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FMRFamide-like Substances in the Leech. III. Biochemical Characterization and Physiological Effects

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FMRFamide-like immunoreactivity has been previously localized to identified neurons in the CNS of the leech, *Hirudo medicinalis* (Kuhlman et al., 1985a). These leech antigens have been characterized biochemically by reverse-phase high-pressure liquid chromatography (HPLC) followed by radioimmunoassay (RIA). The majority of the FMRFamide-like immunoreactivity recovered by HPLC from extracts of leech nerve cords coelutes with authentic FMRFamide. We have tentatively identified this major leech peptide as authentic FMRFamide.

Two neurons that control heartbeat in the leech, the HE motor and HA modulatory neurons, and their neural processes on the heart are FMRFamide-like immunoreactive (Kuhlman et al., 1985a). Single individually dissected HE and HA cells were analyzed by HPLC and RIA. Only 1 FMRFamide-like peptide was found in extracts of HA cells; this peptide was chromatographically indistinguishable from authentic FMRFamide. The FMRFamide-like peptide in HE cells could not be isolated by experimental procedures used. Most of the FMRFamide-like immunoreactivity contained within the neural processes on the heart also coeluted with authentic FMRFamide.

HE motor neurons, which are believed to be cholinergic (Wallace, 1981a, b; Maranto and Calabrese, 1984a, b), were examined for their FMRFamide-like effects on the heart. The presence of curare in the bathing medium did not block the ability of FMRFamide to induce myogenic activity in heart muscle, suggesting that FMRFamide and ACh act at different receptor sites on the heart. Prolonged firing in HE cells in the presence of curare also induced myogenic activity in heart muscle. This FMRFamide-like action of the HE motor neurons may be normally masked by their cholinergic actions.

The tetrapeptide FMRFamide (Phe-Met-Arg-Phe-amide) was first isolated from ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg, 1977a, b). The neuropeptide was initially described as a molluscan cardioexcitatory factor that mimicked the effects of 5-HT (Price and Greenberg, 1977a). FMRFamide and FMRFamide-like peptides have since been characterized in both invertebrates and vertebrates, where they have multiple physiological effects (for review, see O'Shea and Schaffer, 1985).

Thus far, authentic FMRFamide has been isolated only from molluscs (Price and Greenberg, 1977a, b; Lehman et al., 1984). Peptides that have C-terminal homology with FMRFamide, however, have been discovered in the chicken (Dockray et al., 1983), snail (Price, 1982; Price et al., 1985), and lobster (Kobierski et al., 1985; Trimmer et al., 1985). These peptides may be the FMRFamide counterparts in these animals. FMRFamide elicits responses similar to those of the endogenous peptides in the chicken and snail. Other peptides with similar bioactivity and/or immunoreactivity but with chemical properties different than FMRFamide have been partially purified from hydra (Grimmelikhuijzen et al., 1982), horseshoe crab (Watson et al., 1984), frog (Dockray et al., 1981), and rat (Dockray et al., 1981; Dockray and Williams, 1983; O'Donohue et al., 1984).

The nervous system of the leech *Hirudo medicinalis,* because of its inherent simplicity and accessibility, is especially amenable for studies of peptides. Neurons in the CNS can be reproducibly identified from animal to animal. Many of the neurons have been characterized morphologically and electrically and have known physiological roles (see Muller et al., 1981). In particular, the neural circuitry controlling heartbeat in the leech has been elucidated at the cellular level.

FMRFamide-like immunoreactivity has been localized to approximately 50 neurons in a typical segmental ganglion of the leech (Kuhlman et al., 1985a). Two cell types that control heartbeat, the HE motor and HA modulatory neurons, are FMRFamide-like immunoreactive (Kuhlman et al., 1985a). Neural processes on the heart, but not the heart muscle cells themselves, are also immunoreactive; at least some of these immunoreactive processes emanate from the HE and HA neurons (Kuhlman et al., 1985a). Furthermore, application of FMRFamide on hearts and central ganglia reveals that FMRFamide has both central and peripheral effects in the leech (Kuhlman et al., 1985b). The peripheral effects of FMRFamide on heart tissue (induction of myogenicity and modulation of muscle tension) are very similar to those produced by activity in the HA modulatory neurons (Calabrese and Maranto, 1984), suggesting, as does the immunocytochemical data, that FMRFamide or an FMRFamide-like peptide is active at the heart neuromuscular synapse.

In the present study, the FMRFamide-like immunoreactivity in the leech is characterized biochemically, and physiological evidence is presented showing that the HE motor neurons have FMRFamide-like effects on the hearts similar to those of the

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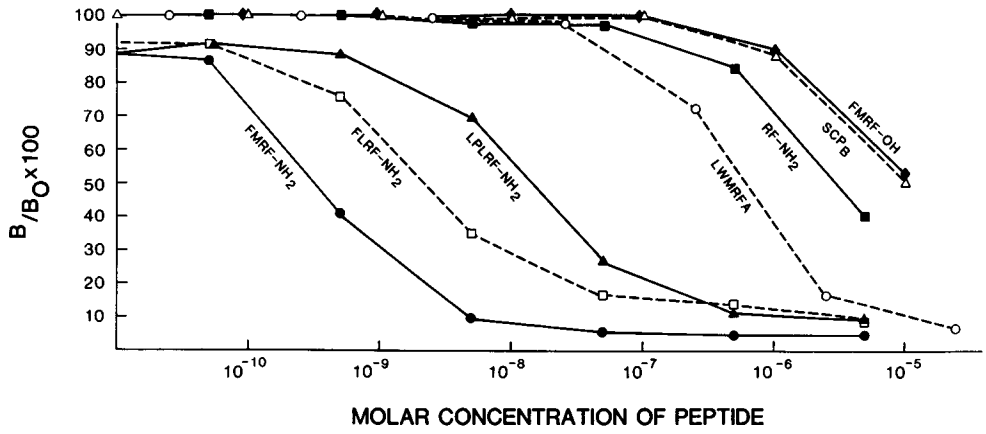

Figure 1. Specificity of anti-FMRFamide antiserum. Cross-reactivity of peptides that have structural similarities to FMRFamide; other homologous peptides were tested by O'Donohue and coworkers (1984). Peptide sequences are indicated by their one-letter symbols. Of the peptides tested, only FLRFamide and LPLRFamide showed any significant cross-reactivity with the antibody. For some peptides, $B = B_0$ at molar concentrations less than 10^{-12} M. B, percentage of tracer binding; B_0, maximum percentage of tracer binding.

