Innervation and control of the heart of a gastropod, *Rapana*

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Summary. The innervation and control of the heart of a prosobranch mollusc, *Rapana thomasiana*, were studied. Acetylcholine was found to be an inhibitory neurotransmitter. Both serotonin and FMRFamide (Phe-Met-Arg-Phe-NH_2) showed excitatory effects on the heart; FMRFamide had greater inotropic and more regulatory chronotropic effects than serotonin. The effects of serotonin were blocked by methysergide, while the effects of FMRFamide and of stimulating the excitatory cardiac nerves were not blocked. Stimulation of circumesophageal ganglia elicited a slow enhancement of heart beat together with body movement. This enhancement was blocked by methysergide. Serotonin was considered to act at the heart as a local neurohormone. Although the mechanism of action of FMRFamide is still not yet clarified, it is possible that FMRFamide plays a physiological role as a cardioregulatory substance, as indicated by the physiological and histological findings.
Key words. Molluscan heart; neurotransmitter; serotonin; FMRFamide.

Introduction

It is known that the molluscan heat is myogenic and that the rhythm of the heart beat is controlled by the action of neurotransmitters. In general, acetylcholine acts as an inhibitory substance, while serotonin is an excitatory substance which enhances cardiac activity[7, 22, 32]. However, this generality cannot be applied to all molluscan hearts, since the mode of action of these substances is quite variable[3, 23, 25]. Moreover, knowledge is accumulating about mechanisms for regulation of the heart beat in which one or more neurosubstances modulate the action of a main neurotransmitter[15, 22, 23].

In the heart of the prosobranch, *Rapana thomasiana*, activity is regulated by the action of cardiac nerves arising from a pair of visceral ganglia[13]. Acetylcholine has been found to be an inhibitory neurotransmitter[9]. With regard to the excitatory effects, both serotonin and FMRFamide (phenylalanyl-methionyl-arginyl-phenylalanine amide) are proposed as putative transmitters but their mechanisms are still unknown[9].

The aim of the present investigation is to clarify the mechanism of function of neurosecretory substances and to understand the heart regulatory system in which the heart beat is controlled by these substances.

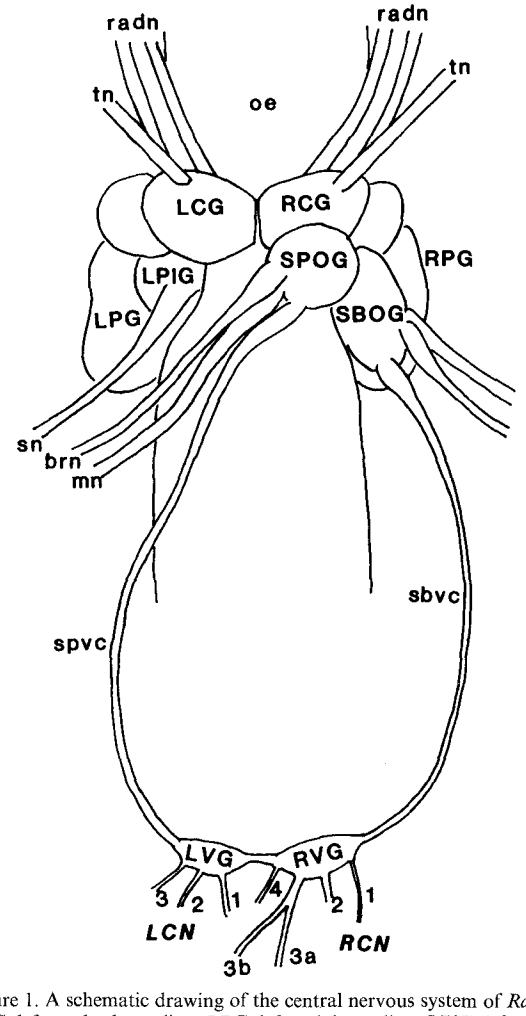

Figure 1. A schematic drawing of the central nervous system of *Rapana*. LCG, left cerebral ganglion; LPG, left pedal ganglion; LPlG, left pleural ganglion; LVG, left visceral ganglion; RCG, right cerebral ganglion; RPG, right pedal ganglion; RVB, right visceral ganglion; SBOG, subesophageal ganglion; SPOG, supraesophageal ganglion; *LCN*, left cardiac nerves; *RCN*, right cardiac nerves; brn, branchial nerve; mn, mantle nerve; oe, esophagus; radn, radula nerves; sbvc, subesophageal-visceral connective; sn, siphon nerve; spvc, supraesophageal-visceral connective; tn, tentacle nerve.

