FMRF-amide-like Substances in the Leech

II. Bioactivity on the Heartbeat System¹

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Abstract

In the preceding paper (Kuhlman, J. R., C. Li, and R. L. Calabrese (1985) *J. Neurosci.* 5: 2301–2309) FMRF-amide-like immunoreactivity was localized to a specific set of neurons in the leech. Three types of these neurons are involved in controlling the animal's heartbeat: HE motor neurons and HA modulatory neurons which directly innervate the hearts, and the swim-initiating interneurons (cells 204) which can accelerate the heartbeat central pattern generator. Application of synthetic FMRF-amide had effects on the hearts and the heartbeat central pattern generator that mimicked the actions of the HA and cell 204 neurons.

Bath application of FMRF-amide (10^{-7} to 10^{-6} M) to the hearts activated their myogenic rhythm and increased their beat tension, thus mimicking the effects of activity in HA cells. Bath application of lower concentrations of FMRF-amide (10^{-9} to 10^{-8} M) to the isolated central nervous system dramatically accelerated the central motor program for heartbeat, thus mimicking the effects of activity in cell 204. These observations suggest that an FMRF-amide-like substance may be used as a chemical signal by HA and cell 204 neurons. The role of the FMRF-amide-like substance contained in HE motor neurons remains unclear, but it may be released along with the HE cell's neuromuscular transmitter, acetylcholine.

In the preceding paper (Kuhlman et al., 1985) we demonstrated that a defined population of central neurons in the leech shows FMRF-amide-like immunoreactivity (FLI). Three types of these neurons play a role in the control of the animal's heartbeat. Two types innervate the heart directly—the heart excitatory (HE) motor neurons and the heart accessory (HA) modulatory neurons, and the other type—cells 204—are swim-initiating interneurons (Weeks and Kristan, 1978) that have acceleratory effects on the heartbeat central pattern generator (Arbas and Calabrese, 1984).

The heartbeat central pattern generator comprises seven pairs of identified HN interneurons that generate a coordinated activity rhythm (Calabrese and Peterson, 1983). Through rhythmic inhibition, the cycling of this oscillatory network is imposed on the HE motor neurons and the HA modulatory neurons. The cycle rate of the

pattern generator and, thus, of the HE and HA cells, is subject to considerable neuromodulation (Arbas and Calabrese, 1984).

A bilateral pair of heart excitator motor neurons, the HE cells, is located on the ventral surface of segmental ganglia 3 to 18, and each HE cell innervates the ipsilateral heart tube in its own segment (Thompson and Stent, 1976a; Maranto and Calabrese, 1984a). HE cells are rhythmically active due to the rhythmic inhibition which they receive from the heartbeat central pattern generator (Thompson and Stent, 1976b; Calabrese and Peterson, 1983). Whereas the leech heart is capable of producing a myogenic polarization and contraction rhythm, bursts of impulses in the HE motor neurons both reset and entrain the heart's myogenic rhythm (Maranto and Calabrese, 1984b).

The myogenic activity of the heart is subject to additional modulation by the heart accessory neurons, or HA cells (Calabrese and Maranto, 1984). Bilateral pairs of HA neurons have been identified in both the fifth and sixth segmental ganglia. Like HE cells, HA cells synapse with heart muscle cells as demonstrated ultrastructurally (Maranto and Calabrese, 1984a). The HA cells also receive rhythmic inhibition from the heartbeat central pattern generator (Calabrese and Maranto, 1984). Activity in the HA neurons induces myogenicity in quiescent hearts and accelerates the rhythm of hearts that are already myogenic. In hearts that are entrained by the rhythmic output of HE motor neurons, HA cell activity increases the beat tension of individual contractions (Calabrese and Maranto, 1984).

In this paper we describe the physiological effects of bath-applied FMRF-amide on the heartbeat system of the leech; low concentrations of FMRF-amide have strong modulatory effects on the leech heart and on the heartbeat central pattern generator. These results indicate that a FMRF-amide-like substance functions in chemical transmission within the leech heartbeat system.

Materials and Methods

Animals. Adult specimens of the leech *Hirudo medicinalis* were purchased from European suppliers (Ricarimpex and Blutegelimport und Versand). Leeches were kept at 15°C in artificial pond water for up to 6 months before use.

Physiological studies. In all physiological studies preparations were perfused with a constant flow of leech physiological saline (Ringer's). Perfusing solutions could be switched from normal Ringer's to Ringer's containing a specified concentration of synthetic FMRF-amide. FMRF-amide concentrations tested ranged from 8×10^{-7} M to 8×10^{-10} M. The volume of solution in the recording chamber (clear Sylgard-coated dish) was kept constant at approximately 5 ml, with both inflow and outflow rates of perfusing solution adjusted to approximately 5 ml/min.