HA modulatory neurons. Radioimmunoassays (RIAs) were used to assess the amounts of FMRFamide-like peptide(s) in the nerve cord of the leech, and reverse-phase high-pressure liquid chromatography (HPLC) was employed to examine the similarity between the leech peptide(s) and authentic FMRFamide. In addition, the FMRFamide-like immunoreactivity within the HE and HA cells, which control the heart, was similarly analyzed. These results indicate that authentic FMRFamide or a peptide chemically very similar to FMRFamide is present in the leech and functions in heartbeat control.

Materials and Methods

Animals. Adult specimens of the leech *H. medicinalis* were purchased from European suppliers (Ricarimpex and Blutegelimport und Versand). The leeches were kept at 15°C for up to several months in artificial pond water.

Tissue extractions. Nerve cords, which included the head ganglia through ganglion 20, and lateral heart tubes were dissected from animals in cold physiological saline (Muller et al., 1981). Tissues were collected and homogenized in either an acidified methanol mixture (90% methanol, 9% acetic acid, 1% water) or 10% acetic acid on ice. The homogenates and, subsequently, the pellets, which were resuspended in the acidified methanol or acetic acid mixture, were spun at 15,000 × *g* three times for 1 min and once for 15 min. The supernatant was pooled after each spin and stored at −10°C until use.

Nerve cords and hearts were alternatively extracted with 100% acetone (Price, 1982). Tissues were dissected in cold physiological saline and placed in 100% acetone at −10°C for a minimum of 2 d. Tissues were removed and discarded, and the acetone extract was stored at −10°C until use.

For RIAs, tissue extracts were dried in a Speed Vac Concentrator (Savant) and reconstituted in RIA buffer (see below). For HPLC analysis, extracts were dried in the Speed Vac, reconstituted in water, and filtered (Millipore Millex GV). Extracts were then applied to a C_{18} Sep-pak cartridge (Waters) that had been preequilibrated with 5 ml each of methanol and water. The sample was eluted with 100% methanol and dried in the Speed Vac.

Cell isolation. HA modulatory, HE motor, and Retzius neurons were identified physiologically in isolated desheathed ganglia and injected with 0.2% Fast green FCF (Harleco) in 0.2 M KCl by repeated pulses of pressure (5–20 psi). Ganglia were then bathed in 70% ethylene glycol (Fisher), 30% physiological saline on dry ice for 0.5–2 hr. The dye-filled cells were removed with fine forceps and collected in a glass tube that had been siliconized with 4% dimethyldichlorosilane (Sigma) in chloroform. Cells were sonicated and extracted with 100% methanol. Cell extracts were dried in a Speed Vac and reconstituted in RIA buffer (see below) for RIAs or in 50 mM ammonium acetate (Baker), pH 4.5, for HPLC analysis.

Peptide iodination. Tyr-Met-Arg-Phe-amide (YMRFamide) (Peninsula), which is recognized with a similar affinity as FMRFamide by the anti-FMRFamide antiserum used in these experiments (O'Donohue et al., 1984), was iodinated by the modified chloramine T method (Greenwood et al., 1963) of Price (1982). The iodinated peptide (specific activity, 50–150 Ci/mmol peptide) was diluted with RIA buffer (see below) and stored at 4°C.

Radioimmunoassay. A polyclonal antibody raised in rabbits against synthetic FMRFamide conjugated to succinylated bovine thyroglobulin (O'Donohue et al., 1984) was used in all RIAs. Standard amounts of FMRFamide (Peninsula or Cambridge Research Biochemicals), Arg-Phe-amide (Peninsula), SCP_B (Peninsula), Leu-Trp-Met-Arg-Phe-Ala (Research Plus), Phe-Leu-Arg-Phe-amide (Cambridge), Leu-Pro-Leu-Arg-Phe-amide (Cambridge), or tissue samples were diluted or resuspended in a total volume of 100 μl of RIA buffer [0.1% BSA, RIA grade (Sigma) in 0.1 M phosphate buffer, pH 7.5]. Fifty microliters of iodinated YMRFamide (containing approximately 5–10,000 cpm) and 50 μl of diluted antiserum (final dilution, 1:25,000–1:100,000), both in RIA buffer, were added to assay tubes. All tubes were vortexed and incubated at 4°C for 12–24 hr. Unbound peptide was precipitated by addition of dextran-coated charcoal (Sigma, MCB). FMRFamide-like equivalents in samples were determined by comparing displacement of tracer from antiserum with standard amounts of FMRFamide. The minimum amount of FMRFamide detectable in the assay was between 10 and 40 fmol. FMRFamide concentrations between 30 and 120 fmol reduced antibody binding of tracer by 50%.

High-pressure liquid chromatography. The HPLC system was composed of 2 Rabbit HP solvent delivery systems (Rainin) controlled by a Gilson 811 dynamic mixer, a Rheodyne 7125 injection valve, and a Pharmacia Frac-100 fraction collector. The column used for reverse-phase HPLC was a 4 mm × 30 cm μBondapak C_{18} column (Waters).

Two chromatographic separations were used. In the first system, the column was equilibrated with 5% acetonitrile (Burdick and Johnson) in 50 mM ammonium acetate (Baker), pH 4.5, and 0–500 μM dithiothreitol (Sigma). A sample was injected and eluted with a 5 min isocratic desalting step of 5% acetonitrile, followed by a 30 min linear gradient of 5–50% acetonitrile at a flow rate of 1 ml/min. In the second separation system, the column was equilibrated with 35% methanol (Omnisolve) in 0.1% trifluoroacetic acid (Aldrich). The sample was injected and eluted with a 35 min linear gradient of 35–100% methanol at a flow rate of 1 ml/min. Fractions of either 0.5 (acetronitrile gradients) or 1 ml (methanol gradients) were collected, dried in a Speed Vac, and reconstituted in RIA buffer. FMRFamide and FMRFamide-like equivalents in the fractions were measured by the RIA as described above.