Innervation

The central nervous system of *Rapana thomasiana* is composed of circum-esophageal (head) ganglia and visceral ganglia[10]; a schematic drawing of these ganglia is shown in figure 1. The supraesophageal ganglion (SPOG) and the subesophageal ganglion (SBOG) in the mass of head ganglia are connected with the left and right visceral ganglia, respectively. The heart is innervated by four cardiac nerves arising from the right visceral ganglion and three from the left. Physiological experiments were carried out on preparations including the heart, the visceral ganglia and the cardiac nerves all interconnected and mounted on the surrounding tissues. In some experiments, a semi-intact preparation of the whole organism was also used. In this preparation, the entire central nervous system was exposed, including the head ganglia, the connectives between them and the visceral ganglia, the visceral ganglia, and the cardiac nerves. The heart was constantly perfused with saline and the heart beat was recorded with a tension transducer.

Stimulation of the right cardiac nerves (RCN) 3a and 4, or the left cardiac nerves (LCN) 1 and 2, increased heart activity. These results were obtained by using preparation 'A', in which the aorta was cut and a cannula was inserted into the ventricle through the cut end of the aorta while the atrium remained intact. These physiological results imply that the four nerves investigated contain predominantly excitatory fibers, and innervate the atrium; this is consistent with our anatomical findings. By using preparation 'B', in which the atrial end was cut and a cannula was inserted into the atrium while the ventricle and aorta remained intact, it was found that RCN 1 and RCN 3b are inhibitory nerves which have conspicuous inhibitory effects on heart beat. These results are also consistent with the anatomical observation that these two nerves innervate the ventricle through the aorta.

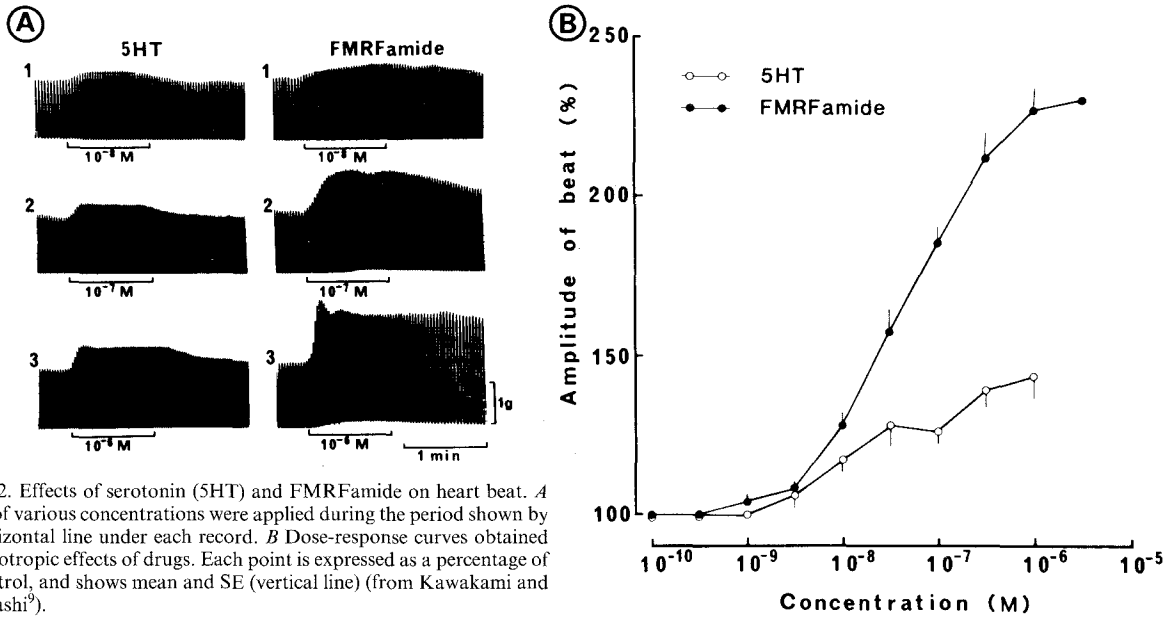

Figure 2. Effects of serotonin (5HT) and FMRFamide on heart beat. *A* Drugs of various concentrations were applied during the period shown by the horizontal line under each record. *B* Dose-response curves obtained from inotropic effects of drugs. Each point is expressed as a percentage of the control, and shows mean and SE (vertical line) (from Kawakami and Kobayashi[9]).

