°C, and fed seaweed.

Animals were anesthetized by intra-abdominal injection of 0.33 mol l^{-1} $MgCl_2$. Approximately one third of the proximal region of the anterior aorta was dissected out from the anesthetized animal, and was kept in artificial seawater (ASW). The composition of ASW was (mmol l^{-1}): NaCl 445, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55, Tris-HCl 10, pH 7.9. In some experiments, ASW containing either low K^+ or low Cl^- was used. 10% K^+-ASW was made by replacing 9 mmol l^{-1} KCl with NaCl. 50% Cl^--ASW was made by replacing 222.5 mmol l^{-1} NaCl with sodium methylsulfate. All the experiments were performed at room temperature (20–23°C).

Immunohistochemistry

The anterior aorta was excised from the anesthetized *Aplysia*, and fixed by 4 % paraformaldehyde for 24 h at 4°C or for 2–3 h at room temperature. The fixed tissue was immunostained by using an anti-enterin antibody (kindly provided by Dr Vilim) as described previously (Furukawa et al., 2001). The antibody was visualized by diluted fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (Organon Teknika, Durham, NC, USA). 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 version 5 (Adobe Systems, San Jose, CA, USA).

Physiological recordings

To record the contraction of the anterior aorta, the proximal end of the anterior aorta was pinned to the bottom of a chamber (0.5–1 ml volume). The other end of the aorta was connected to a force-transducer (Type 45196A, NEC San-ei Instrument Ltd., Tokyo, Japan). The signal from the transducer was monitored on a chart recorder (FBR-251A, TOA Electronics Ltd, Tokyo, Japan). In some experiments, the vulvar nerve was left attached to the anterior aorta to permit electrical stimulation of the nerve.

The membrane potential of the muscle fibers in the aorta was measured using a conventional microelectrode. The preparations were grounded directly using an Ag/AgCl electrode or *via* a 3 mol l^{-1} KCl–agar bridge connected to a reservoir containing 3 mol l^{-1} KCl and an Ag/AgCl electrode. To restrict the movement of the anterior aorta, 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 membrane potentials were amplified by the Duo 773 electrometer (World Precision Instruments, USA), and 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) for later analysis. The data analysis and the compilation of figures were done using Origin (version 6, Microcal Software Inc., Northampton, MA, USA). The results are expressed as means ± S.E.M., except where indicated.

To analyze a concentration–response relationship of peptides, the data were fitted to an equation of the form $y=r_1+(r_2-r_1)/[1+10^{(\log x0-x)n}]$ by the Levenberg–Marquardt algorithm using Origin. y is a response, x is a power for the concentration of the peptide (i.e. the concentration used is 10^x), n is a Hill coefficient, r_1 is a minimum response, r_2 is a maximum response, and $10^{\log x0}$ is EC_{50} (the concentration of peptide at which 50% response is expected). Because n values for our data were in the range 1–1.3, we fixed a value of 1 for the fittings presented in this paper. Other constraints and parameters obtained by the fittings are described in the figure legends.

Peptides and chemicals

All the peptides and drugs were applied by bath perfusion. 4-aminopyridine (4-AP, Sigma, USA) and tetraethylammonium (TEA, Katayama, Japan) were dissolved in ASW just before use. The enterins and an *Aplysia* cardioactive peptide, NdWFamide (Asn-D-Trp-Phe-NH_2; Morishita et al., 1997), were synthesized with an automated solid-phase peptide synthesizer (PSSM8, Shimadzu, Kyoto, Japan) and purified by reversed-phase high performance liquid chromatography (HPLC). The enterins used in the present study were as follows (nomenclature is based on Furukawa et al., 2001): ENa, VSPKYGHNFVamide; ENe, ADLGFTHSFVamide; ENk, APGYSHSFVamide; ENl, ELNFQHAFVamide; ENpa, APSFGHSFVamide; ENr, DPGFNHAFVamide. ENpa is a novel enterin purified from the CNS extract of *A. kurodai* (Y. Fujisawa, unpublished). Although ENpa is not coded on the enterin precursor of *A. californica* (Furukawa et al., 2001), it was found to be coded on the enterin precursor of *A. kurodai* (Y. Furukawa, unpublished observation). In this paper, we designate APSFGHSFVamide as ENpa because the peptide is identical

