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Regulatory Actions of 5-Hydroxytryptamine and Some Neuropeptides on the Heart of the African Giant Snail, *Achatina fulica* Férussac

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ABSTRACT—Effects of putative neurotransmitters and modulators on the atrium preparation of the African giant snail, *Achatina fulica* were observed to investigate the regulatory mechanisms of these substances on the heart beat. Application of 5-hydroxytryptamine (5-HT) as well as stimulation of a heart excitatory neuron, PON, resulted in the enhancement of the heart beat. The enhancement of the beat was blocked by a 5-HT blocker, methysergide. A neuropeptide FMRFamide potentiated the excitatory responses of the atrium to both PON stimulation and 5-HT application, whereas small cardioactive peptide B (SCP_B) depressed them.

A burst of impulses in the cerebral neuron, d-RCDN or d-LCDN, evoked excitatory response in the heart excitatory neurons, TAN, TAN-2 and TAN-3, as well as in PON. It depolarized the membrane and increased the frequency and duration of spikes in three TANs. Similar responses were elicited by 5-HT application. On the contrary, FMRFamide hyperpolarized the membrane and shortened the spike duration in three TANs and PON. The experiment of current measurement showed that 5-HT and FMRFamide act antagonistically in the heart excitatory neurons.

INTRODUCTION

It has been known that several neurotransmitters, such as acetylcholine (ACh) and 5-hydroxytryptamine (5-HT), are involved in the control of the molluscan heart beat, and that some invertebrate neuropeptides show powerful modulatory actions in the synaptic transmission [1, 2]. The modes of action of the transmitters as well as the modulators are not uniform in general but quite variable depending on species and organs [3–5].

In the African giant snail, *Achatine fulica,* several heart regulatory neurons have been identified in the central nervous system [6, 7]. Among them, two cerebral ganglion cells, the dorsal right cerebral distinct neuron (d-RCDN) and the dorsal left cerebral distinct neuron (d-LCDN), produce a slow depolarization and prolong the duration of action potential in the periodically oscillating neuron (PON), which is the most effective heart excitor. The transmitter of the two cerebral neurons is suggested to be 5-HT [8]. The direct action of 5-HT on the heart has also been examined by using the isolated ventricle and dual effects, enhancing and arresting, on the beat have been demonstrated [9]. However, in *Achatina* heart, responses to putative neurotransmitters and modulators are not always similar between the ventricle and atrium [10].

In the present study, to demonstrate the regulatory mechanisms on the heart beat, the effects of application of several putative neurotransmitters including neuropeptides and stimulation of heart excitatory neurons on the isolated atrium preparation were observed. In addition, the mode of action of the cerebral neurons (d-RCDN and D-LCDN) on the heart excitors were also investigated.

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MATERIALS AND METHODS

The African giant snail, *Achatina fulica* Férussac, which was captured in Okinawa, transported by air to Hiroshima and bred in our laboratory at 24°C, was used. Circumoesophageal ganglia and heart connected with the intestinal nerve were dissected from the animal. The connective capsule and the inner sheath covering the dorsal surface of the cerebral ganglia and the right parietal ganglion were completely removed by dissection. Most of the ventricle was cut off leaving the atrium intact. The preparation was pinned to the bottom of an experimental chamber coated with silicone resin. The chamber consisted of two compartments (ganglia compartment and heart compartment) which could be perfused separately [6].

The composition of normal physiological solution was as follows (mM/l): NaCl, 61.0; KCl, 3.3; $CaCl_2$, 10.7; $MgCl_2$, 13.0; glucose, 5.0; and Hepes, 10.0 (pH adjusted to 7.5 by titration with NaOH). High magnesium solution was prepared by merely adding extra $MgCl_2$ to normal saline.

For the application of 5-HT to the preparation a definite volume of the drug was introduced into the solution in the chamber through a small pipette, which was rapidly spread throughout the solution by means of air bubbles released from the bottom of the chamber. The concentration of the drug was stated as final concentration in the solution. Duration of 5-HT application was usually less than 2 min, except for cases stated otherwise, during which perfusion of the solution was stopped, and interval of at least 20 min was allowed between the two applications. The applicaton of 5-HT antagonist, methysergide, and several neuropeptides for a longer period was made by perfusing the solution cntaining the chemical at a given concentration.