Inhibitory effects on heart beat

The heart beat of *Rapana* was markedly inhibited by the application of acetylcholine (ACh). When the concentration of ACh was increased, both the amplitude and frequency of heart beat were decreased, and finally the beat stopped in diastole. The duration of arrest of the beat was also dependent on the ACh concentration.

To test the possibility that ACh acts as an inhibitory neurotransmitter in the heart, effects of several blockers of ACh receptors were examined. Of five drugs tested, benzoquinonium was the most potent in blocking the inhibitory action of ACh; treatment with benzoquinonium for only 1 min was effective in blocking ACh action. Electrical stimulation of either of the right cardiac neves, RCN 1 or RCN 3b, caused an extreme inhibition of heart beat. This inhibitory effect was also blocked by treatment with benzoquinonium. Another blocker of ACh receptors, propantheline, similarly prevented the inhibitory effects both of ACh application and of nerve stimulation. However, atropine, curare or hexamethonium showed no significant action on either the inhibitory effects of ACh or on nerve stimulations.

Generally, the remarkable inhibitory effects of ACh in molluscan hearts have led to its being considered as an inhibitory neurotransmitter[8, 14]. In the present investigation, both the inhibition of heart beat by the direct application of ACh, and that by the stimulation of the cardiac nerves RCN 1 and RCN 3b, were similarly prevented by the same antagonists, benzoquinonium and propantheline. These results indicate that ACh is the inhibitory neurotransmitter at the neuromuscular junction in the heart of *Rapana*.

Excitatory effects on heart beat

Comparison of serotonin and FMRFamide

The heart beat of *Rapana* is enhanced by electrically stimulating the cardiac nerves, RCN 3a, RCN 4 and LCN 1, and by applying a drug such as serotonin and FMRFamide. Both serotonin and FMRFamide enhanced heart beat, and at each concentration tested, FMRFamide showed greater and longer-lasting effects than serotonin (fig. 2A). Dose-response curves, obtained from inotropic effects of both drugs, also illustrate that FMRFamide shows a lower threshold and greater maximum effects than serotonin and that the two curves are not parallel (fig. 2B). These results indicate that FMRFamide has greater enhancing effects on heart beat than serotonin and that the mechanisms of action of these two drugs may be different.

On the frequency of heart beat, however, FMRFamide showed diverse effects in different specimens. In specimens with a relatively slow beat, FMRFamide produced positive chronotropic and inotropic effects, whereas in relatively

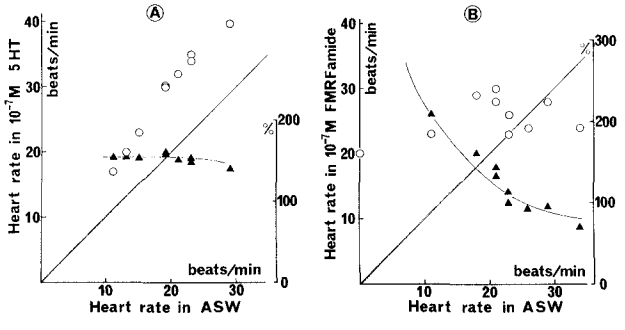

Figure 3. Effects of serotonin (5HT) and FMRFamide on heart rate. Heart rate in serotonin (*A*) and in FMRFamide (*B*) is plotted against the heart rate in ASW as determined by changing the perfusion rate. White circles show beat frequency per min and black triangles show percent of the control.