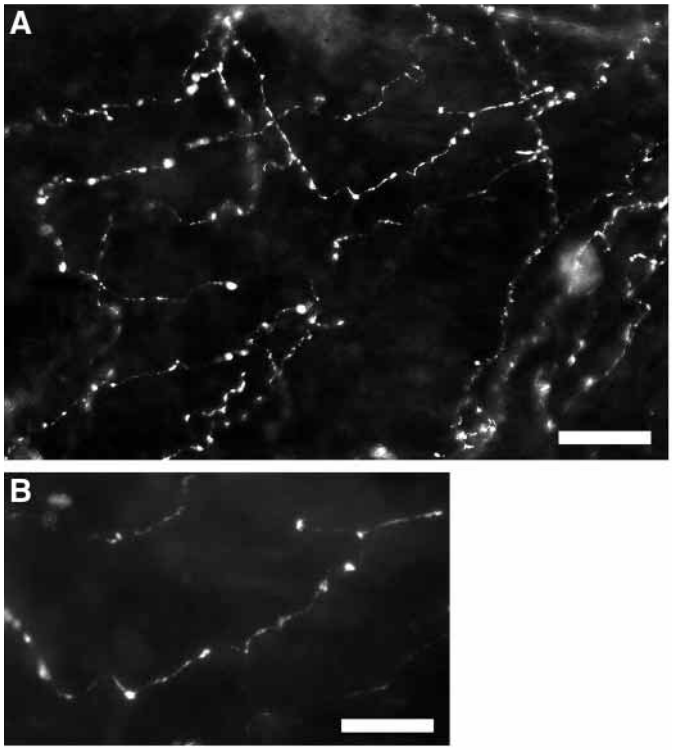

Fig. 1. The enterin-immunopositive fibers and varicosities in the proximal region of the anterior aorta. The upper middle region in A is shown at higher magnification in B. Scale bars, 100 μm (A); 50 μm (B).

to ENp of *A. californica* (VPSFGHSFVamide), except for the N-terminal amino acid. The peptides were dissolved in distilled water to make a concentrated stock solution (10^{-2} mol l^{-1}). The stock solution was diluted appropriately just before use.

Results

The enterin-immunopositive fibers in the anterior aorta

Distribution of the enterin-containing nerve fibers in the anterior aorta was examined immunohistochemically. Although any immunohistochemical results are affected by non-specific signals, our previous study shows that the anti-enterin antibody used here is rather specific to the enterins (Furukawa et al., 2001). A typical immunostained anterior aorta is shown in Fig. 1. Thin immunopositive fibers and varicosity-like structures are seen in the muscular tissues of the aorta. The enterin-immunopositive fibers were abundant in a proximal region of the anterior aorta that is close to the heart, and were scarce in a distal (rostral) region beyond the mantle artery (on the arterial system of *Aplysia*; see Skelton and Koester, 1992). The enterin-immunopositive fibers were also observed in other two large arteries (the gastroesophageal artery and the abdominal aorta; data not shown). The results were confirmed in eight preparations, and the immunohistochemical evidence suggests that the enterins might be involved in regulation of the contractility of the *Aplysia* arterial system.

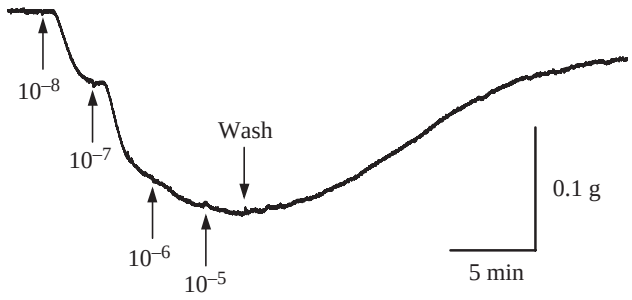

Fig. 2. Effect of the enterin on the basal tonus of the anterior aorta. Sequential application of increasing concentrations of ENpa (mmol l^{-1}) induced a concentration-dependent relaxation of the anterior aorta. After washing out ENpa, the muscle tonus was recovered gradually.

Action of the enterins on the quiescent anterior aorta

An isolated anterior aorta of *Aplysia* can show spontaneous contractile activities and even regular beating in some cases (e.g. Morishita et al., 1997). In the present study, however, the isolated anterior aorta rarely showed spontaneous contractions. When the enterin was applied to an isolated aorta by bath perfusion, the aorta more or less relaxed, but the extent of the relaxation was quite variable, depending on the preparations, and some preparations did not respond at all even after the application of high concentrations of the peptide (10^{-6} mol l^{-1}). Fig. 2 shows an example of the enterin-induced relaxation of the anterior aorta. In this preparation, 10^{-8} mol l^{-1} ENpa induced a clear relaxation of the basal tonus, and maximum relaxation was observed at >10^{-6} mol l^{-1}. The enterin-induced relaxation of the aorta could be reversed after washing out the enterin. In some preparations, however, a reduced tonus of the aorta was never recovered after application of high concentrations of the enterins (>10^{-6} mol l^{-1}). Because the relaxation of the aorta by the enterins seems to be dependent on the level of basal tonus of the aorta, we next examined the actions of the enterins on the contracted aorta.

Action of the enterins on the contraction of the anterior aorta

To examine the action of the enterins on the contracted aorta, we used an *Aplysia* cardioactive peptide, NdWFamide (Morishita et al., 1997), to raise the basal tonus of the anterior aorta. Although other transmitters can evoke contraction of the anterior aorta (Sawada et al., 1982, 1984a,b), NdWFamide is preferable because NdWFamide evokes a tonic contraction of the aorta with little desensitization (Morishita et al., 2001). 10^{-7} mol l^{-1} NdWFamide evoked a tonic contraction of the anterior aorta, and the extent of contraction usually changed little in the presence of NdWFamide (see Fig. 3Ai). After washing out NdWFamide, the developed tension of the anterior aorta returned slowly to the initial level during the next 20–30 min. When 10^{-7} mol l^{-1} ENk was applied in the presence of NdWFamide, the contraction of the anterior aorta evoked by NdWFamide was inhibited and the aorta showed a clear relaxation (Fig. 3Aii), which was more prominent at higher concentrations of ENk (Fig. 3Aiii).