The following drugs were used: 5-hydroxytryptamine creatinine sulfate (5-HT, Sigma), methysergide-hydrogenmaleinate (methysergide, Sandoz), 3-hydroxytyramine hydrochloride (dopamine, Katayama), DL-octopamine hydrochloride (octopamine, Nakarai), FMRFamide, YGGFMRFamide, pQDPFLRFamide, small cardioactive peptide A (SCP_A) and SCP_B (Peninsula Laboratories Inc.), and FLRFamide (Cambridge Research Biochemicals).

Intracellular recording and stimulation of neurons were carried out using glass microelectrodes filled with 3 M potassium acetate, having a resistance of 5–10 MΩ. Heart beat was recorded by a strain gauge. In a few experiments, the intestinal nerve was cut off from the ganglia and it was stimulated by an Ag-AgCl bipolar electrode at the point just before entering the pericardium.

When interconnections between two neurons or effects of chemicals on neurons were investigated, only the preparation of circumoesophageal ganglia was used. Membrane current was measured using a voltage-clamping method by two microelectrodes as described previously [8].

The data were stored in an FM tape recorder (Sony, DFR3515) for later analysis and redisplayed on an ink-writing pen-recorder (Nihon Kohden, RJG 4024).

All the experiments were carried out at room temperature of 23–25°C.

RESULTS

Effects of 5-HT application and nerve stimulation on heart beat

Isolated atrium usually repeats regular beating in the experimental chamber. When 5-HT at concentrations above 10^{-8} M was applied to the atrium, the frequency and amplitude of heart beat were enhanced. Stimulation of the intestinal nerve produced potentiation of heart beat similar to that obtained by 5-HT application (Fig. 1). The potentiation by nerve stimulation was abolished when the preparation was perfused with high magnesium ($3\times Mg^{2+}$) solution which may block the neuromuscular synapses. The enhancement of heart beat by 5-HT application, however, was not blocked in $3\times Mg^{2+}$ solution, suggesting that 5-HT acts postsynaptically. Dopamine exhibited effects similar to 5-HT on the atrium, but the threshold concentration was 10^{-6} M. Octopamine showed no significant effects at concentrations up to 10^{-5} M.

The action of heart excitatory neurons

A neuron in the right parietal ganglion, PON, has been shown to be the most potent heart excitor

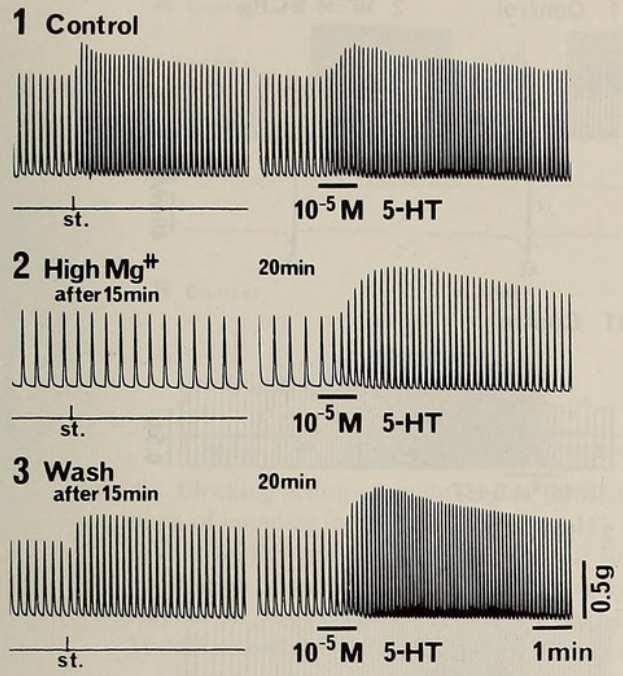

FIG. 1. Effects of high-Mg^{2+} in solution on the excitatory responses of the atrium to nerve stimulation (st.) and 5-HT application. The intestinal nerve was stimulated with electrical pulses of 3 V, 1 msec at 5 Hz for 1 sec. 5-HT was applied during the period shown by the horizontal line under each record.