FMRFamide and oxidized forms of FMRFamide were similarly injected and eluted to mark the fractions in which the synthetic and oxidized peptides eluted. To produce the sulfoxide form of FMRFamide, 5 μl of 3% hydrogen peroxide (Mallinckrodt) was added to a standard amount (1.25 pmol) of FMRFamide in 50 mM ammonium acetate or water (G. Guidotti, personal communication). This mixture was reacted for at least 1 hr before injection onto the HPLC system. To produce the sulfone form of FMRFamide, performic acid was made according to the method of Hirs (1967). A standard amount (1.25 pmol) of FMRFamide in 100 μl of water was reacted with an equivalent volume of performic acid reagent for 2 hr on ice. Two hundred microliters of water was added to the reaction tube, and the entire contents were dried in a Speed Vac. The sulfone form was reconstituted in 50 mM ammonium acetate or water and injected onto the HPLC system.

Physiological recordings and application of pharmacological agents.

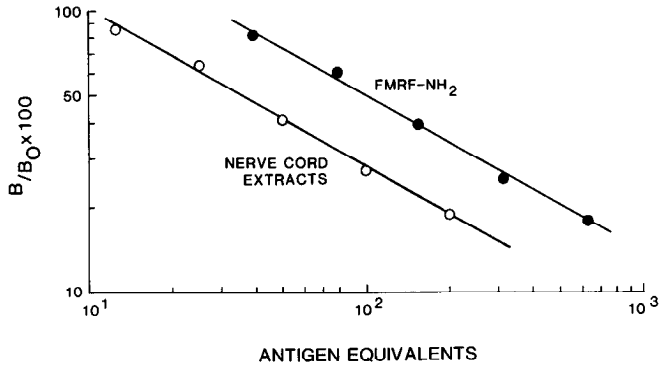

Figure 2. Inhibition of tracer binding by nerve cord extracts. Inhibition of tracer binding to anti-FMRFamide antibody by serial dilutions of nerve cord extracts parallels the inhibition by serial dilutions of synthetic FMRFamide, indicating a similar recognition of leech extracts and synthetic FMRFamide by the antibody. Lines were fitted by least-squares regression analysis. Slope of FMRFamide line is −0.653; slope of nerve cord line, −0.652. Antigen equivalents refer to leech extracts and synthetic FMRFamide. B, percentage of tracer binding; B_0, maximum percentage of tracer binding.

Heart preparations and recording methods employed have been described (Calabrese and Maranto, 1984; Maranto and Calabrese, 1984a, b). Pharmacological methods used have also been described (Kuhlman et al., 1985b; Calabrese and Maranto, 1986).

Results

Characterization of FMRFamide-like material in nerve cords

Radioimmunoassay

As a first step towards the biochemical characterization of the FMRFamide-like immunoreactivity, extracts of leech nerve cords were measured by RIA for their FMRFamide-like content. The anti-FMRFamide antiserum used had been previously characterized as being specific for the C-terminus of FMRFamide and shown not to cross-react appreciably with -Arg-Tyr-amide, -Asp-Arg-Phe-amide, -Met-Asp-Phe-amide, or -His-Phe-amide (O'Donohue et al., 1984). In particular, a C-terminal -Phe-amide is necessary for any significant binding to the antibody.

Other peptides homologous to FMRFamide were examined for their degree of cross-reactivity. The degree of cross-reactivity with FMRFamide was calculated by comparing the concentration of peptide needed to inhibit tracer binding by 50%. Among the peptides tested, only Phe-Leu-Arg-Phe-amide (FLRFamide) and Leu-Pro-Leu-Arg-Phe-amide (LPLRFamide), which is the putative chicken analog to FMRFamide (Dockray et al., 1983), showed any significant cross-reactivity (Fig. 1). FLRFamide showed approximately 14.5% cross-reactivity and LPLRFamide approximately 2.3%.

Table 1. FMRFamide-like immunoreactivity

Tissue or cell extract	n	FMRFamide-like equivalents (mean ± SD)/tissue or cell extract
Nerve cord		
Head ganglion thru ganglion 20	6	12.80 ± 3.58 pmol
Ganglia 1–20		
Homogenized	5	8.16 ± 0.66 pmol
Acetone extracted	5	2.51 ± 1.86 pmol
Head ganglion	3	3.98 ± 0.87 pmol
Heart	12	1.28 ± 0.96 pmol
HA cell	5	32.47 ± 14.03 fmol

FMRFamide-like immunoreactivity, as measured by RIA, in extracts of nerve cords, hearts, and isolated cells. Nerve cords were homogenized and extracted in acidified methanol, unless otherwise noted. Hearts and isolated cells were extracted in acetone or methanol, respectively.

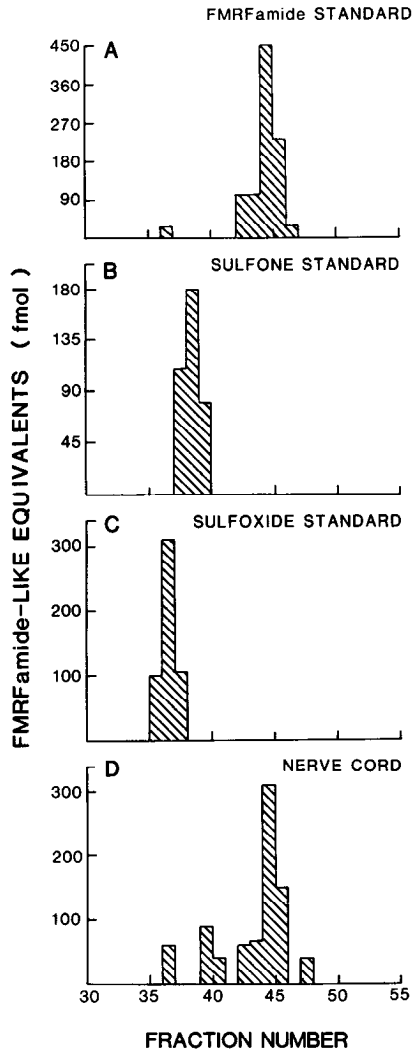

Figure 3. Analysis of nerve cord extracts by HPLC: acetonitrile separation. Peptides were eluted with a linear gradient of 5–50% acetonitrile. Approximately 1.25 pmol FMRFamide (*A*), sulfone form of FMRFamide (*B*), sulfoxide form of FMRFamide (*C*), and FMRFamide-like equivalents from nerve cord extracts were injected onto the HPLC system. The majority of FMRFamide-like immunoreactivity recovered from nerve cord extracts coelutes with FMRFamide or one of its oxidized forms. Smaller peaks of immunoreactivity sometimes elute in fractions more (as seen here) or less hydrophobic than FMRFamide. Minimal detectable amount on the RIA was 20 fmol FMRFamide.