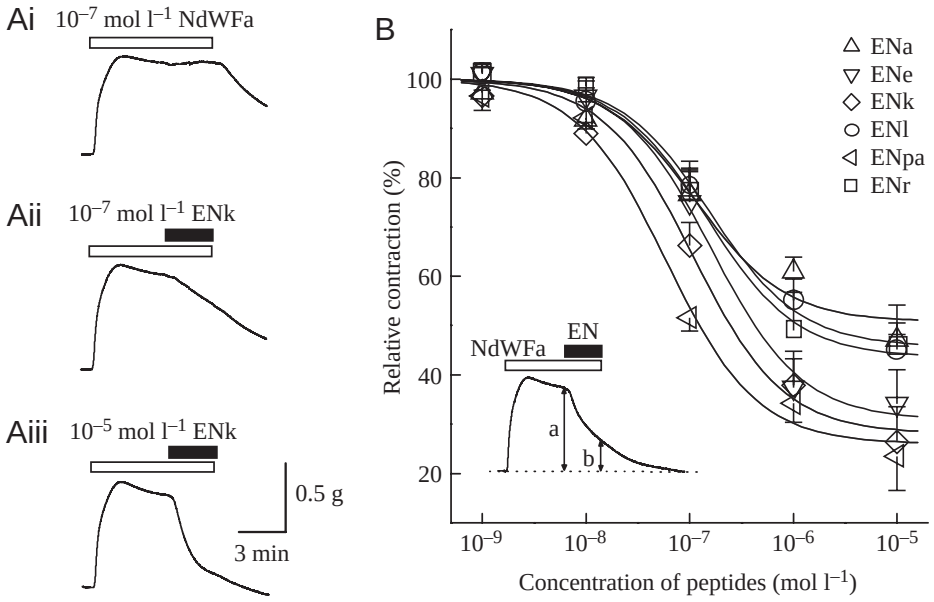

Fig. 3. The enterins inhibit the NdWFamide-evoked tonic contraction of the anterior aorta. (Ai) The NdWFamide evoked contraction. 10^{-7} mol l^{-1} NdWFamide was applied for 8 min as indicated, before washing out. (Aii) Effect of 10^{-7} mol l^{-1} ENk on the NdWFamide-evoked contraction. 10^{-7} mol l^{-1} ENk was co-applied with NdWFamide 5 min after the initial NdWFamide application. (Aiii) Effect of 10^{-5} mol l^{-1} ENk on the NdWFamide-evoked contraction. (B) The concentration–response relationships of the enterins. The effects of six enterins (ENa, ENe, ENk, ENl, ENpa, ENr) on the NdWFamide-evoked contraction were tested. Amplitude of the contraction at the end of the application of the enterin (b in inset) was normalized to the one just before the application of the enterin (a in inset). Values are means ± S.E.M. (N=3–5) plotted against concentration of enterins. Smooth lines are best-fits of the equation described in Materials and methods. r_2 and n were fixed to 100 and –1, respectively. r_1 and log$x$0 for each fitting were as follows, respectively: ENa, 50.7, –6.9; ENe, 30.8, –6.8; ENk, 28.1, –7.0; ENl, 45.7, –6.8; ENpa, 26.0, –7.2; ENr, 43.5, –6.8.

To quantify the inhibitory action of the enterins, we normalized the minimum amplitude of the contraction in the presence of the enterin by the amplitude of the control contraction (see Fig. 3 legend). The concentration–response relationships obtained for six enterins (ENa, ENe, ENk, ENl, ENpa, ENr) are shown in Fig. 3B. Although the amino acid sequences of the enterins used are rather diverse (see Materials and methods), the inhibitory actions of the enterins were quite similar. A threshold concentration was close to 10^{-9} mol l^{-1}, and EC_{50} was approximately 10^{-7} mol l^{-1} (see Fig. 3 legend). Although the curve-fittings showed different levels of maximum inhibition among the enterins (see Fig. 3 legend), the differences between relative contractions at 10^{-5} mol l^{-1} were statistically marginal but not significant (ANOVA, $F_{5,18}$=2.7, P>0.05). For the experiments in Fig. 3, we applied the enterins for 3 min, although the inhibitory action was obviously not saturated during that time (see Fig. 3Aii). A longer application of the enterins produced a larger relaxation of the aorta than shown in Fig. 3, especially at the lower concentration range (data not shown). Complete recovery of the enterin-induced inhibition was only seen after a short application time, which was also necessary for the reproducible contracture by the following NdWFamide applications. Because the enterins tested had a similar potency, ENpa was used in most of the following experiments.