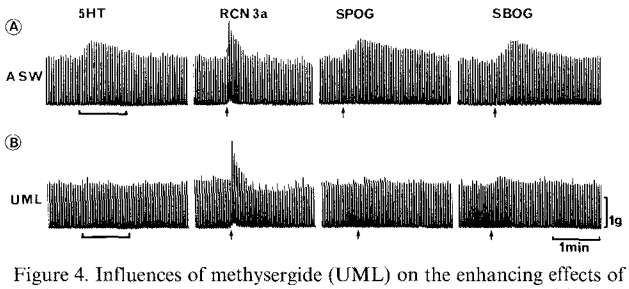

Figure 4. Influences of methysergide (UML) on the enhancing effects of applying serotonin (5HT) and of stimulating RCN 3a, SPOG and SBOG. Results (*A*) before and (*B*) after treatment with methysergide are illustrated. Serotonin was applied during the period shown by the horizontal line under each record. Stimulation (2 ms, 8 V, at 10/s) was applied for 1 s, at each arrow.

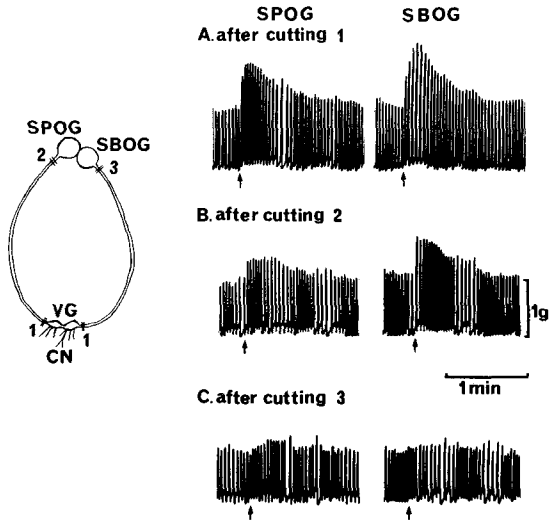

Figure 5. Effects of stimulating SPOG or SBOG on heart beat after cutting nerve connectives between SPOG or SBOG and visceral ganglia. Schematic drawing on the left illustrates the places at which the nerves were cut. Stimulation (2 ms, 8 V, at 10/s) was applied for 1 s at each arrow.

rapidly beating hearts, the beat frequency was either not changed significantly or was reduced by FMRFamide. The reduction in frequency was especially prominent when a higher dose of FMRFamide was applied which caused a greater enhancement of the amplitude of the beat.

To clarify these relationships a more systematic study was performed. Although *Rapana* heart is myogenic, internal pressure seems to be essential for spontaneous beating[13]. In our experiments, the heart was constantly perfused with artificial seawater (ASW) through a cannula, and the frequency of the heart beat was positively correlated with the perfusion rate.

In the experiments illustrated in figure 3, the effects of drugs on beat frequency were compared at different heart rates caused by changing the perfusion rate. In figure 3A, the heart rate in serotonin is plotted against the heart rate in ASW. As shown by white circles, serotonin increased beat frequency at each heart rate examined, and the percent of the control, shown by black triangles, was almost equal; i.e. 150–160%. On the contrary, FMRFamide increased beat frequency when heart rate in ASW was low, but it reduced the frequency when heart rate in ASW was high (fig. 3B). As a result, the beat frequency in FMRFamide became roughly constant irrespective of the heart rate in ASW. These results appear to indicate that FMRFamide is acting as a modulator for regulating the beat frequency within a normal range.

Next, the effects of a blocker of serotonin, methysergide[33], were examined. The enhancing effects of serotonin were completely blocked by perfusion with methysergide for 1 min, but this drug did not block the effects of FMRFamide; this showed that the receptors for serotonin and FMRFamide are different. An enhancement of heart beat was also elicited by electrically stimulating the cardiac nerves, RCN 3a, RCN 4 and LCN 1. The excitatory effects of nerve stimulation were not affected by methysergide applied for 60 min or more, and there were no significant differences among the three nerves in the results. These results suggest that serotonin is not involved in nervously induced excitation in the heart of *Rapana*.