Standard glass microelectrodes (Haer) backfilled with 4 M potassium acetate were used for all intracellular recordings. Activity of heart tubes was monitored by a tension transducer (Grass FT 30 B) connected to one end of a section of heart tube by a small hook and a short length of thread. Output of the transducer was calibrated statically. Tension during a contraction was integrated by cutting the tension trace out of chart paper and weighing it. All tension integrals for a particular experiment were normalized

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Figure 1. FMRF-amide increases the beat tension when the heart is normally entrained by the HE motor neuron's central activity rhythm. The CNS was separated from the heart and body wall by a petroleum jelly dam so that FMRF-amide application was restricted to the periphery. Four beats before, during, and after FMRF-amide (8×10^{-8} M) application are illustrated.

by dividing them by the maximum integral for that experiment and expressing them as a percentage of the maximum.

Heart tube preparations. A preparation (Maranto and Calabrese, 1984a) consisting of one segment of heart tube innervated by a nerve cord containing the essential elements of the heartbeat central pattern generator (segmental ganglia 2 to 7) (Calabrese and Peterson, 1983) was used to determine the bioactivity of FMRF-amide on the heart muscle. When a stable base line of heart tube activity was established, the HE cell ipsilateral to the innervated segment was penetrated with a microelectrode and was either allowed to burst normally or was hyperpolarized by injection of steady negative current (0.5 to 2 nA). The preparation was then perfused with Ringer's containing FMRF-amide for up to several minutes while monitoring both heart tube and HE cell activity, followed by a wash with normal Ringer's perfusion until the heart tube resumed its previous base line state of activity. This procedure of FMRF-amide application was repeated up to five times in a stable preparation. For some experiments, a petroleum jelly dam was constructed between the body wall segment and nerve cord to prevent the FMRF-amide perfusion from producing central effects.

Isolated nerve cords. Isolated nerve cords (Miller et al., 1981) including segmental ganglia 2 to 7 were used to investigate the bioactivity of FMRF-amide on the heartbeat central pattern generator. The heartbeat central pattern generator comprises a set of HN interneurons for which the rhythmic activity was monitored by recording their IPSPs in the HE motor neurons (Calabrese and Peterson, 1983). Penetration of HE rather than HN cells prevents damaging any elements of the oscillatory network. Once the HE motor neuron was monitored for several minutes to determine its activity period, the perfusing solution was then changed to Ringer's with FMRF-amide for a period of 30 sec to a few minutes, followed by washing with normal Ringer's until the rhythm of IPSPs in the HE cell returned to its previous state. FMRF-amide application at various concentrations was repeated many times for a given nerve cord, although not necessarily while recording from the same HE cell.

Results

Peripheral effects of FMRF-amide

Since both the HE motor neurons and HA modulatory neurons and their processes on the heart show intense FLI (Kuhlman et al., 1985), we sought physiological effects of FMRF-amide at the heart muscle. Experiments involving the simultaneous monitoring of a segment of heart tube and the innervating HE motor neuron revealed effects of FMRF-amide on the heart muscle which were independent of changes in neural activity. Such effects occurred both when the central activity of the HE cell was expressed and when this activity was suppressed experimentally with injected current.

Central activity expressed. All of the experiments described in this section were performed with a wall of petroleum jelly separating the nerve cord from the perfusing solution, thereby eliminating possible central changes in the HE cell's activity.

With the normal central activity of the HE cell expressed, application of FMRF-amide (8×10^{-8} M) increased the peak tension and, to a lesser extent, the total tension integral of individual contractions of the heart produced by discharge of the HE motor neuron (Figs. 1 and 2, A and B). Moreover, such application decreased the latency from the start of the HE motor neuron's burst to the beginning of the heart's contraction, suggesting an underlying acceleration of the heart's myogenic rhythm (Fig. 2C). Similar concentrations of FMRF-amide sometimes caused the heart's contraction rhythm to break away from entrainment by the HE motor neuron (Fig. 3), again suggesting an acceleration of the heart's myogenic rhythm. Washing with normal Ringer's reversed all of the effects caused by FMRF-amide application. To test directly whether FMRF-amide accelerates the heart's myogenic rhythm, we applied it to a segment of heart with the central activity of the innervating HE motor neuron experimentally suppressed.