Action of the enterins on the phasic contraction of the anterior aorta

We next examined the action of the enterins on the phasic contraction of the anterior aorta, which is evoked by the electrical stimulation of the vulvar nerve. The vulvar nerve is one of the nerve bundles arising from the abdominal ganglion, and innervates the anterior aorta (Sawada et al., 1981a). Although a single electrical stimulus of the nerve can evoke a phasic contraction of the anterior aorta, we used a repetitive stimulation (10 Hz for 1 s) to obtain a reproducible phasic contraction of the aorta. Fig. 4A shows the effect of 10^{-8} mol l^{-1} ENpa on the phasic contraction of the anterior aorta. In the presence of ENpa, the evoked contraction was reduced to about 40% of the control. The phasic contraction was recovered after washing out ENpa. The peak amplitude of the phasic contraction in the presence of ENpa was normalized to the one obtained in the absence of ENpa, and plotted against the concentration of ENpa (Fig. 4B). The concentration–response relationship showed that a threshold for the inhibitory action is approximately 10^{-10} mol l^{-1} and EC_{50} is close to 10^{-8} mol l^{-1}. In most cases, the phasic contraction was completely abolished by 10^{-7} mol l^{-1} ENpa.

Action of the enterins on the membrane potential of the aortic muscle fibers

As described above, the enterins inhibit both the basal tonus and the evoked contraction of the anterior aorta, suggesting their myoinhibitory roles. To clarify the mechanisms of the inhibitory action, we next examined the effect of the enterins on the membrane potential of the muscle fibers in the anterior aorta. The resting membrane potentials of the muscle fibers were –59.0±1.3 mV (N=38). The values are consistent with those published by others (Sawada et al., 1981a).

The enterins induced a hyperpolarization of the muscle fibers in a dose-dependent manner (Fig. 5A). The response turn-on was slow, and it took several minutes to reach the peak. A threshold concentration was <10^{-8} mol l^{-1}, and EC_{50} was close to 10^{-7} mol l^{-1}. A maximum hyperpolarization evoked by

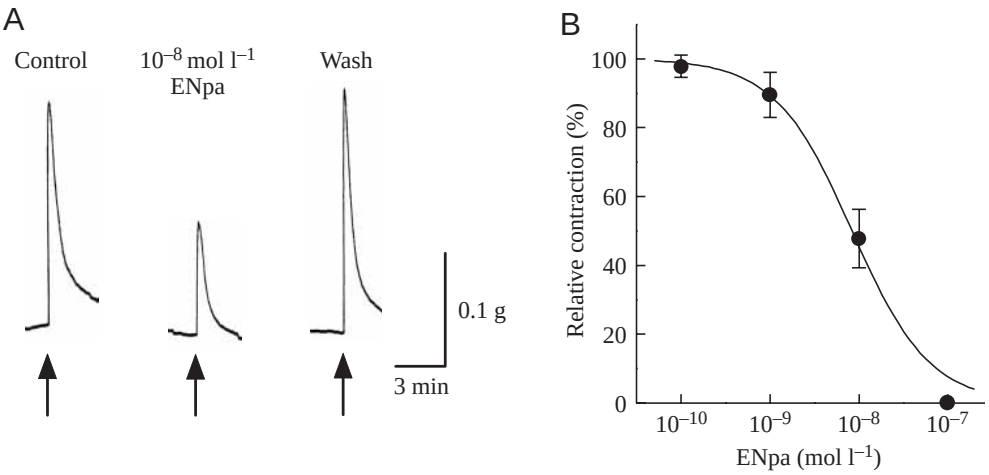

Fig. 4. The enterin inhibits the phasic contraction of the anterior aorta evoked by electrical stimulation of the vulvar nerve. (A) Effect of 10^{-8} mol l^{-1} ENpa on the phasic contraction. To evoke a phasic contraction of the aorta, the vulvar nerve was stimulated by repetitive pulses (5 V, 1 ms, 10 Hz) for 1 s. (B) The concentration–response of ENpa. The amplitude of the evoked contraction in the presence of ENpa was normalized to the one obtained before the application of the peptide. Values are means ± S.E.M. (N=4) plotted against the concentration of the peptide. The smooth line is a best-fit of the equation described in Materials and methods. r_1, r_2 and n were fixed to 0, 100 and –1, respectively. log$x$0 was estimated to be –8.1.

>10^{-6} mol l^{-1} ENpa (or ENe) was more than 10 mV from the resting potential (Fig. 5B).

Considering the resting potential level of the muscle fibers, the enterin-induced hyperpolarization can be due to the increase of either K^+ conductance or Cl^- conductance, or both. To determine the ionic mechanisms, we next examined the ENpa-induced hyperpolarization in K^+ or Cl^--deficient condition. When the anterior aorta was bathed in 10% K^+-ASW, the hyperpolarizing response increased markedly (Fig. 6A). In ASW, ENpa induced the hyperpolarization of 10.7±1.0 mV, whereas it became 21.0±1.0 mV in 10% K^+-ASW (N=3). The difference between the two conditions is statistically significant (t-test, P<0.01). When the anterior aorta was bathed in 50% Cl^--ASW, the ENpa-induced hyperpolarization increased slightly, and became 14.6±2.6 mV compared to 12.2±3.2 mV in ASW (N=3). This small change is in the opposite direction to that expected if increased Cl^- conductance is involved, and is perhaps due to a depolarized membrane potential of the muscle fibers in low Cl^- solution (see Fig. 6B). These results suggest that the hyperpolarizing response of the muscle fibers by the enterins is mainly caused by an increase in K^+ conductance.