Serotonin

Since serotonin receptors are considered to be present in the *Rapana* heart, a question arises as to how serotonin acts in this system. The following experiments (figs 4 and 5) were performed using semi-intact perparations. The two tracings on the left of figure 4A are typical pictures illustrating effects of serotonin application and stimulation of the excitatory cardiac nerve RCN 3a. When a circumesophageal ganglion (SPOG or SBOG) was electrically stimulated after all cardiac nerves had been cut off from the visceral ganglia, a slower and long-lasting enhancement of heart beat was produced (fig. 4A). This increment in contraction was always preceded by a vigorous movement of the whole body. After perfusing methysergide for 40–50 min, these enhancing effects were abolished like serotonin effects, in contrast to the effects of cardiac nerve stimulation (fig. 4B). These results suggest that serotonin may be involved in the excitation induced by the stimulation of SPOG or SBOG. If this is true, how is serotonin released and transported to the heart?

To search for the pathways of serotonin action, effects of stimulating SPOG or SBOG on heart beat were examined after successive cutting of the nerve connectives between SPOG or SBOG and the visceral ganglia (fig. 5). After cutting at point 1, shown by the schematic drawing in the figure, stimulation of SPOG or SBOG still enhanced heart beat. After cutting at point 2 the enhancement was reduced, and it was eliminated after cutting at point 3.

Serotonin has been detected in most of the major invertebrate phyla, and it is known to have diverse effects on the excitable tissues of molluscs, including heart, noncardiac muscles, and neurons[15,22]. In *Rapana* buccal mass, serotonin acts as an excitatory neuromodulator for the radula protractor but as an inhibitory one for the retractor[11,20]. It is noteworthy that the heart beat of *Rapana* was enhanced together with the movements of the whole body by the stimulation of SPOG or SBOG, and that this enhancing action was blocked by methysergide, a blocker of serotonin receptors.

Considering these results, it is assumed that serotonin is released from neurons of SPOG and/or SBOG. Since serotonin does not seem to be transported by blood against the flow of perfusate, it is possible that small unknown nerve fibers originating from SPOG and/or SBOG come near the heart without passing through the visceral ganglia. These fibers may contribute to the regulation of heart beat, probably by releasing serotonin as a local neurohormone.

FMRFamide

FRMRamide, extracted and purified from clam ganglia, is a potent cardioexcitatory neuropeptide[28,29], and is known to function physiologically in many molluscan species[1,4,5,16,31]. Unfortunately, however, a good blocker for FMRFamide is not yet known. To search for substances having FMRFamide-blocking activity and to analyze the structure-activity relations of FMRFamide, the effects of FMRFamide and related peptides on the *Rapana* heart were investigated. From the analyses of dose-response relations of FMRFamide (cf. fig. 2B) and related peptides, the following results were obtained:

FMRFamide was the most potent of the 14 peptides tested. Replacement of the N-terminal Phe by the D-isomer or by Tyr resulted in a comparatively small loss in activity. Nle could be substituted for Met. Replacement of Met with Leu also had little effect on activity. In contrast, substitution of the D-isomer for Met, Arg or the C-terminal Phe reduced activity markedly. A peptide without the C-terminal amide was inactive at doses up to 10^{-5} M. Removal of the N-terminal Phe or Met similarly resulted in loss of activity.

From these results it could be said that the C-terminal amide is essential to FMRFamide-like effects and Arg and the C-terminal Phe are also critical for such activity. The finding that changes at, or near, the C-terminal remarkably reduce FMRFamide-like activity appears to be a general feature of molluscan hearts and buccal muscles[24,30]. Furthermore, the