extending axons directly to the heart [6]. In the experiments illustrated in Figure 2, the effects of methysergide, a potent blocker of 5-HT receptor in the gastropod heart muscles, on the action of PON stimulation and 5-HT application were examined. The intracellular stimulation of PON evoked impulses in PON, which, in turn, enhanced the frequency and amplitude of heart beat. This enhancement of the beat was almost completely blocked by perfusing the atrium preparation with methysergide (Fig. 2A). Similarly, the potentiation of heart beat by 5-HT application was blocked by methysergide, although methysergide showed no significant direct effects on the beat (Fig. 2B). These results suggest that the potentiation of heart beat by PON stimulation may be mediated by 5-HT.

To obtain a better understanding on the mode of action of PON, modulatory effects of several neuropeptides on the heart excitatory action of this neuron as well as direct effects of those substances on the heart beat were examined. Of six neuropeptides (FMRFamide, FLRFamide, YGGFMRFamide, pQDPFLRFamide, SCP_A and

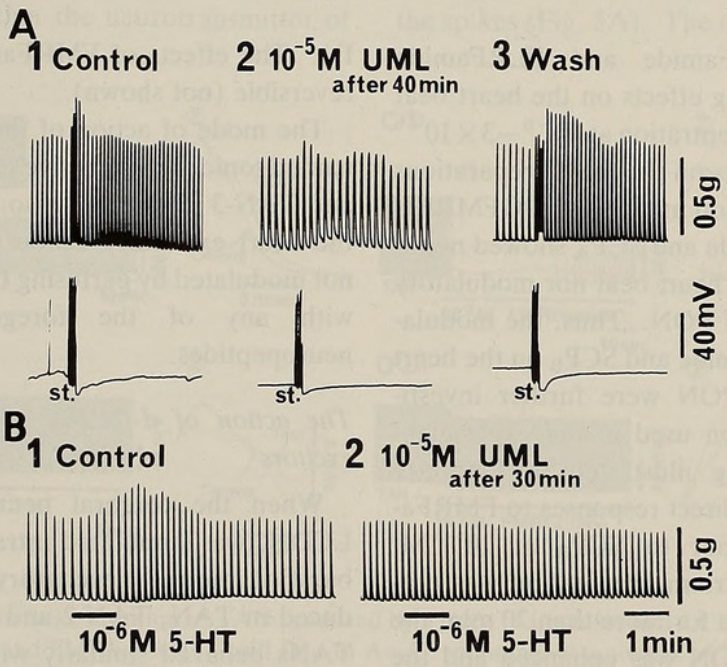

FIG. 2. Effects of a 5-HT blocker, methysergide (UML), on responses of the atrium to PON-stimulation (A) and 5-HT application (B). A and B are records from different preparations. In A, the heart beat (upper tracings) induced by the spikes of PON (lower tracings) are recorded simultaneously. PON was made to fire by current injection (st.) at 5 Hz for 10 sec. In B, 5-HT was applied during the period shown by the horizontal line under each record.