Extracts of homogenized nerve cords, which included head ganglia through ganglion 20, contained 12.80 ± 3.58 pmol (mean ± SD, n = 6) of FMRFamide-like equivalents per nerve cord (Table 1). Head ganglia contained 3.98 ± 0.87 pmol (n = 3) of FMRFamide-like equivalents per supra- and subesophageal complex, or about 31% of the total amount in a nerve cord. Homogenization and extraction with either acidified methanol or acetic acid yielded approximately 30–80% more FMRFamide-like material than the acetone extraction procedure (Table

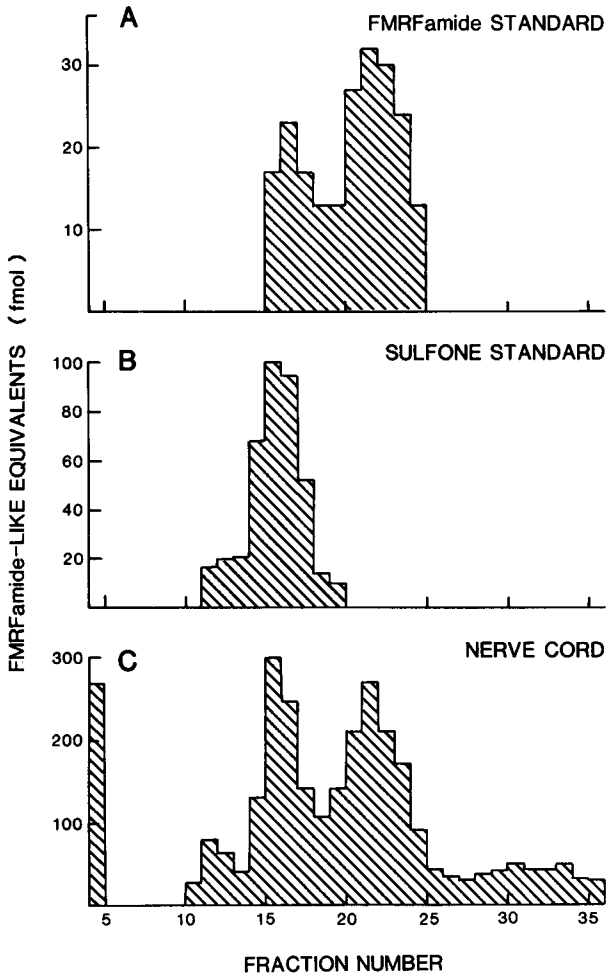

Figure 4. Analysis of nerve cord extracts by HPLC: methanol separation. Peptides were eluted with a linear gradient of 30–100% methanol. Approximately 1.25 pmol of standards and 4.2 pmol of FMRFamide-like equivalents from nerve cord extracts were injected onto the HPLC system. The majority of immunoreactivity recovered from nerve cord extracts coelutes with either FMRFamide or the sulfone form of FMRFamide. Other peaks of immunoreactivity elute in fractions more and less hydrophobic than FMRFamide. (The peak of immunoreactivity in fraction 4 corresponds to immunoreactivity that does not bind to the column.) The sulfoxide form of FMRFamide does not elute from the column with this gradient. Minimal detectable amount on the RIA was 10 fmol FMRFamide.

1). Inhibition of tracer binding to the antibody by serial dilutions of whole nerve cord or head extracts paralleled the inhibition by serial dilutions of synthetic FMRFamide (Fig. 2, only nerve cord data shown), indicating a similar recognition by the antibody to tissue extracts and synthetic FMRFamide.

High-pressure liquid chromatography

To extend the biochemical characterization of the FMRFamide-like immunoreactivity, extracts of nerve cords were fractionated into component peptides by reverse-phase HPLC and then assayed by RIA. Two solvent systems (see Materials and Methods), which had different patterns of peptide elution, were used. The methionine in FMRFamide can oxidize to either a sulfoxide or sulfone form (Roberts and Caserio, 1964). These 2 oxidation forms of FMRFamide eluted separately from each other and from FMRFamide on the HPLC gradients. Both oxidized forms

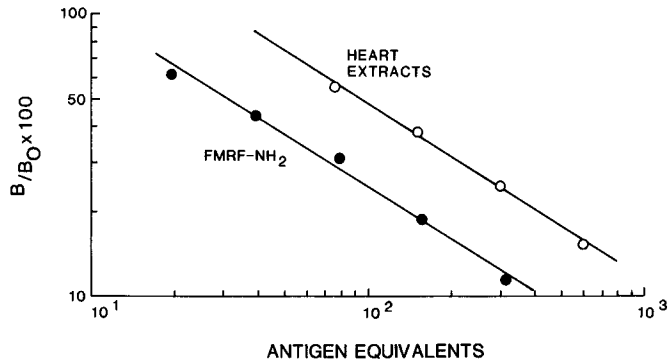

Figure 5. Inhibition of tracer binding by heart extracts. Inhibition of tracer binding to anti-FMRFamide antibody by serial dilutions of heart extracts parallels the inhibition by serial dilutions of synthetic FMRFamide, indicating a similar recognition of heart extracts and FMRFamide by the antibody. Lines were fitted by least-squares regression analysis. Slope of FMRFamide line is −0.611; slope of heart line, −0.620. *B*, percentage of tracer binding; B_0, maximum percentage of tracer binding.

of FMRFamide, however, were immunoreactive. Standard amounts (1.25 pmol) of FMRFamide and its sulfoxide and sulfone forms were included in addition to tissue samples on all HPLC runs. The elution pattern of the FMRFamide-like immunoreactivity in tissue extracts was compared with the elution patterns of synthetic FMRFamide and its oxidized forms. In the acetonitrile solvent system, the sulfoxide form generally peaked in fraction 36 (Fig. 3*C*), the sulfone form in fraction 38 (Fig. 3*B*), and FMRFamide in fraction 44 (Fig. 3*A*). In the methanol solvent system the sulfone form generally peaked in fraction 16 and FMRFamide in fraction 22; the sulfoxide form was apparently not eluted from the column with this solvent system (Fig. 4). Between any injection of standards (FMRFamide or oxidized forms of FMRFamide) and samples, control injections containing only running buffer were made. The eluted fractions were tested by RIA for carry-over of FMRFamide from previous injections. No amount of immunoreactivity above background levels was ever recovered from these control fractions. FMRFamide standards were routinely run at both the beginning and end of the day's series of runs. In many instances, the peak of the FMRFamide standard injected at the beginning of the day was 1 fraction earlier/later than the peak of the standard injected at the end of the day. One fraction differences between peaks of standards and samples, therefore, were considered within the bounds of normal variability.