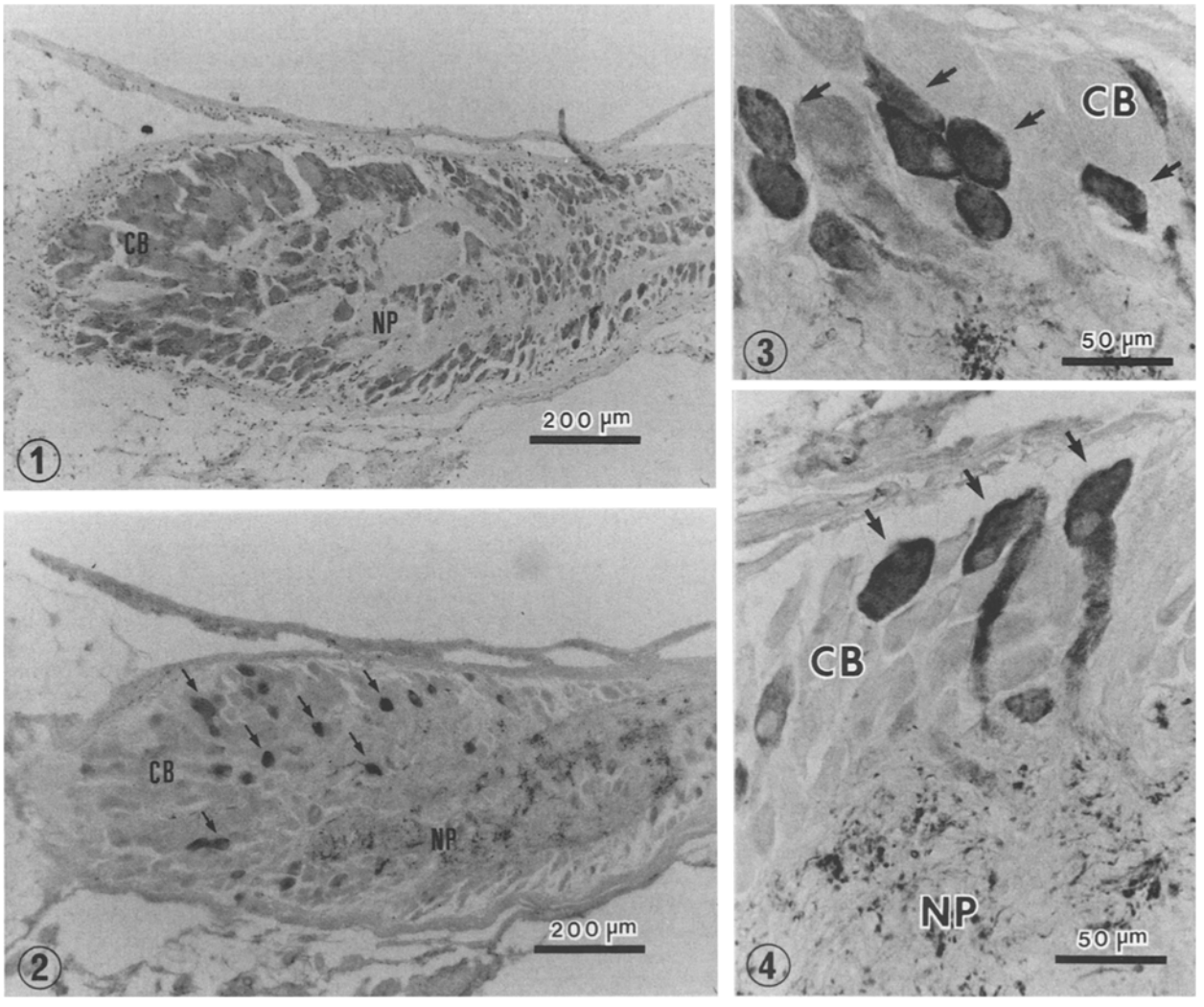

Figure 6. Histological pictures of a visceral ganglion. Sections stained with hematoxylin-eosin (1) and with FMRFamide antiserum (2) are compared. 3 and 4 are enlarged pictures of sections stained with FMRFamide antiserum. Black materials indicated by arrows show the presence of FMRFamide-like peptides. CB, cell bodies; NP, neuropile (from Saitoh, unpublished).

results above indicate that the four amino acid residues and the C-terminal amide interact as a whole with the FMRFamide receptor, rather than individually. Similar results have been obtained in the contracture-producing activity of FMRFamide on the anterior byssus retractor muscle (ABRM) of *Mytilus*[21].

Out of 14 peptides examined, 7 peptides were tested for FMRFamide-blocking activity. But none of these analogs antagonized FMRFamide.

Small cardioactive peptide B (SCP_B; Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2), which has recently attracted attention as an active substance in molluscs[17–19], was only slightly effective in reducing the frequency of heart beat in *Rapana*. When the heart was perfused with 10^{-5} M SCP_B for 3–4 min the beat frequency usually decreased to 60–70% of the control, and then recovered 7–8 min later. After more than 10 min perfusion with SCP_B, the positive inotropic effect of FMRFamide was significantly reduced. This means SCP_B has FMRFamide-inhibiting activity.

