Matching Neural and Muscle Oscillators: Control by FMRFamide-like Peptides

Pierre Meyrand^a and Eve Marder

Biology Department, Brandeis University, Waltham, Massachusetts 02254

Stomatogastric nervous systems of the shrimp, *Palaemon serratus*, were stained with antisera raised against the peptide FMRFamide. FMRFamide-like immunoreactivity was found in fibers in the input nerve to the stomatogastric ganglion (STG), in several STG somata, in dense neuropil in the STG, in the motor nerves that innervate the dilator muscles of the pyloric region, but not in the pyloric dilator (PD) motor neurons. FMRFamide and several FMRFamide-like peptides elicited sequences of rhythmic depolarizations and contractions of the pyloric dilator muscle. As peptide concentrations were increased, a discrete threshold for contraction was found, above which contractions were initiated with a decreasing latency in an all-or-none fashion. Muscles stopped rhythmically contracting after many seconds to several minutes of activity; the duration of spontaneous oscillatory activity in peptide was proportional to the concentration of applied peptide. In the absence of peptide, each motor neuron discharge evoked small graded muscle contractions. During peptide-induced oscillations, motor neuron activity did not always entrain muscle oscillations. After spontaneous oscillations had stopped, when the motor neurons were stimulated in the presence of the peptide, each motor neuron burst evoked large amplitude contractions as a result of the peptide-induced regenerative properties of the muscle membrane.

Rhythmic movements often result from rhythmic activity in central neural networks. In many cases, though the neural output may be oscillatory, the muscles activated by these rhythmic motor systems contract only when driven. Because most vertebrate skeletal muscle fibers generate action potentials, muscle contraction is not critically sensitive to the amplitude of the postsynaptic response as long as it is sufficient to depolarize the muscle to threshold. In contrast, many invertebrate muscle fibers do not routinely display regenerative membrane properties, but the amplitude of muscle contraction is a function of the size of the postjunctional response (Atwood, 1976). Under these conditions movement amplitude can be extremely sensitive to

the pattern of activity in the motor neurons, as activity-dependent processes such as facilitation (Dixon and Atwood, 1989) play an important role in controlling contraction amplitude. In recent years it has become clear that some muscles display conditional regenerative properties (Calabrese, 1989), because modulatory substances such as amines and peptides (Lingle, 1981; Glusman and Kravitz, 1982; Meyrand and Moulins, 1986) can elicit myogenic activity.

Rhythmic movements that depend on myogenic properties, such as those of the vertebrate heart and gut, can continue without precise timing information from the CNS. Many oscillators can only be entrained at a frequency close to their natural one. Therefore, if a rhythmic motor discharge varies over a large frequency range, the presence of strong intrinsic oscillatory activity in a muscle could, in principle, lead to an inability of the muscle to be entrained over the full frequency range of rhythmic neural inputs. Thus, understanding how rhythmic neural inputs interact with a rhythmically active muscle requires understanding how these oscillators interact.

In the heartbeat system of the leech the peptide FMRFamide enhances the amplitude and frequency of small myogenic oscillations in the muscles of the heart tube (Kuhlman et al., 1985; Li and Calabrese, 1987), and the excitatory motor neurons to these muscles can entrain this myogenic rhythm (Maranto and Calabrese, 1984; Calabrese, 1989). In a previous study on the dilator muscle of the shrimp stomatogastric system, Meyrand and Moulins (1986) showed that dopamine induces myogenic contractions of the dilator muscle that can also be entrained by rhythmic neural activity.

The stomatogastric nervous systems of the large decapod crustaceans such as lobsters and crabs contain several neuropeptides thought to have neuromodulatory functions (Hooper and Marder, 1984; Marder, 1987; Marder et al., 1987; Marder and Nusbaum, 1989). Among these are one or more FMRFamide-like peptides (Hooper and Marder, 1984; Callaway et al., 1987; Marder et al., 1987). Immunocytochemical studies of the shrimp stomatogastric nervous system show FMRFamide-like peptides are present in the motor nerves of the pyloric chamber, suggesting a neuromuscular target for one or more FMRFamide-like peptides in shrimp.

In control saline the dilator muscle of the pyloric chamber of the stomach is inactive, and responds to nerve stimulation with a small, graded contraction (Meyrand and Moulins, 1986). In this paper we show that FMRFamide-like peptides evoke sequences of rhythmic depolarizations and contractions, followed by a state in which the muscle does not spontaneously contract but is close to threshold for the generation of rhythmic activity. Under these conditions intrinsic regenerative properties of the muscle itself are easily triggered by neural inputs. Some of these data have been presented in abstract form (Meyrand et al., 1987).

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Correspondence should be addressed to Dr. Eve Marder, Department of Biology, Brandeis University, Waltham, MA 02254.