Central activity suppressed. When the central activity of HE motor neurons in body wall preparations is experimentally suppressed, a peripheral neurogenic rhythm is usually observed (Maranto and Calabrese, 1984b). This rhythm, which consists of antidromic bursts of action potentials recorded in the HE soma and associated contractions of the heart muscle (Fig. 4), was seen in all but one of the preparations studied ($n = 27$). In the absence of externally applied FMRF-amide we occasionally observed a myogenic rhythm in such preparations, either alone or in conjunction with peripheral neurogenic bursts, as previously described by Maranto and Calabrese (1984b).

In hearts which were beating solely in response to the peripheral neurogenic rhythm, bath application of FMRF-amide induced a tonic contracture of the heart, elicited a strong myogenic rhythm, and suppressed the peripheral neurogenic rhythm (Fig. 4). All three of these effects occurred shortly after the beginning of FMRF-amide perfusion and were reversed by perfusion of normal Ringer's solution (usually within 2 to 5 min). Myogenic beats and tonic contractures could be repeatedly elicited with FMRF-amide concentrations in the perfusing solution as low as 8×10^{-8} M (Fig. 5) and were once observed with perfusion of 8×10^{-9} M FMRF-amide.

Both the strength of the tonic contracture and the frequency of contractions varied in a dose-dependent fashion. In the experiment illustrated (Fig. 5), a 10-fold increase in FMRF-amide concentration increased the strength of the tonic contracture by about 140% and the frequency of myogenic contractions by about 20%. With higher concentrations of FMRF-amide ($\geq 10^{-7}$ M), the individual myogenic beats were often obscured by the tonic contracture of the heart, suggesting that the heart muscle was in a state of maximal contraction and could generate no further tension. The effect of externally

Figure 2. FMRF-amide increases the peak and integral tension and decreases the latency of heart contractions entrained by the HE motor neuron's central activity rhythm. Records from the same preparation as Figure 1 are analyzed quantitatively. Peak tension (A), percentage of the maximum tension integral (B), and latency from the beginning of the heart motor neuron's burst to the start of the heart's contraction (C) were averaged over five successive cycles for a hundred cycles and the averages (with their standard errors) were plotted as bar graphs. Two successive periods of FMRF-amide (8×10^{-8} M) application are indicated by bars. The records of Figure 1 correspond to cycles 46 to 49, 66 to 69, and 89 to 92, respectively, in the graph.

applied FMRF-amide on the peripheral neurogenic rhythm was similarly dose dependent (Fig. 6). Lower concentrations of FMRF-amide caused a decrease in the duration of each peripheral HE terminal burst and an increase in their period, whereas higher concentrations suppressed the peripheral neurogenic rhythm entirely (Fig. 6).

In the single preparation in which a myogenic rhythm accounted for each contraction of the heart tube (no peripheral neurogenic

rhythm present), application of 8×10^{-8} M FMRF-amide increased the rate of the myogenic rhythm by about 70%.

Application of FMRF-amide to the heart with a wall of petroleum jelly isolating the nerve cord or with all innervating connections to the heart severed produced all of the effects described above, thereby indicating a peripheral effect.

Central effects of FMRF-amide

Since cell 204 shows FLI (Kuhlman et al., 1985) and since it can accelerate the cycle rate of the heartbeat central pattern generator (Arbas and Calabrese, 1984), we sought effects of FMRF-amide on the pattern generator. Bath application of FMRF-amide to the nerve cord greatly increased the pattern generator's cycle rate, determined from the rhythm of IPSPs recorded in an HE motor neuron (Fig. 7). FMRF-amide was a potent accelerator of the heartbeat central pattern generator, producing effects at concentrations as low as 8×10^{-10} M (lowest concentration tested) and often doubling the frequency of the rhythm. The onset of the effect was rapid, and the acceleration was reversed within minutes by normal Ringer's perfusion (Fig. 7).

As seen in Figure 7, the increase in the cycle rate of the inhibitory output of the HN cell network leads to a decrease in the duration of the HE cell's impulse bursts. When higher concentrations of FMRF-amide ($\geq 10^{-7}$ M) were applied to a nerve cord containing the central pattern generator, inhibitory input to the HE cells was often continuous and they could not maintain cyclic bursting (Fig. 8). Perfusion of normal Ringer's reversed this effect, although longer wash times were generally required.

Recordings were also made from HA modulatory neurons during application of FMRF-amide to an isolated nerve cord. The acceleration of the heartbeat central pattern generator was apparent in the HA cells, which also receive inhibitory input from the HN cell network (Calabrese and Maranto, 1984), but no other changes in the activity of the HA cells were detected.