Three distinct peaks of FMRFamide-like material were found consistently in acetone and acidified methanol extracts of nerve cords with the acetonitrile separation. These 3 peaks accounted for 80–100% of the immunoreactivity recovered. Each of these tissue peaks comigrated with either FMRFamide or 1 of the oxidized forms of FMRFamide (Fig. 3*D*). The size of the 3 peaks varied from sample to sample, presumably depending on the varying amounts of oxidation that occurred to the sample during purification procedures. Addition of a reducing agent (dithiothreitol) to the running buffer during HPLC decreased the amounts of oxidized FMRFamide recovered. Other peaks of immunoreactivity that did not coelute with FMRFamide or 1 of its oxidized forms were found in 70–80% of the runs (Fig. 3*D*).

With the methanol separation three distinct peaks of

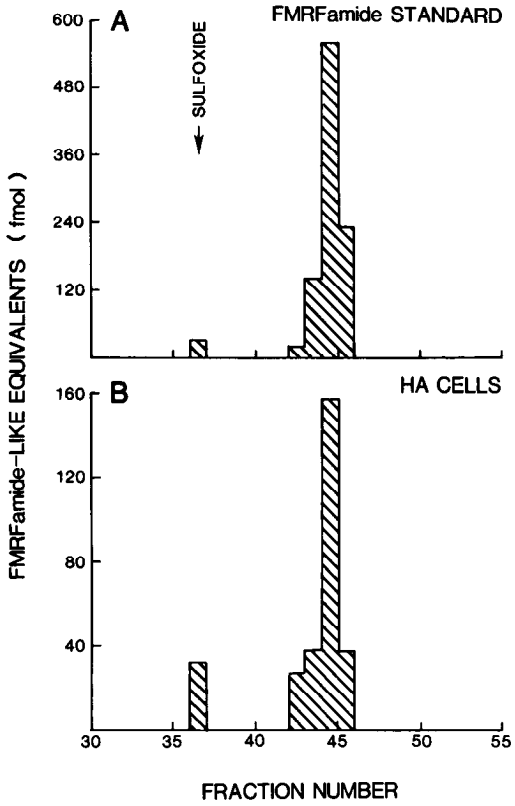

Figure 6. Analysis of HA cell extracts by HPLC: acetonitrile gradient. Peptides were eluted with a linear gradient of 5–50% acetonitrile. Extracts from 24 HA modulatory neurons and 1.25 pmol of standards were injected onto the HPLC system. The FMRFamide-like immunoreactivity in HA cells coelutes with FMRFamide and the sulfoxide form of FMRFamide (elution fraction indicated by *arrow*). No immunoreactivity was detected in any other fraction. Minimal detectable amount on the RIA was 20 fmol of FMRFamide.

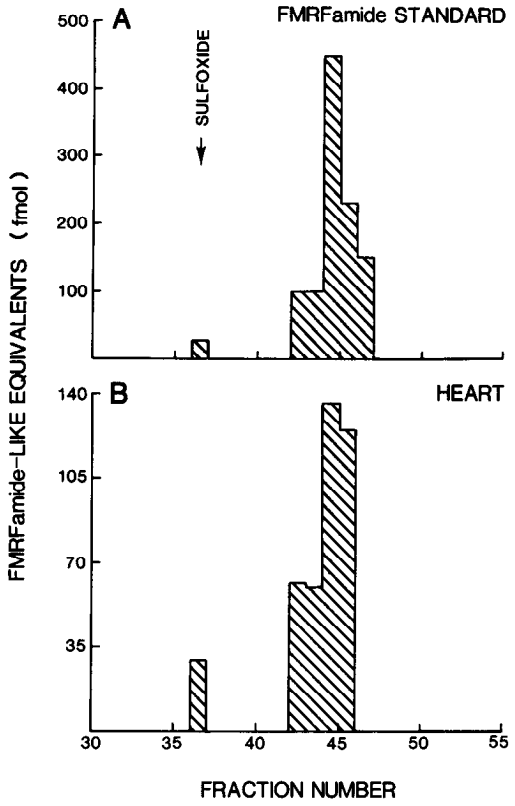

Figure 7. Analysis of heart extracts by HPLC: acetonitrile gradient. Peptides were eluted with a linear gradient of 5–50% acetonitrile. Approximately 1.4 pmol of heart extract and 1.25 pmol of standards were injected onto the HPLC system. The FMRFamide-like immunoreactivity in heart extracts coelutes with either FMRFamide or the sulfoxide form of FMRFamide (elution fraction indicated by *arrow*). Rarely, some small amount of immunoreactivity was detected in other fractions. Minimal detectable amount on the RIA was 20 fmol FMRFamide.

FMRFamide-like immunoreactivity were observed in acetone extracts of nerve cords. The majority (about 70%) of immunoreactivity was localized to 2 peaks, which comigrated with either FMRFamide or the sulfone form of FMRFamide (Fig. 4). The third peak may correspond to 1 of the other FMRFamide-like immunoreactive peaks seen in the acetonitrile solvent system. The recovery of immunoreactivity from samples or standards in both solvent systems ranged between 15 and 100%; recovery of immunoreactivity from the acetonitrile solvent system was consistently higher than that from the methanol solvent system.