^a Present address: Laboratoire de Neurobiologie, et Physiologie Comparées, CNRS et Université de Bordeaux I, Arcachon, France 33120.

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Materials and Methods

Animals. Pink shrimp, *Palaemon serratus*, were obtained from fishermen in Royan, France, and maintained in circulating aerated sea water until used. Seventy-six animals 7–10 cm in length were used.

Movement recordings. Single bundles of the shrimp dilator muscle were used. Single bundles are too small for commercially available force-movement transducers, so movements were recorded using a transducer made by M. O'Neil with a modified design from W. Chapple. Briefly, this transducer was constructed using 4 Kulite Semiconductor Products semi-conductor strain gages model AGP-1000-300 in a bridge configuration and an Analog Devices High Performance Economy Strain Gage/RTD Conditioner model 2B31.

Cardiopyloric valve (cpv) muscle. Cpv1 muscles with attached nerve were dissected as described in Meyrand and Moulins (1986). The cpv1 muscle has 3 bundles of 10–12 electrically coupled muscle fibers. Fibers within a bundle contract synchronously, but the 3 bundles are not coupled and therefore contract asynchronously unless they receive synchronous neural inputs. Control experiments involving simultaneous intracellular recordings from 2 or more muscle fibers within a bundle showed that the entire muscle bundle is virtually isopotential.

Intracellular recordings. Conventional electrophysiological methods were used. Microelectrodes were filled with 2.5 M KCl, and had resistances of 15–25 M Ω . All experiments requiring current injection were done with 2 independent microelectrodes.

Immunocytochemistry. Whole-mount immunocytochemistry was done as previously described for FMRamide-like peptides (Marder et al., 1987). Antiserum 231 (O'Donohue et al., 1984; Watson et al., 1984) was a gift of T. O'Donohue and W. Watson III and was used at 1:300. Antiserum 671 was described in Marder et al. (1987) and was used at 1:600. Secondary antibodies were 1:25 goat anti-rabbit IgG labelled with fluorescein or rhodamine obtained from either Boehringer-Mannheim or Cappel. Preparations were viewed and photographed on a Zeiss IM-35 epifluorescence microscope.

Control experiments in which the antisera were preincubated with known peptides were performed to test the specificity of the antisera for FMRamide-like peptides in the shrimp tissue. Preincubation with the peptide, proctolin (10^{-5} M) produced no block of the staining. In contrast, preincubation of the antiserum with (10^{-6} – 10^{-4}) M FMRamide and related peptides such as YGGFMRamide abolished staining.

Double labels. Pyloric dilator (PD) motor neurons were identified electrophysiologically on the basis of their ability to evoke excitatory junctional potentials in the cpv1 muscle. Neurons were filled by intracellular injection of Lucifer yellow CH, fixed in paraformaldehyde, and then treated for immunocytochemistry (antibody 231), as previously described (Marder et al., 1987), using rhodamine-labeled secondary antibodies. Double labels were viewed and photographed by switching between fluorescein (Lucifer yellow) and rhodamine filter sets.

Peptides. TNRNFLRFamide was synthesized and purified by James Wiemann at Brandeis University. All other peptides were purchased from commercial sources (Peninsula Labs, Bachem, or Sigma).

Results

Localization of FMRamide-like immunoreactivity

The stomatogastric nervous system of crustaceans, including the shrimp, consists of 4 ganglia, the paired commissural ganglia (CoGs), the esophageal ganglion (OG), and the stomatogastric ganglion (STG; Fig. 1). The STG is connected to the OG and the CoGs via a single input nerve, the stomatogastric nerve (stn; Fig. 1). The shrimp STG contains about 25 neurons, some of which are motor neurons that innervate muscles of the pyloric region (Meyrand and Moulins, 1988a,b).

Previous work in lobsters and crabs (Marder et al., 1987) showed FMRamide-like immunoreactive neurons in the CoGs and OG. In these species, staining was seen in the stn and in neuropilar projections in the STGs. When we stained whole-mount preparations of the stomatogastric nervous systems of the shrimp with 2 different FMRamide antisera we found patterns of staining similar, but not identical, to those seen in larger decapods.

Figure 1. Stomatogastric nervous system of the shrimp, *Palaemon serratus*. This schematic drawing shows the positions of the 4 ganglia of the stomatogastric nervous system. The stomatogastric ganglion (STG) is connected to the paired commissural ganglia (CoG) and an unpaired esophageal ganglion (OG) by the stomatogastric nerve (stn). The somata of the pyloric dilator (PD) motor neurons are in the STG. The PD neurons innervate the cpv1 (cardio pyloric valve 1) muscle via the dorsal ventricular nerve (dv) and the lateral ventricular nerve (lv). Other abbreviations: ion, inferior esophageal nerve; ivn, inferior ventricular nerve; on, esophageal nerve; PDn, pyloric dilator nerve; son, superior esophageal nerve.