FMRF-amide (10^{-8} to 10^{-6} M) had a general stimulatory effect on ganglionic motor activity monitored extracellularly from dorsal posterior nerve (Payton, 1981) with a suction electrode; prolonged bursts of activity in motor neurons alternated with relatively quiet periods, but no behaviorally identifiable motor pattern was observed. These effects were also reversed by perfusion with normal Ringer's.

Discussion

Peripheral activity of FMRF-amide. The most pronounced effects of FMRF-amide application on the hearts of the leech are in activation or acceleration of the myogenic rhythm of an undriven heart and an increase in the beat tension of a heart entrained to an HE motor neuron's central activity. These effects are very similar to those produced by activity in the HA modulatory neurons (Calabrese and Maranto, 1984). FLI is seen in both the HA neurons and their processes which innervate the hearts; it is, therefore, likely that the HA neurons produce their modulatory effects through release of an FMRF-amide-like peptide. The lack of any known FMRF-amide antagonist prevents testing this proposition fully. FMRF-amide similarly stimulates the myogenic rhythm of the heart in a variety of bivalve molluscs (Greenberg and Price, 1979; Painter and Greenberg, 1982).

Application of FMRF-amide to heart muscle in which rhythmic input from the CNS has been experimentally suppressed produces not only HA-like effects but also additional effects not previously reported in conjunction with HA cell activity. These include a tonic contracture of the heart tube and inhibition of the peripheral neurogenic bursts. The magnitude of each effect is dependent upon the concentration of FMRF-amide applied, but their significance remains unknown. FMRF-amide causes similar tonic contractures in a variety of molluscan muscles (Greenberg and Price, 1979; Painter, 1982; Cottrell et al., 1983).

The HE motor neurons and their processes on the heart also show intense FLI. The HE cells provide rhythmic excitation to the heart muscle via classical chemical synapses, and several obser-

Figure 3. FMRF-amide can accelerate the heart's myogenic rhythm so that it breaks from entrainment by the HE motor neuron's central activity rhythm. Records are from the same preparation as Figures 1 and 2. Application of FMRF-amide (solid bar, concentration indicated) induced myogenic contractions (indicated by arrowheads) not associated with the HE cell's bursts. The effect reversed quickly once FMRF-amide application was terminated.

Figure 4. Peripheral effects of FMRF-amide on the heart muscle. In this and the subsequent four figures the contractile activity in a segment of heart tube and the electrical activity in the innervating HE motor neuron were simultaneously monitored while the preparation was alternatively perfused with Ringer's and Ringer's containing FMRF-amide. The central activity of the HE cell was suppressed by injection of a steady hyperpolarizing current. Peripheral neurogenic bursts were recorded in the HE soma, and each of these bursts gave rise to a contraction of the heart tube. Upon bath application of 8×10^{-8} M FMRF-amide (solid bar), a tonic contracture of the heart ensued, the myogenic rhythm of the heart was activated, and the peripheral neurogenic rhythm was suppressed. All of these effects were reversed upon termination of FMRF-amide application.

variations indicate that acetylcholine (ACh) is the primary excitatory transmitter of the HE cell. First, low concentrations of iontophoretically or bath-applied ACh cause depolarizations and contractions of individual muscle cells (Maranto, 1983). Second, the ACh antagonist curare prevents bursts of impulses in the HE motor neuron from resetting the heart's myogenic rhythm (Maranto, 1983). Third, the enzymes choline acetyltransferase and acetylcholinesterase have been detected at significant levels in the HE cell body (Wallace, 1981a, b; Wallace and Gillon, 1982). Finally, acetylcholinesterase has been detected on the heart tubes (Maranto, 1983). Based on these findings and the present finding of FLI in the HE soma and peripheral processes, we propose that the HE motor neuron may release both ACh and an FMRF-amide-like peptide. The modulation already produced by peptide release from the HE motor neurons would be enhanced by peptide release from the HA modulatory neurons.

Central activity of FMRF-amide. Nanomolar concentrations of FMRF-amide cause a reversible acceleration of the heartbeat central pattern generator. Similarly, FMRF-amide accelerates the pyloric central pattern generator of the lobster (Hooper and Marder, 1984). The acceleration of the heartbeat pattern generator may be due to either direct effects of FMRF-amide on the HN oscillatory neurons or indirect effects on neurons which synapse onto the HN cells. No effect of the HA cell's or the HE cell's activity on the rhythm of the heartbeat central pattern generator has been found (Calabrese and Maranto, 1984).