Characterization of FMRFamide-like material in single cells and heart tubes

Since HA modulatory neurons, HE motor neurons, and their neural processes on their peripheral target, the hearts, are FMRFamide-like immunoreactive (Kuhlman et al., 1985a), the FMRFamide-like material within the heartbeat system was characterized more thoroughly. Pooled extracts of 7–56 isolated cell bodies of HA, HE, and Retzius (which are not FMRFamide-like immunoreactive and were used as control cells) neurons were measured by RIA for their FMRFamide-like content. HA cells contained 32.47 ± 14.03 fmol (mean ± SD, n = 5) of FMRFamide-like equivalents per cell (Table 1). Neither extracts of HE cells (n = 5) nor of Retzius cells (n = 7) consistently showed any measurable amount of FMRFamide-like immunoreactivity with the RIA.

Acetone extracts of isolated lateral hearts contained 1.28 ± 0.96 pmol (n = 12) of FMRFamide-like equivalents per heart (Table 1). The extraction procedure with acidified methanol yielded approximately 10% more FMRFamide-like material than the acetone extraction in 1 experimental comparison. Inhibition of tracer binding to the antibody by serial dilutions of hearts paralleled the inhibition by serial dilutions of synthetic FMRFamide (Fig. 5), indicating, as with the nerve cord extracts, a similar recognition by the antibody to heart extracts and synthetic FMRFamide.

To determine its structural similarity to authentic FMRFamide, the FMRFamide-like immunoreactivity contained in extracts of HA cells and hearts was also analyzed by reverse-phase HPLC (Figs. 6 and 7). With both solvent systems, extracts of HA cells contained only peaks of FMRFamide-like immunoreactivity that comigrated with either synthetic FMRFamide or 1 of the oxidized forms of FMRFamide (Fig. 6, acetonitrile separation shown). The majority of FMRFamide-like immunoreactivity in heart extracts also comigrated with either synthetic FMRFamide or one of the oxidized forms of FMRFamide. With an acetonitrile separation, 2 peaks of FMRFamide-like immunoreactivity were detectable; 1 peak comigrated with FMRFamide and 1 with the sulfoxide form of FMRFamide (Fig. 7). Occasionally, acetone extracts of heart tissue contained

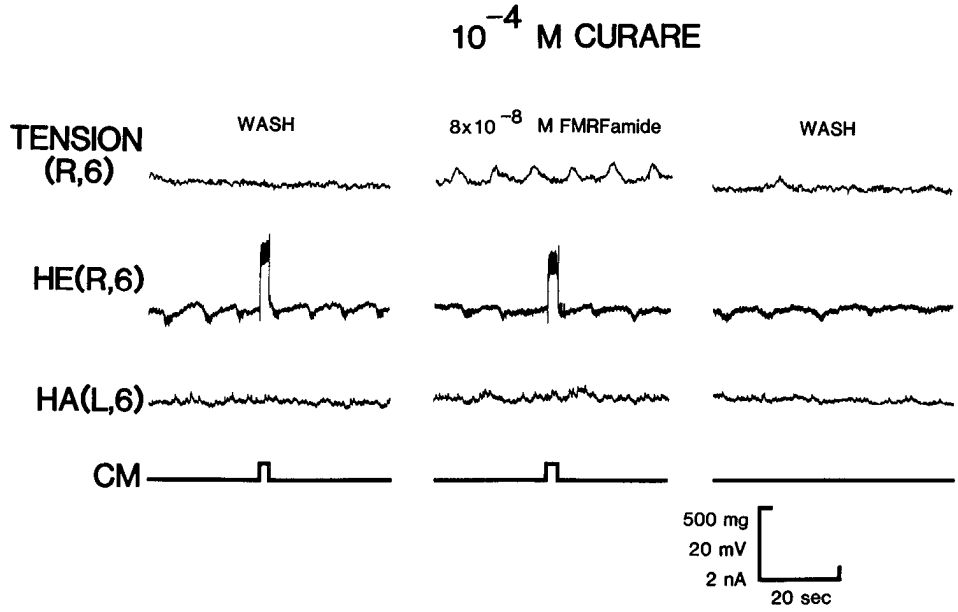

Figure 8. The ability of FMRFamide to induce myogenic activity in the heart is not blocked by curare. Activity in HE and HA neurons (neurons are indexed according to segment and body side) were monitored by intracellular recordings; the section of heart innervated by these neurons was monitored with a force transducer. The neurons were rendered inactive with a steady hyperpolarizing current injected through the recording electrode. The preparation was constantly superfused with either leech Ringer's containing 10^{-4} M curare (*d*-tubocurarine) or the same superfusate supplemented with 8×10^{-8} M FMRFamide. Before addition of FMRFamide (*right panel*), a burst of activity in the HE motor neuron, which was caused by briefly releasing the cell from hyperpolarization, induced no contraction in the heart, and no myogenic activity was discernible. Superfused FMRFamide induced a strong myogenic rhythm in the heart that was not reset by a burst of activity in the HE motor neuron, which was again caused by briefly releasing the cell from hyperpolarization (*middle panel*). Return to the normal superfusate reversed the effects of FMRFamide (*right panel*). *Middle panel* was recorded 200 sec after the beginning of FMRFamide application, and *right panel* was recorded 150 sec after the end of FMRFamide application. *CM*, current monitor for the HE motor neuron.

additional immunoreactive peaks that eluted in similar positions as nerve cord non-FMRFamide peaks (data not shown). With the methanol separation, 3 peaks of immunoreactivity were recovered from heart extracts (data not shown). Two peaks, which accounted for greater than 70% of the immunoreactivity recovered, coeluted with either FMRFamide or the sulfone form of FMRFamide. The third peak eluted in a fraction more hydrophobic than FMRFamide.