To characterize the K^+ conductance activated by the enterins, we examined the effects of two conventional K^+ channel blockers, 4-AP and TEA. TEA had little effect on the ENpa-induced hyperpolarization even at 10 mmol l^{-1} (data not shown). On the other hand, the K^+ conductance was quite sensitive to 4-AP. Fig. 7 shows the blocking action of 4-AP on the ENpa-induced hyperpolarization. A threshold concentration for the blocking action was <10^{-7} mol l^{-1}, and the hyperpolarizing response was completely blocked by 1 mmol l^{-1} 4-AP (Fig. 7B). The 4-AP block can be completely washed out if the concentration of 4-AP was <10^{-4} mol l^{-1}. These results suggest that the enterins activate the 4-AP sensitive K^+ channels.

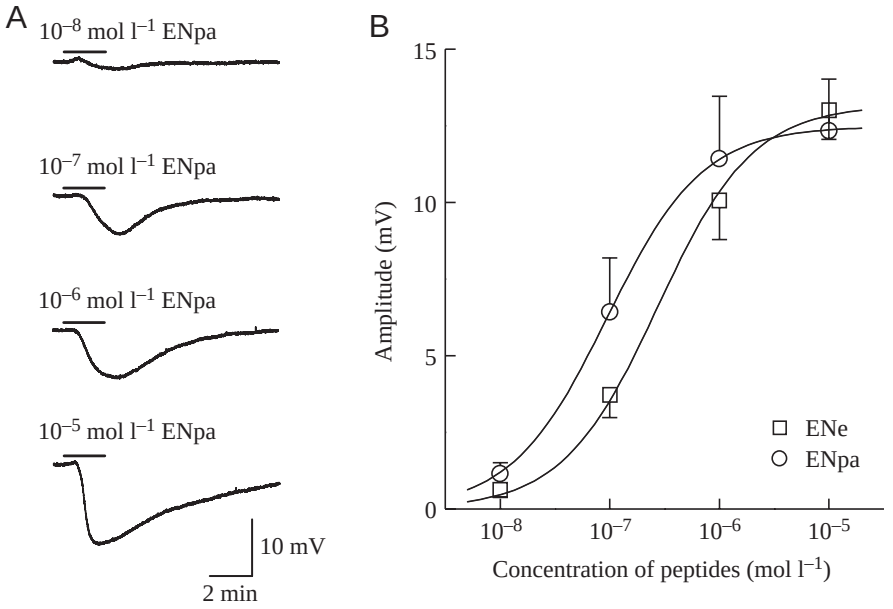

Fig. 5. The enterins induce a hyperpolarization of the arterial muscle fibers. (A) Effect of ENpa on the membrane potential of the muscle fiber. ENpa was applied for 2 min at the concentrations indicated. (B) The concentration–response relationships of two enterins. Values are means ± S.E.M. (N=4–9) of the hyperpolarization plotted against the concentrations of enterins. The smooth lines are best-fits of the equation described in Materials and methods. r_1 and n were fixed to 0 and 1, respectively. r_2 and log$x$0 for each fitting were as follows, respectively: ENe, 13.2, –6.6; ENpa, 12.5, –7.0.

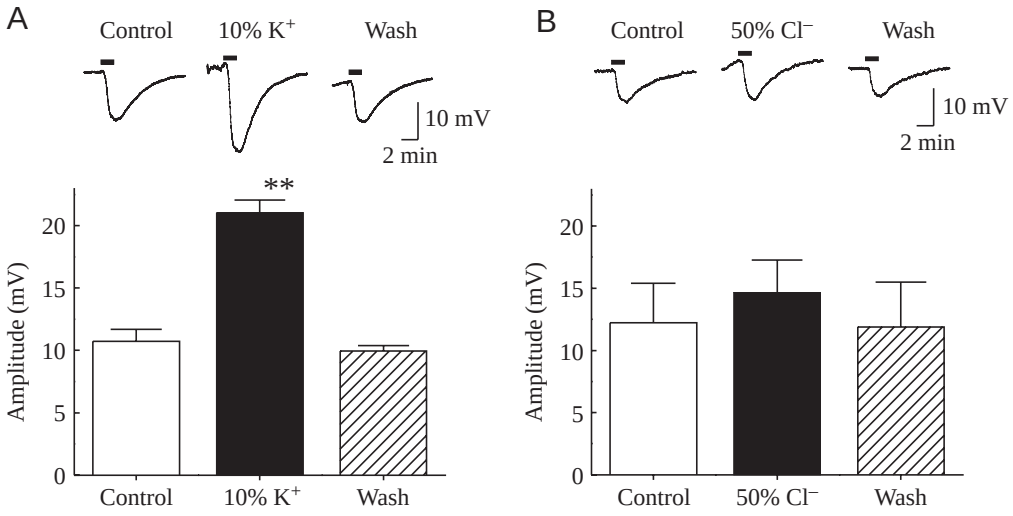

Fig. 6. The enterin-induced hyperpolarization of the muscle is dependent on the external [K^+], but not on the [Cl^-]. (A) Effect of 10% K^+-ASW on the ENpa-induced hyperpolarization. The traces show examples of the response. Histograms show means ± S.E.M. (N=3). (B) Effect of 50% Cl^--ASW on the ENpa-induced hyperpolarization. Histograms show means ± S.E.M. (N=3). **P<0.01 (t-test).