Then, with the hope of utilizing SCP_B as a FMRFamide antagonist, the effects of SCP_B on the excitation caused by stimulation of the cardiac nerve were examined. In two preparations the excitatory action of the nerve was prevented by SCP_B, but irreversibly. In the other three preparations SCP_B had no effects. Thus, it must be said at present that we do not yet have enough data to determine the mechanism of action of FMRFamide. However, it is very possible that FMRFamide plays a physiological role as an active cardioregulator in *Rapana*[12].

The following histological data also support this idea. The distribution of FMRFamide-like substances in the visceral ganglia and the heart was investigated histologically. Tissues were fixed and cut in sections, then incubated with FMRFamide antiserum. The sections of the visceral ganglia are illustrated in figure 6. The section shown at the top left (1) is stained with hematoxylin-eosin, and the others were treated with FMRFamide antiserum. Black materials indicated by arrows show the location of FMRFamide-like peptides, i.e., peptides having Arg, Phe and amide at the C-terminal. These pictures demonstrate that visceral ganglia have many FMRFamide-like substances.

Figure 7 shows sections of the heart. The atrium includes FMRFamide-like materials, but the ventricle has little. These results are consistent with the anatomical and physio-

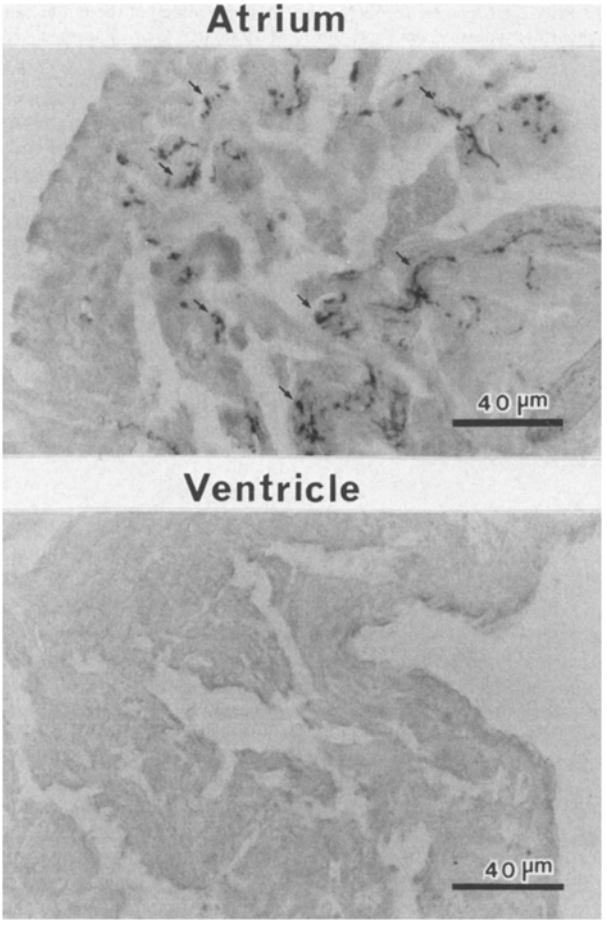

Figure 7. Comparison of sections of atrium and ventricle stained with FMRFamide antiserum. Arrows indicate the presence of FMRFamide-like peptides (from Saitoh, unpublished).

logical evidence that excitatory nerves are all atrial nerves; that is, they innervate the atrium through the ventral wall of the pericardium and a few of their branches travel through the atrium to the ventricle[13]. It may be that excitatory nerves containing FMRFamide innervate the atrium for regulating the heart beat and also contribute to enhancement of cardiac activity.

Concluding remarks

The present study considers the mechanism of function of the peptide FMRFamide, as well as serotonin and ACh, in the cardioregulation of *Rapana*. The possibility that FMRFamide is an active cardioregulator was found to be supported by physiological and histological evidence. Comparative studies have demonstrated that FMRFamide and the related peptides are present not only in molluscs but also in other phyla and may play roles in the transmission of information or its regulation[2, 6, 27]. Such investigation from the comparative viewpoint should be one of the most reliable approaches to the understanding of cardioregulatory mechanisms in the individual organism, and could also contribute to studies of evolutionary aspects[26]. The present results may be conducive to the development of the comparative physiological view in this field.