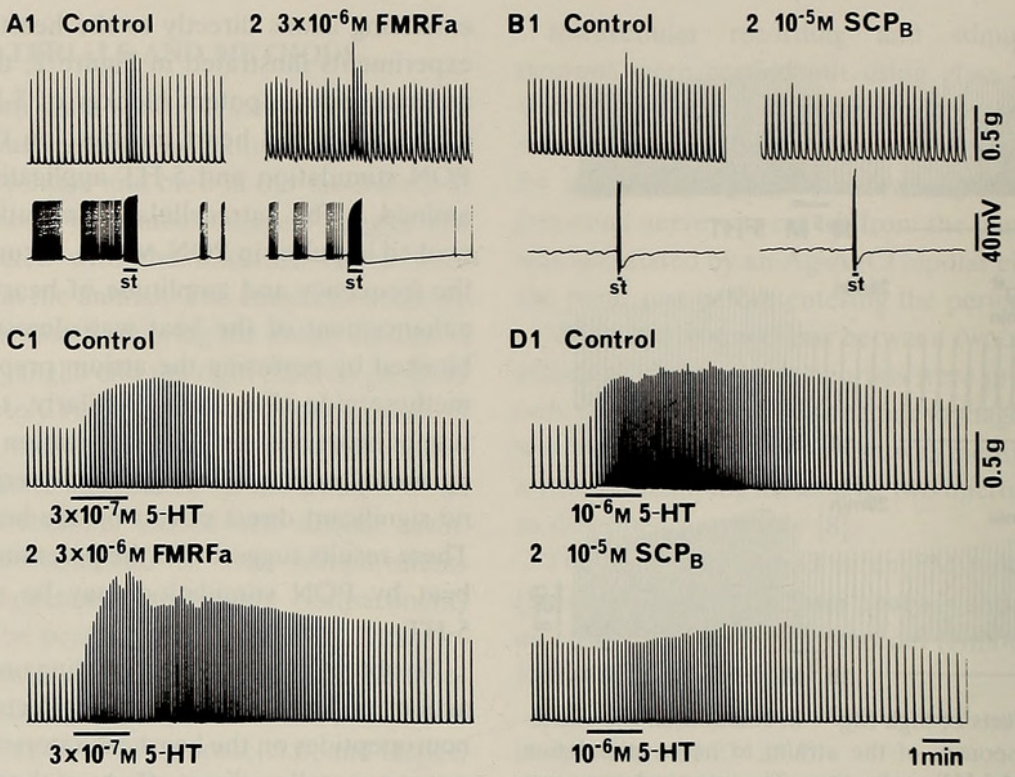

Fig. 3. Effects of neuropeptides on the enhancement of heart beat induced by the spikes of PON (A and B) and 5-HT application (C and D). A, B, C and D are records from different preparations. In A and B, PON was stimulated (st) at 5 Hz for 20 sec and 10 sec, respectively. A_2 was recorded 20 min after application of FMRFamide (FMRFa), and B_2 was 30 min after application of SCP_B. In C and D, 5-HT was applied during the period shown by the horizontal line under each record. C_2 and D_2 were recorded 20 min after application of FMRFa and SCP_B, respectively.

SCP_B) tested, FMRFamide and FLRFamide showed slight enhancing effects on the heart beat with the threshold concentration at $10^{-6}\sim3\times10^{-6}$ M. SCP_B had no effects on the most preparations but exhibited potentiation in a few. YGGFMRFamide, pQDPFLRFamide and SCP_A showed neither direct effects on the heart beat nor modulatory effects on the action of PON. Thus, the modulatory effects of FMRFamide and SCP_B on the heart excitatory action of PON were further investigated. The preparation used in the experiment with the results being illustrated in Figure 3 showed no significant direct responses to FMRFamide at 3×10^{-6} M nor to SCP_B at 10^{-5} M. However, when the atrium preparation was perfused with FMRFamide for more than 20 min, the excitatory action of PON was enhanced and the response to 5-HT was also potentiated (Fig. 3A, C). On the contrary, perfusion with SCP_B resulted in depression of the excitatory responses to both PON stimulation and 5-HT application (Fig. 3B, D). The effects of FMRFamide and SCP_B were reversible (not shown).

The mode of action of the other heart excitors named tonically autoactive neurons, TAN, TAN-2 and TAN-3 [6, 11] was also examined. However, the heart excitatory action of these neurons was not modulated by perfusing the atrium preparation with any of the foregoing six kinds of neuropeptides.

The action of d-RCDN and d-LCDN on heart excitors

When the cerebral neuron, d-RCDN or d-LCDN, was stimulated intracellularly to evoke a burst of impulses, excitatory responses were produced in TAN, TAN-2 and TAN-3. These three TANs behaved similarly with no different properties. Figure 4A shows an example in the case between d-RCDN and TAN-2. By the stimulation of d-RCDN at 10 Hz, TAN-2 which had previously been hyperpolarized to stop firing was depolarized