Action of the enterins in the presence of 4-AP

The results presented above suggest that the enterins inhibit the contraction of the muscles in the anterior aorta *via* the activation of the 4-AP-sensitive K^+ channels. The extra K^+ currents can hyperpolarize the membrane potential of the muscles, and keep it below the threshold depolarization required for the muscle contraction. Because 1 mmol l^{-1} 4-AP completely blocked the ENpa-induced hyperpolarization, we tested the hypothesis by examining the action of the enterins in the presence of 1 mmol l^{-1} 4-AP. Fig. 8A shows an example of the effect of 10^{-6} mol l^{-1} ENpa on the phasic contractions of the aorta in the absence (Fig. 8Ai) or the presence of 4-AP (Fig. 8Aii) obtained in the same preparation. In the presence of 4-AP, a basal tonus as well as the phasic contraction of the aorta was increased. The result can be explained by the blockade of K^+ channels, which would enhance the excitability of the muscle membrane, and/or increase the excitatory transmitter release (Molgo et al., 1977). Although 10^{-6} mol l^{-1} ENpa almost completely blocked phasic contraction in the control (Fig. 8Ai), the contraction was not affected in the presence of 4-AP (Fig. 8Aii). In this series of experiments (N=4), 10^{-6} mol l^{-1} ENpa reduced the phasic contraction to 14.8±4.5% of the control in the absence of 4-AP, compared to 93.4±6.5% in the presence of 4-AP, suggesting that inhibition of the phasic contraction by the enterins is mostly due to activation of the 4-AP-sensitive K^+ channels. However, it is noteworthy that ENpa also diminishes the enhanced basal contraction by 4-AP (see Fig. 8Aii). The result implies that the inhibitory action of the enterins is not entirely due to the activation of the 4-AP-sensitive K^+ channels.

We next examined the effect of 4-AP on the enterin-induced inhibition of the NdWFamide-evoked contraction (Fig. 9). In the presence of 4-AP, NdWFamide evoked oscillating contractions superimposed on the tonic contraction, but the amplitude of the tonic contraction was not affected so much (compare Fig. 9Ai and Aii). Because the contraction of the aorta *via* the excitatory synaptic transmission is enhanced by 4-AP (see above), the result favors an hypothesis that NdWFamide evokes the muscle contraction directly. The oscillating contraction of the aorta is probably due to the enhanced excitability of the muscle membrane by 4-AP. In contrast to the nerve-evoked phasic contraction, the action of the enterins on the NdWFamide-evoked contraction was not affected by 4-AP (Fig. 9B).

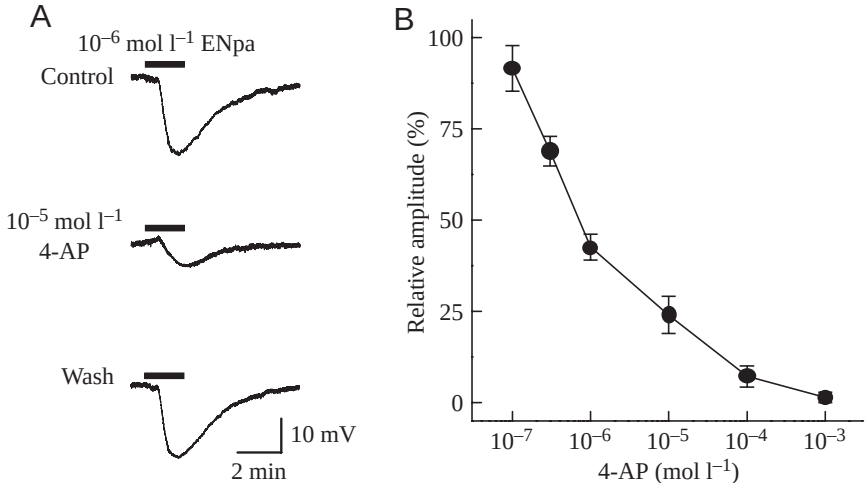

Fig. 7. 4-AP blocks the enterin-induced hyperpolarization. (A) Effect of 10^{-5} mol l^{-1} 4-AP on the 10^{-6} mol l^{-1} ENpa-induced hyperpolarization of the muscle. (B) The concentration–response relationship of the 4-AP block. Amplitude of the ENpa-induced hyperpolarization in the presence of 4-AP was normalized to that seen in the absence of 4-AP. Values are means ± S.E.M. (N=3–5) of the ENpa-induced hyperpolarization plotted against [4-AP].