Acknowledgments. The author would like to acknowledge the co-operation of Dr Yojiro Muneoka, Ms T.K. Hasimoto, Ms M.F. Nakamura and Ms H.O. Kawakami in the physiological and anatomical experiments, and the help of Mr Hitoshi Saitoh for his contribution to making histological preparations. The author is also grateful to Sandoz Ltd. for supplying a sample of methysergide.

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0014-4754/87/090981-06$1.50 + 0.20/0

Inhibitory neural control of the myocardium in opisthobranch molluscs

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Key words. Opisthobranch gastropods; molluscan hearts; acetylcholine responses; inhibitory junctional potentials.

Introduction

Acetylcholine (ACh) has long been known as a cardioinhibitory agent in molluscs[4,8,11]. There was some evidence, however that ACh produced excitatory effects on the heart in a few species of bivalve[3]. In gastropods, the excitatory effect of ACh has been observed in *Strophocheilus*[7], and in *Helix*[18]. In the heart of *Helix*, the amplitude and tone are reduced although the frequency of action potentials may be increased[18]. Hill[5] demonstrated that ACh (2×10^{-8} to 2×10^{-3} M) depolarized the isolated ventricle of the opisthobranch *Dolabella auricularia*.

It has also been reported by Hill and Yantorno[6] that the ventricles of both *Dolabella auricularia* and *Aplysia dactylomela* were depolarized by ACh. Kuwasawa[13] showed that ACh may have different effects in different sites of the heart of *Dolabella*. The auriculo-ventricular valve (A–V valve) is usually hyperpolarized and the ventricle is depolarized.

Although the effects of ACh on the heart thus vary widely, even among gastropod species[8], it has been demonstrated in the *Dolabella* heart that neurally mediated cardiac inhibition itself may be cholinergic[13].

In *Aplysia californica*, the role of ACh as the transmitter of a heart inhibitor neuron has been established by biochemical and electrophysiological criteria[15]. On the other hand, clear evidence showing cholinergic inhibition has been obtained from the A–V valve of *Dolabella*. Muscle cells from which inhibitory junctional potentials (IJPs) are recorded are confined to the A–V valve[13]. Kuwasawa and Yazawa[14] showed that IJPs, induced by stimulation applied to the cardiac nerve, were clearly blocked by tubocurarine.

Materials and methods

The physiological significance of the responses of the myocardium to bath-applied ACh, and to ionophoretic application of ACh, were investigated with an intracellular recording technique. The purpose of the investigation was to elucidate what type of ACh response is involved in the physiological mechanisms of cardiac inhibition which are induced by regulatory nerves in opisthobranch molluscs.

Opisthobranch molluscs of the species *Dolabella auricularia*, belonging to the anaspidea, were collected on the seashore at Shimoda, and *Pleurobranchaea novaezealandiae*, belonging to the notaspidea was obtained from fishing boats in Yokohama. Both animals were stored separately at 15 °C in aquaria with natural seawater.

The experimental preparation was dissected in natural seawater from an animal injected with 5–15 ml isotonic $MgCl_2$ solution 5 min before the dissection. This preparation consisted of the heart alone or together with the cardiac nerve. In the case of *Pleurobranchaea*, the visceral (= abdominal) ganglion from which the cardiac nerve arises was included. The heart was cut longitudinally and pinned out flat in a small Sylgard-lined bath filled with artificial seawater

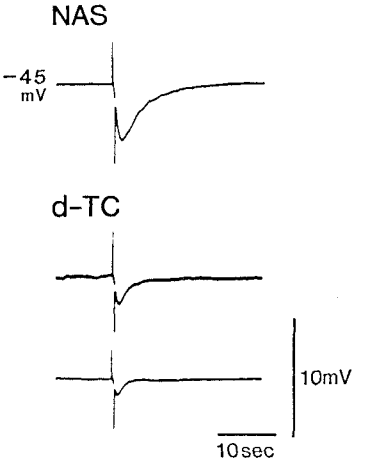

Figure 1. Effect of tubocurarine on the hyperpolarizing response to ACh of A–V valve muscle cells in *D. auricularia*. Top, control; middle and bottom, respectively, at 1 min and 3 min after the application of tubocurarine 2×10^{-5} M. In this and the following figures, numerals are membrane potentials at the beginning of each record.