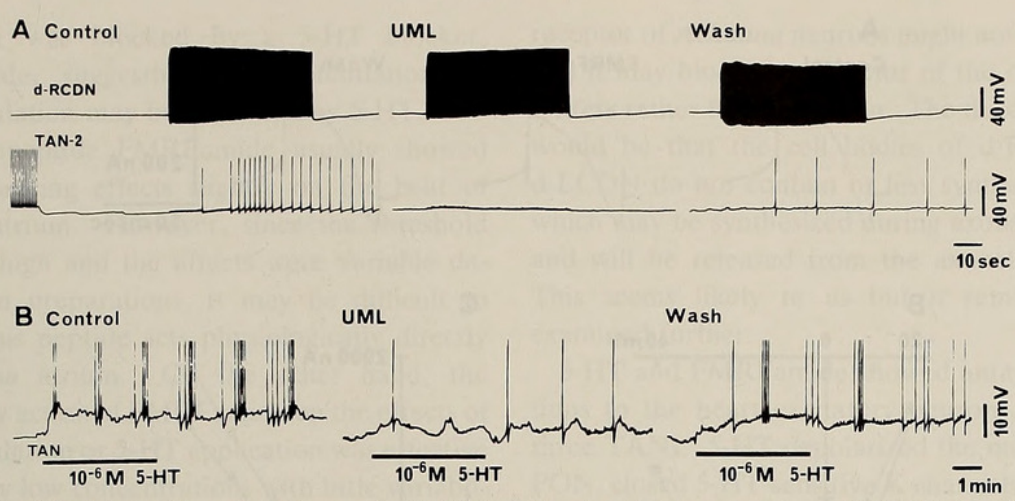

FIG. 4. Blocking action of methysergide (UML) on the depolarizing responses of TAN induced by a burst of impulses in d-RCDN (A) and 5-HT application (B). A. TAN-2 was hyperpolarized by 10 mV. d-RCDN was stimulated at 10 Hz for 50 sec. Middle record (UML) was obtained 60 min after application of methysergide. B. TAN was hyperpolarized by 30 mV. The top of action potentials was cut off. 5-HT was applied during the period shown by the horizontal line under each record. Middle record (UML) was obtained 30 min after application of methysergide.

and began to fire. These excitatory responses were found to be depressed reversibly by 5-HT antagonist, methysergide. Similarly, as shown in Figure 4B, application of 5-HT produced spikes superimposed on a slow depolarization in TAN, which were also depressed by methysergide. These results are essentially the same as those obtained in PON [8], suggesting that the neurotransmitter of the two cerebral neurons is 5-HT.

In the experiments shown in Figure 5, the effects of a burst of impulses in the cerebral neuron or the application of putative neurotransmitters on the activities of three TANs were examined. A burst of impulses in d-LCDN increased the spike frequency in TAN-2 and produced a broadening of the spikes (Fig. 5A). The application of 5-HT also