Discussion

The enterins are nona/decapeptides recently identified in

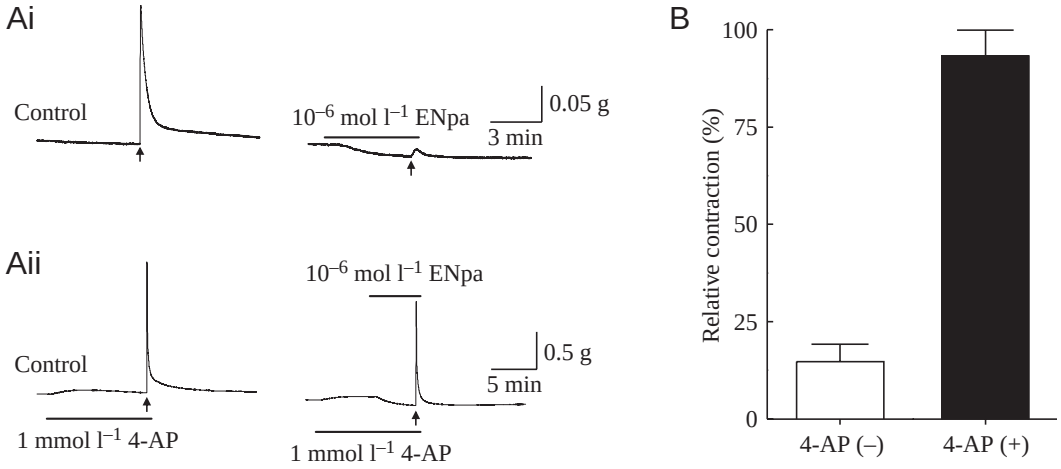

Fig. 8. 4-AP blocks the inhibitory action of the enterin on the phasic contraction of the anterior aorta. (A) The action of 10^{-6} mol l^{-1} ENpa on the phasic contraction in the absence (Ai) or presence (Aii) of 1 mmol l^{-1} 4-AP. The phasic contraction of the aorta was evoked by four repetitive stimuli (2 V, 1 ms, 10 Hz) of the vulvar nerve. (B) The inhibition of the nerve-evoked contraction by ENpa in the absence [4-AP(–)] or the presence [4-AP(+)] of 1 mmol l^{-1} 4-AP. Histograms show means ± S.E.M. (*N*=4) of the relative amplitude of the contraction in the presence of ENpa.

both the central and peripheral nervous systems of *Aplysia* (Furukawa et al., 2001). Previous studies suggest that the enterins are inhibitory peptides in the gut, and that the enterins may change the feeding motor programs. Because enterin-positive neurons are found throughout the nervous system, the peptides are thought to have other functions in *Aplysia*. In the present study, we show that enterin-immunopositive fibers and varicosity-like structures are abundant in the anterior aorta of *Aplysia*, and that the enterins inhibit both the basal tonus and the evoked contraction of the anterior aorta. Both the immunohistochemical and physiological results suggest that the enterins are inhibitory neuropeptides for the contractile activity of the aorta. Despite some structural differences between the six enterins used in this study, their inhibitory potencies on the NdWFamide-evoked contraction were almost identical. The results are in accord with the previous observation that the inhibitory actions of the different enterins are similar in the gut of *Aplysia* (Furukawa et al., 2001), supporting the previous suggestion that the enterins are functionally redundant. A structural consideration of the present pharmacological evidence as well as previous evidence suggests that a common structure in the C-terminal half of the enterins (i.e. (Y/F)XH(S/A)F(V/L)amide) is sufficient to exert the action of the enterins. Indeed, a preliminary experiment shows that a synthetic analogue peptide, YSHSFVamide, has an inhibitory action in the *Aplysia* gut (O. Matsushima, unpublished observation).

The anterior aorta of *Aplysia* is innervated by nerves arising from the abdominal ganglion (Sawada et al., 1981a). The vulvar nerve is one of the nerves innervating the anterior aorta,