The localization of FLI to the swim-initiating interneuron cell 204 (Kuhlman et al., 1985) suggests that FMRF-amide may act centrally on both the swimming and the heartbeat pattern generators. Activity in cell 204 leads to both an activation of the swimming motor program and an acceleration of the heartbeat central pattern generator (Arbas and Calabrese, 1984). Bath application of FMRF-amide at concentrations that dramatically accelerate the heartbeat pattern generator, however, did not activate the swimming motor pattern in an isolated nerve cord. Although FMRF-amide may not be the transmitter involved in initiating swimming, co-release of FMRF-amide and another transmitter by cell 204 could result in simultaneous swim initiation and heartbeat acceleration.

Conclusion. The localization and identification of FMRF-amide-like immunoreactive neurons in the leech CNS (Kuhlman et al. 1985) have laid the foundation for *in vivo* studies of peptidergic transmission. Initial investigations of FMRF-amide bioactivity in the leech revealed that FMRF-amide is cardioexcitatory, increasing output of the hearts by both central modulation of the neural pattern generator and peripheral modulation of the cardiac muscle. As with the molluscan heart (Painter and Greenberg, 1982), FMRF-amide produced both chronotropic and inotropic effects on the myogenic rhythm.

The peripheral effects of FMRF-amide on the leech heart are very similar to those effects produced by an identified FMRF-amide-like-immunoreactive neuron, the previously characterized HA cell (Calabrese and Maranto, 1984). Peptidergic transmission can thus be studied in a system in which the presynaptic central neuron and

Figure 5. The effects of FMRF-amide on the heart muscle are repeatable and dose dependent. The central activity of the innervating HE cell was suppressed by injection of a steady hyperpolarizing current. Peripheral neurogenic bursts were recorded in the HE soma (records not shown; see Fig. 4), and each of these bursts gave rise to a contraction of the heart tube. The application of FMRF-amide (solid bars, concentrations indicated) caused a tonic contracture of the heart, the amplitude of which was dose dependent, and induced a myogenic rhythm the frequency of which was dose dependent (cf. traces 1 and 2 with trace 3). Repeated application of the same concentration of FMRF-amide gave comparable responses (cf. traces 1 and 2). During FMRF-amide application the peripheral neurogenic rhythm was suppressed; the period of suppression is indicated by the arrowheads. All records are from the same preparation, and traces 1 and 2 form a continuous record.

Figure 6. The effects of FMRF-amide on the peripheral neurogenic rhythm are dose dependent. The central activity of an innervating HE cell was suppressed by injected current and the CNS was separated from the heart and body wall by a petroleum jelly dam so that FMRF amide application was restricted to the periphery. A regular peripheral neurogenic rhythm was recorded in the HE soma. The application of FMRF-amide (solid bars, concentrations indicated) affected the peripheral neurogenic rhythm in a dose-dependent fashion. The lower concentration extended the burst period and shortened the burst duration, whereas the higher concentration suppressed peripheral bursts entirely. The traces form a continuous record. Arrowheads indicate the peripheral neurogenic bursts.

Figure 7. FMRF-amide increases the cycle rate of the heartbeat central pattern generator. These data were obtained by recording intracellularly from an HE motor neuron in an isolated nerve cord (ganglia 2 to 9), which was alternately perfused with Ringer's and Ringer's containing FMRF-amide (solid bars, concentrations indicated). Burst periods were calculated as the interval between successive barrages of IPSPs in the HE cell. The two FMRF-amide applications were separated by more than 100 cycles. The graph shows that low concentrations of FMRF-amide have a rapid acceleratory effect on the heartbeat central pattern generator, and this effect is quickly reversed by washing. The record of the HE cell's activity corresponds to the second FMRF-amide application as indicated. Shorter bursts in the HE cell were observed during acceleration of the cycle rate.

HE(R,7)

NORMAL RINGER

$$[FMRF_{AMIDE}] = 8 \times 10^{-8} \text{ M}$$

NORMAL RINGER, 6 MINUTE WASH

10 mV

20 sec

Figure 8. Higher concentrations of FMRF-amide disrupt the cyclic activity of the heartbeat central pattern generator. An HE motor neuron's activity was monitored in an isolated nerve cord containing ganglia 2 to 9, while the preparation was alternately perfused with Ringer's and Ringer's containing 8×10^{-8} M FMRF-amide. This concentration of FMRF-amide disrupted the cyclic activity of the heartbeat central pattern generator, producing nearly continuous IPSPs in the HE cell. After prolonged washing, the cyclic activity of the central pattern generator returned but at an increased rate.

postsynaptic target are readily identified and well characterized. Leech behaviors other than heartbeat may also require transmission of an FMRF-amide-like substance, as motor neurons innervating the body wall musculature and genitalia also show FLI (Kuhlman et al., 1985).

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