FMRFamide-like effects of HE motor neurons on the heart

Although no measurable amounts of FMRFamide-like immunoreactivity were detected by RIA in HE cell extracts, despite pooling of as many as 56 cell extracts, previous immunocytochemical data (Kuhlman et al., 1985a) suggest the presence of a FMRFamide-like peptide in the HE motor neurons. HE neurons, therefore, were examined for their possible FMRFamide-

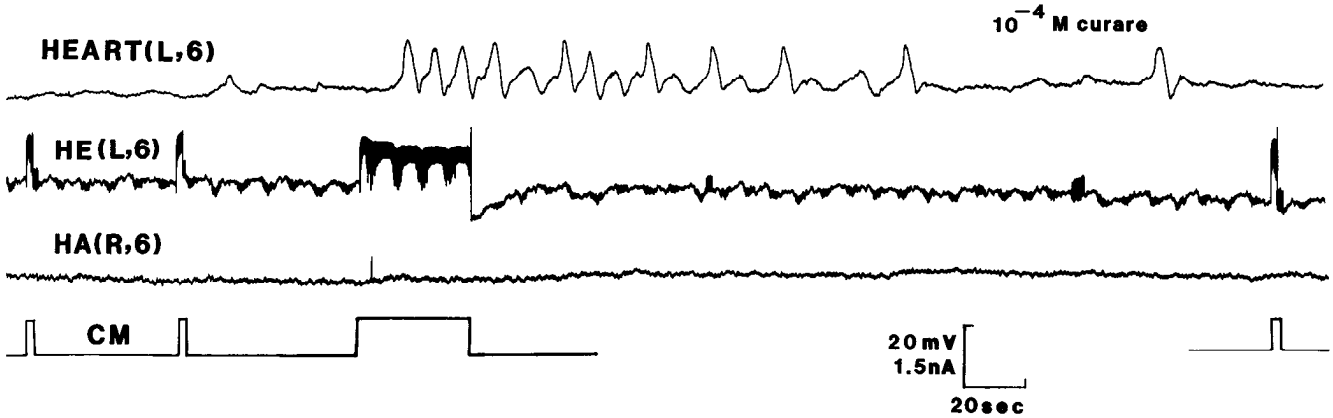

Figure 9. Prolonged activity in an HE motor neuron in the presence of curare (10^{-4} M *d*-tubocurarine) causes FMRFamide-like effects on heart muscle. Preparation, recordings, and experimental conditions were similar to Figure 8, except that heart activity was monitored by an intracellular recording of a heart muscle cell, and no FMRFamide was added to the superfusate. Short bursts of activity in the heart motor neuron caused no discernible phasic effect on the electrical activity of the heart, but prolonged activity induced a strong myogenic rhythm that far outlasted the activity in the HE cell. Activity in the HE cell was caused by releasing it from hyperpolarization. Stray bursts of impulses in the HE cell not associated with release from hyperpolarization were initiated spontaneously in the periphery (Maranto and Calabrese, 1985b). *CM*, current monitor for the HE motor neuron.

like effects on the heart. Application of FMRFamide to hearts produces 2 effects: an induction or acceleration of myogenic activity and an increase in beat tension (Kuhlman et al., 1985b). Activity in HA modulatory neurons have similar effects on the heart (Kuhlman et al., 1985b). Demonstration of the FMRFamide-like effects produced by HE motor neurons was complicated by their cholinergic properties (Wallace, 1981a, b) and strong cholinergic actions on the heart (Calabrese and Maranto, 1986). A brief burst of impulses in an HE motor neuron causes a rapid depolarization of the heart muscle that resets any ongoing myogenic activity (Maranto and Calabrese, 1984b); these effects are readily blocked by 10^{-4} M curare (*d*-tubocurarine) (Calabrese and Maranto, 1986). The ACh receptors on heart muscle appear to be nicotinic (Calabrese and Maranto, 1986).

To unmask an FMRFamide-like effect associated with HE motor neuron activity, the cholinergic actions of the HE neuron were blocked with curare. The preparation consisted of a section of 1 lateral heart innervated by only 1, either the fifth or sixth, segmental ganglion (Maranto and Calabrese, 1984b; Calabrese and Maranto, 1986). Since each fifth and sixth ganglion contains a pair of HE neurons and a pair of HA neurons, the attached heart section was innervated by 1 of each cell type. Preparations were constantly superfused with leech Ringer's that contained 10^{-4} M curare (standard superfusate). In some experiments, 8×10^{-8} M FMRFamide was added to and alternated with standard superfusate. The effectiveness of the curare blockade of the HE cell's cholinergic actions on the heart was tested in each experiment by inducing brief bursts of impulses in the HE motor neuron with depolarizing current pulses. We considered blockade to be complete when these bursts had no discernible phasic effect on the heart. The brief bursts caused neither a phasic contraction when tension was monitored nor a phasic depolarization when membrane potential was monitored.

All efferent activity from the HE and HA neurons to the innervated heart section was controlled by altering the membrane potentials of these neurons with injected current. The HA neuron was hyperpolarized to render it inactive and to prevent any changes in its activity that might be caused by our experimental manipulations. Such changes in HA cell activity could be expected to exert FMRFamide-like effects on the heart. The HE motor neuron was similarly held hyperpolarized so that its activity was under experimental control. Heart activity was monitored by recording either beat tension or electrical activity of a heart muscle cell (Calabrese and Maranto, 1984).

Curare did not block the ability of FMRFamide to induce myogenic activity in heart muscle (Fig. 8). Superfusion of 8×10^{-8} M FMRFamide induced a strong myogenic rhythm in the presence of 10^{-4} M curare. This observation suggested that FMRFamide acts on a receptor other than an ACh receptor and indicates that curare would most likely not interfere with any FMRFamide-like effects of the HE motor neuron. In the presence of curare, prolonged firing in an HE motor neuron induced myogenic activity in heart muscle (Fig. 9). Brief bursts (<5 sec) of action potentials in the HE motor neuron caused no discernible effect on the heart muscle. Prolonged firing in the HE motor neuron, however, induced strong myogenic bursting in the heart muscle that commenced some 10 sec after the onset of firing in the HE neuron and that persisted, although gradually waning in frequency, for more than 2 min after cessation of firing in the HE neuron. The HE motor neuron thus appears to have a tonic FMRFamide-like action that is normally masked by its phasic cholinergic action.

Discussion

FMRFamide-like immunoreactivity has been previously localized to a specific subset of neurons in the CNS of the leech (Kuhlman et al., 1985a). This set of immunoreactive neurons included 2 cell types, the HE motor and HA modulatory neurons, that control heartbeat in the animal (Kuhlman et al., 1985a). Moreover, application of FMRFamide on the heart produced cardiac effects similar to those produced by activity in the HA modulatory neurons (Kuhlman et al., 1985b). In the present study, the FMRFamide-like substances in the leech were characterized biochemically, particularly with respect to neurons controlling heartbeat. In addition, the possible role of a FMRFamide-like peptide in the HE motor neurons was examined physiologically.