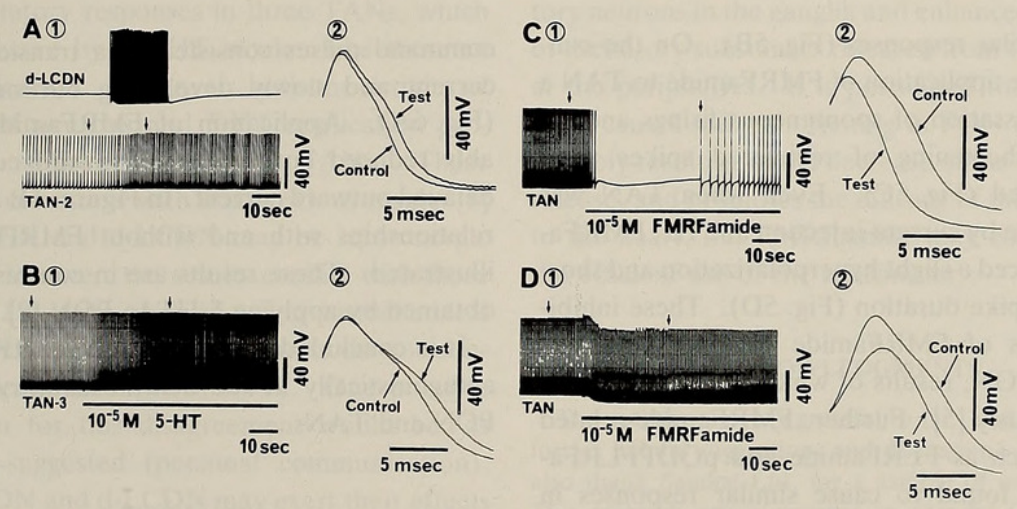

FIG. 5. Change in spike duration of TAN produced by a burst of impulses in d-LCDN (A), application of 5-HT (B) and FMRFamide (C and D). In A~C, spontaneous activities of TAN-2 (A), TAN-3 (B) and TAN (C) were recorded. In D, TAN was driven to fire by a depolarizing current injection at 2 Hz. In A, d-LCDN was stimulated at 10 Hz for 20 sec. In B~D, 5-HT (B) or FMRFamide (C and D) was applied during the period shown by the horizontal line under each record. Arrows in A_1, B_1, C_1 and D_1 indicate selected spikes which are displayed at expanded time scale in A_2, B_2, C_2 and D_2.

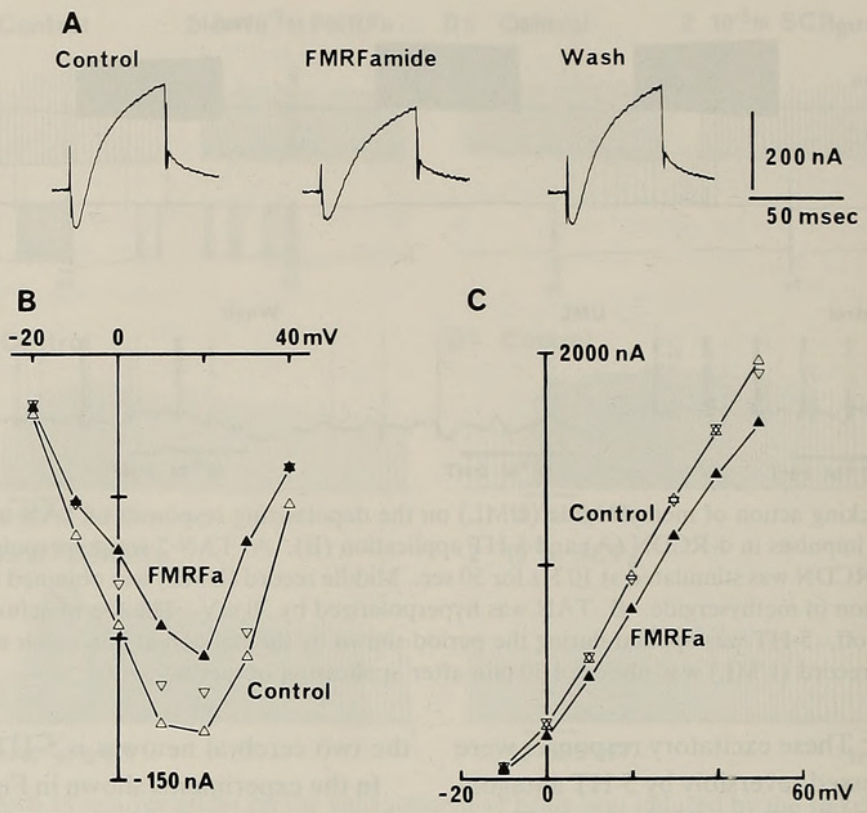

Fig. 6. Effects of FMRFaminde on the membrane currents of TAN. A. Membrane currents with and without 1.25×10^{-5} M FMRFamide. Holding potential was -40 mV. The command pulse was 50 msec in duration and depolarized to 0 mV. B. I-V relationships of peak inward currents with (closed triangles) and without (open triangles) FMRFamide (FMRFa). Open upright (△) and upside-down (▽) triangles denote values before application of FMRFamide (Control) and after wash (Wash), respectively. C. I-V relationships of oupward currents measured at the end of the pulse with and without FMRFamide. Symbols mean the same with B.

elicited similar responses (Fig. 5B). On the contrary, by the application of FMRFamide to TAN a tentative cessation of spontaneous firings and remarkable shortening of recovered spikes were demonstrated (Fig. 5C). Even when TAN was driven to fire by current injection at 2 Hz, FMRFamide produced a slight hyperpolarization and shortened the spike duration (Fig. 5D). These inhibitory actions of FMRFamide were also demonstrated in PON, results of which were reported in part previously [5]. Further, FMRFamide-related peptides such as FLRFamide and pQDPFLRFamide were found to cause similar responses in TANs.