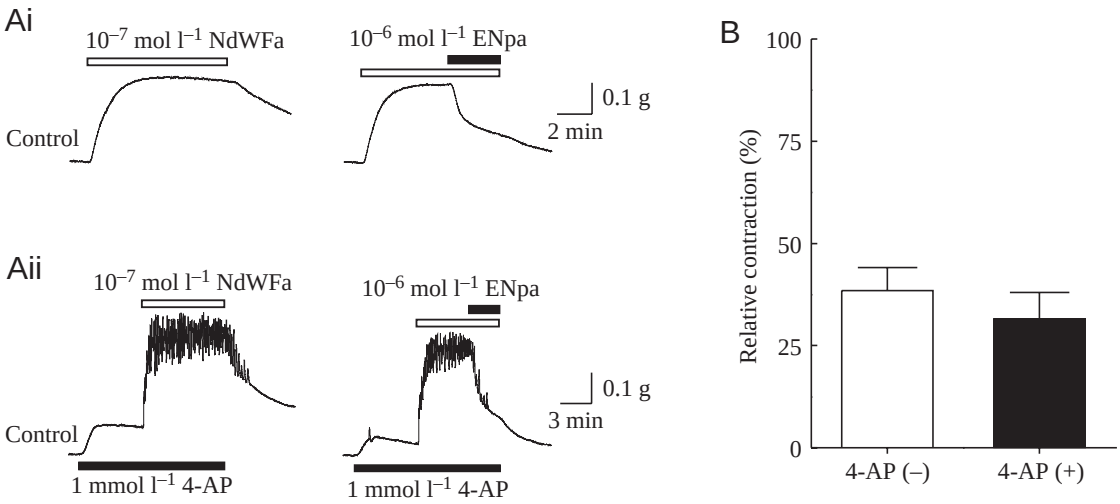

Fig. 9. 4-AP does not affect the inhibitory action of the enterin on the NdWFamide-evoked contraction of the anterior aorta. (A) Relaxation of the NdWFamide-induced contraction by ENpa in the absence (Ai) or presence (Aii) of 1 mmol l^{-1} 4-AP. The aorta was contracted by 10^{-7} mol l^{-1} NdWFamide, and the action of 10^{-6} mol l^{-1} ENpa was tested. (B) The inhibition of the NdWFamide-induced contraction by ENpa in the absence [4-AP(–)] or the presence [4-AP(+)] of 1 mmol l^{-1} 4-AP. Histograms show means ± S.E.M. (*N*=4) of the normalized NdWFamide-induced contraction in the presence of ENpa.

in which axons of some identifiable motoneurons or modulatory neurons for the anterior aorta are contained (Sawada et al., 1981a, 1984c). The phasic contraction evoked by the vulvar nerve stimulation was inhibited by the enterins. At least, one of the mechanisms for the inhibition seems to be activation of K^+ conductance of the muscle membrane. We found that the enterins hyperpolarize the membrane potential of the muscle fibers *via* the activation of 4-AP-sensitive K^+ channels. Sensitivity of the K^+ channels to 4-AP is quite high, and EC_{50} of 4-AP was $<10^{-6}$ mol l^{-1}. The value is comparable to another highly 4-AP-sensitive K^+ channel described in the accessory radula closer muscle of *Aplysia* (Brezina et al., 1994). The enterin-induced hyperpolarization of the muscle membrane should, in principle, reduce the excitability of the muscle, and inhibit the nerve-evoked contraction. The excitatory transmitter release may also be reduced if the K^+ channels that are activated by the enterins are present in the presynaptic terminals. The explanation is straightforward and in accordance with the results in Fig. 8, in which the inhibitory action of the enterins on the nerve-evoked contraction is almost completely blocked by 4-AP.

However, some other results are difficult to explain completely by the hyperpolarizing action of the enterins. A discrepancy may be noticed if the two concentration–response relationships for the action of the enterins (Figs 4 and 5) are compared. The phasic contraction of the aorta by the vulvar nerve stimulation can be inhibited by $>10^{-10}$ mol l^{-1} of ENpa, and was mostly abolished by 10^{-7} mol l^{-1} ENpa. By contrast, ENpa-induced hyperpolarization was rarely seen at 10^{-8} mol l^{-1}, and EC_{50} was approximately 10^{-7} mol l^{-1}. Therefore, the phasic contraction is noticeably inhibited by ENpa at levels well below the threshold concentration to evoke a detectable hyperpolarization of the resting membrane potential. A plausible explanation for this discrepancy may be that the K^+ channel that is activated by the enterins may show outward rectification like the S-K^+ channel of *Aplysia* (Siegelbaum et al., 1982), or may be voltage-dependent, as for the FMRFamide activated K^+ channel in *Lymnaea* neurons (Kits et al., 1997). In either case, more K^+ currents would flow during a rising phase of the excitatory junctional potential than in the resting state, which may result in inhibition of the nerve-evoked contraction of the aorta even though there is little hyperpolarization of the resting potential. Strong evidence suggesting the K^+ channel-independent mechanism for the inhibitory action of the enterins is provided by the results shown in Fig. 9, in which the NdWFamide-evoked contraction of the aorta was inhibited by the enterin even in the presence of 1 mmol l^{-1} 4-AP. Because 1 mmol l^{-1} 4-AP blocks the enterin-induced hyperpolarization completely (Fig. 7), there must be a membrane potential-independent action of the enterins. Taken together, our working hypothesis is that the enterins activate at least two pathways leading to the inhibition of the aortic muscle contraction: (1) indirectly by the activation of the 4-AP-sensitive K^+ channels in the muscle membrane, and (2) direct inhibition of the contractile machinery.

Because *Aplysia* lacks a rigid skeleton, the blood vessels can easily be stretched and/or twisted during a change of posture or locomotion. The anterior aorta is one of the largest arteries of the animal, and it encompasses approximately two thirds of the body length, from the cristae aorta near the heart to the head ganglia. The anterior aorta, therefore, is considered to receive a large external force during several types of animal behavior. To accommodate to such stress, it seems reasonable that the stiffness and the length of the aorta be under neuronal regulation. In fact, Skelton and Koester (1992) have shown that two identifiable neurons in pedal ganglia of *Aplysia* regulate the length of the rostral anterior aorta and the right and left pedal parapodial arteries to meet the shortening of the head and neck region during various behaviors.

In this study, the enterins were found to relax the basal tonus of the aorta and inhibit the contraction. The action of the enterins may counterbalance the stretch posed on the blood vessels in some behavioral states to protect the vessels and maintain the blood flow. An *Aplysia* cardioactive peptide, NdWFamide, seems to have an opposite function because NdWFamide enhances the basal tonus of the anterior aorta. The present study also showed that the action of NdWFamide is antagonized by the action of the enterins. These two types of peptides may be physiological antagonists to regulate the basal stiffness of the blood vessels of *Aplysia*.

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