The RIA data indicate that nerve cord extracts contain FMRFamide-like substances that have a similar recognition by the antibody as synthetic FMRFamide. Analysis of the nerve cord extracts by HPLC revealed 3–6 distinct peaks of FMRFamide-like immunoreactivity (Figs. 3 and 4). Most of the immunoreactivity (80–100%) is localized to 3 peaks, each of which corresponds to either FMRFamide or an oxidized form of FMRFamide. Extracts were analyzed with 2 separation systems that have different peptide elution patterns; each system yielded similar results. The leech, therefore, has at least 1 peptide (peptide FLI) that is antigenically and chromatographically indistinguishable from synthetic FMRFamide, as well as a few peptides that are chromatographically distinct, but antigenically similar, to FMRFamide. Thus far, peptide FLI can be deduced to contain an amidated phenylalanine at the carboxy terminus, based on antisera specificities, and a methionine residue, based on comigration of tissue peaks with oxidized forms of FMRFamide. If peptide FLI is FMRFamide, then FMRFamide is present in a phylum other than mollusca. If peptide FLI is not FMRFamide, it may be the leech counterpart for FMRFamide. Such molecules have already been purified from chicken (Dockray et al., 1983), lobster (Trimmer et al., 1985), and snail (Price, 1982; Price et al., 1985). These homologous peptides, however, have significantly different chromatographic properties than FMRFamide. We tentatively conclude, based on the chemical similarities we see thus far, that peptide FLI is FMRFamide.

The heartbeat system of the leech has been extensively characterized physiologically (Thompson and Stent, 1976a, b; Calabrese and Peterson, 1983; Calabrese and Maranto, 1984; Maranto and Calabrese, 1984a, b). To determine whether peptide FLI or one of the other FMRFamide-like peptides was contained in the heartbeat control neurons, extracts of HE and HA neurons and hearts were examined in greater detail (Figs. 6, 7). Extracts of HA cells consistently contained only peptide FLI. Nearly all of the FMRFamide-like immunoreactivity recovered from heart extracts is attributable to peptide FLI. As the heart muscle cells themselves do not stain for FMRFamide (Kuhlman et al., 1985a), most of peptide FLI is likely to be contained within the processes running along the heart muscle cells. Many of these processes emanate from the HA and HE cells (Kuhlman et al., 1985a).

Surprisingly, although HE cells stain intensely for FMRFamide (Kuhlman et al., 1985a), little or no FMRFamide-like immunoreactivity was measured in HE cell extracts by RIA. Several reasons may account for this result. HE cells are smaller than HA cells and may be more sensitive to multiple electrode impalements. Cell death during isolation procedures may cause

release of proteolytic enzymes that digest the FMRFamide-like peptides. Or, multiple pressure injections of dye to mark the cells may distend the HE cell enough to cause leakage of the peptides. A different procedure for isolating HE cells may yield more positive results. Alternatively, the FMRFamide-like peptide in HE cells may be recognized by the antibody only following fixation of the tissue.

Although we were unable to detect any FMRFamide-like immunoreactivity in extracts of isolated HE cells, our physiological data show that HE cells exert FMRFamide-like effects on the heart. In the presence of curare both superfused FMRFamide and prolonged activity in an HE motor neuron can induce myogenic activity. Since curare blocks all detectable effects of iontophoretically applied or superfused ACh on the heart, and since muscarinic agonists are not effective on the heart (Calabrese and Maranto, 1986), this effect of the HE motor neuron is unlikely to be mediated by ACh, the HE motor neuron's small molecule transmitter (Wallace, 1981a, b; Calabrese and Maranto, 1986). We suggest instead that the HE motor neuron coreleases ACh as well as a substance that has FMRFamide-like effects on the heart. Since HE motor neurons and their peripheral processes on the heart are FMRFamide-like immunoreactive (Kuhlman et al., 1985a), and since heart extracts contain peptide FLI, which we have tentatively identified here as authentic FMRFamide, this substance is likely to be FMRFamide or a FMRFamide-like peptide. ACh and FMRFamide have also been shown to coexist in molluscan neurons (Greenberg et al., 1983; Schaefer et al., 1985). The HE cell is thus a member of the growing class of neurons known to use a peptide and a small molecule transmitter together (Schultzberg et al., 1983; O'Shea and Schaffer, 1985).

The HE motor neuron subserves 2 functional roles in heartbeat control, each attributable to a different transmitter. The HE motor neurons normally are rhythmically active, paced by a network of inhibitory interneurons, the heartbeat central pattern generator (Calabrese and Peterson, 1983). Release of an FMRFamide-like peptide by the HE motor neuron stimulates myogenic activity in the heart. The myogenic action caused by the peptide released by the HE motor neuron is long-lasting (Fig. 9), as is the action of FMRFamide (Kuhlman et al., 1985b), and acts tonically over several heartbeat cycles. Release of ACh from the rhythmically active HE motor neuron phasically depolarizes the heart muscle (Calabrese, 1984; Calabrese and Maranto, 1986) and entrains the myogenic activity of the heart (Maranto and Calabrese, 1984b).

The HA neurons regulate the myogenic rhythm and beat tension of the heart but exert no phasic effects (Calabrese and Maranto, 1984). Their ability to induce myogenic activity in the heart is unaffected by curare (Calabrese and Maranto, 1986) and is mimicked by FMRFamide both in the presence (Fig. 9) and absence (Kuhlman et al., 1985b) of curare. The immunocytochemical evidence (Kuhlman et al., 1985a) and the chemical evidence we present here (Figs. 6 and 7) indicate that they contain FMRFamide. We conclude, therefore, that HA neurons use FMRFamide, but not ACh, as a transmitter on the heart. The above reasoning suggests that HE motor neurons in the presence of curare should act like HA neurons. The action of the HE motor neuron in the presence of curare (Fig. 9) is indeed HA-like. In light of the ability of the HE motor neurons to induce myogenic activity as uncovered here, the ability of HA neurons to exert a similar myogenic effect seems redundant. Perhaps the main function of HA neurons is to regulate beat tension tonically.

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