Finally, the effects of FMRFamide on the membrane currents were examined in TAN, which was axotomized to get better conditions for space-clamp. Holding potential was set at -40 mV. The membrane currents measured using depolarizing command pulses consisted of a transient inward current and slowly developing outward current (Fig. 6A). Application of FMRFamide remarkably reduced both the peak inward current and delayed outward current. In Figures 6B and C, I-V relationships with and without FMRFamide are illustrated. These results are in contrast to those obtained by applying 5-HT to PON [8].

It is concluded that 5-HT and FMRFamide act antagonistically in the heart excitatory neurons, PON and TANs.

DISCUSSION

The present study demonstrated that application of 5-HT produced potentiation of the beat in the atrium preparation like stimulation of a heart excitatory neuron, PON. The potentiation of heart beat by both 5-HT application and PON

stimulation was blocked by a 5-HT blocker, methysergide, suggesting that potentiation by PON stimulation may be mediated by 5-HT.

A neuropeptide FMRFamide usually showed direct enhancing effects slightly on the beat of *Achatina* atrium. However, since the threshold was quite high and the effects were variable depending on preparations, it may be difficult to consider this peptide acts physiologically directly to *Achatina* atrium. On the other hand, the modulatory action of FMRFamide on the effects of PON stimulation or 5-HT application was effective at relatively low concentrations with little variability. FMRFamide is known to show powerful modulatory effects on the synaptic transmission in molluscs [12, 13]. In the cerebral and suboesophageal ganglia of *Achatina* there have been shown a number of FMRFamide immunoreactive neurons [14]. By using a immunohistochemical method, we have also observed FMRFamide-containing nerve terminals in the atrium as well as FMRFaminergic neurons in the ganglia (unpublished data). Thus, the excitatory modulation by FMRFamide (or FMRFamide-related peptide) at the synapse from PON to the heart seems to be probable physiologically.

In the present experiment, the activity of two cerebral neurons, d-RCDN and d-LCDN, produced excitatory responses in three TANs, which were depressed by a 5-HT blocker like those to 5-HT application. Moreover, both the activity of the cerebral neurons and 5-HT application produced the spike broadening in TANs. These results are consistent with our previous results [8] that d-RCDN and d-LCDN may be serotonergic neurons. However, the results conflict with those by Croll [15], who showed using histochemical methods that these cerebral neurons do not contain significant amounts of 5-HT. One possible explanation for this disagreement would be, as Croll has suggested (personal communication), that d-RCDN and d-LCDN may exert their effects upon PON and TANs via a polysynaptic pathway with the last cell in the chain being serotonergic. However, we have considered that the pathway could be monosynaptic from our results of physiological experiments [7]. The second possibility would be that the specificity of methysergide to the receptor of *Achatina* neurons might not be so strict and it may block the receptor of the other transmitters rather than serotonin. The third possibility would be that the cell bodies of d-RCDN and d-LCDN do not contain or less synthesize 5-HT, which may be synthesized during axonal transport and will be released from the axonal terminals. This seems likely to us but it remains to be examined further.

5-HT and FMRFamide showed antagonistic actions to the heart excitatory neurons, PON and three TANs. 5-HT depolarized the membrane of PON, closed 5-HT-sensitive K channels, increased the voltage-dependent Ca^{2+} current and produced spike broadening [8, 16]. The present experiment showed that spike broadening by 5-HT also occurred in TANs. Contrary to these, FMRFamide hyperpolarized TAN membrane, produced spike shortening and decreased inward current possibly by increasing background K^+ current. These antagonistic actions between 5-HT and FMRFamide are similar to those found in *Aplysia* sensory neurons [17–20].

It is well known that actions of FMRFamide are variable on the same organ in different species as well as on different organs in one species [5, 12]. Thus, it may not be surprising that in *Achatina* FMRFamide inhibited the action of heart excitatory neurons in the ganglia and enhanced the effect of excitatory substances released from the neurons at the peripheries. It is postulated that FMRFamide causes spike shortening in PON and TANs, possibly resulting in the decrease of the transmitter release, and promotes the efficacy of the substance to the heart, i.e. FMRFamide may contribute to the efficient use of the transmitter.

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