Figure 2A shows a whole-mount STG preparation stained with antiserum 231. Note the dense staining throughout the neuropil, as seen in the other crustaceans studied (Hooper and Marder, 1984; Callaway et al., 1987; Marder et al., 1987), and the stained fibers (3–5) in the stn. However, in contrast to the large crustaceans, the shrimp STG showed 2 or 3 stained somata in whole-mount preparations (Fig. 2A). One of the stained somata is large and was seen in all the preparations. One or 2 smaller somata were also seen in 20 preparations using antiserum 231 and in 7 preparations using antiserum 671.

Stained fibers were also seen in some of the motor nerves of the stomatogastric nervous system. The unpaired dorsal ventricular nerve (dv) showed at least 2 well stained fibers (Fig. 2B), one of which can be traced to each lateral ventricular nerve (lv; Fig. 2C). These fibers were visible the length of the lv, and were also seen in the nerves innervating the cpv1 dilator muscles of the pyloric chamber (Fig. 2D).

To determine whether the PD motor neurons themselves contained FMRamide-like immunoreactivity, PD somata were filled with Lucifer yellow, the dye was allowed to diffuse, and the preparations were subsequently processed to visualize FMRamide-like immunoreactivity (Fig. 3). In the preparation shown in Figure 3, the 2 PD somata were injected with Lucifer yellow. The 2 somata were partially overlapping and in this plane of focus they appear as 1 large, irregular shape (Fig. 3A). Figure 3A shows the filled cells and 2 large processes extending into the dv (1 from each filled neuron). Figure 3B shows a higher magnification view of the dv as it extends towards the periphery. The 2 large motor axons each branch to send an axon into each lv and a total of 4 large Lucifer yellow stained axons are seen just anteriorly to the point at which the dv splits to form the lv. Figures 3, C and D are the same positions and magnifications as Figures 3, A and B, now viewed with the rhodamine filter set to visualize the location of the FMRamide-like immunoreactivity. Figures 3, C and D, illustrate that the FMRamide-like immunoreactivity is not present in the PD somata or their axons. The open downward arrow in Figure 3C shows the position of the unstained PD somata. A comparison of the pattern in Figure 3, B and D, shows that the FMRamide-

Figure 2. Distribution of FMRFamide-like immunoreactivity in the STG and motor nerves. Preparations were reacted as whole-mounts as in Marder et al. (1987). *A*, Whole-mount of an STG of *Palaemon serratus* stained with 1:300 dilution of antiserum 231. Note the dense neuropilar staining (black arrow). Two stained somata (one large and one small; arrowheads) are visible. Stained fibers in the dvN are out of focus. *B*, Two stained fibers (arrowhead) in the dvN. *C*, Stained fiber (arrowhead) in the lvN. *D*, PDn shows stained fiber (arrowhead). Calibration bars are 100 μ m in all panels.

like fibers are smaller in diameter than the PD axons, and that they run in a different portion of the nerve. Indeed, their position is indicated by the small upward arrow in Figure 3*B*, which shows a faint signal from the rhodamine stain “breaking through” into the fluorescein filter. A small amount of “break through” is also seen in Figure 3*A* where faint fibers in the stn (left of ganglion) are seen. These data demonstrate that the PD axons are not the source of the FMRFamide-like immunoreactivity in the peripheral nerves of the pyloric region. We have not been able to ascertain the source of the cells that give rise to these FMRFamide-like projections.

Physiological effects of FMRFamide related peptides on the PD muscles

When isolated, the PD muscle (cpv1) is frequently quiescent and passive. However, on rare occasions spontaneous rhythmic contractions are seen subsequent to isolation. Previous work showed that dopamine applications evoke rhythmic contractions associated with repeating depolarizations of membrane potential that show all the properties of endogenous oscillations (Meyrand and Moulins, 1986). These oscillatory properties are not always expressed, but are conditional. The presence of FMRFamide-like immunoreactive fibers in the motor nerves to the pyloric region suggested that FMRFamide-like peptides might also influence the expression of the oscillatory properties of these muscles.

A large number of FMRFamide-like peptides have been isolated from a variety of species (Price and Greenberg, 1989). Because we do not know the sequence of the shrimp FMRFamide-like peptides, we were interested to determine the relative potencies of several different FMRFamide-like peptides on this preparation.

Cpv1 muscles with a small piece of motor nerve were dissected from the animal, placed in a small dish, and attached to a movement transducer. Peptides were superfused over the mus-

cle, and movement recorded in response to a variety of peptides at different concentrations. Figure 4 shows the response of a muscle to a just threshold concentration of 2.2×10^{-7} M YGGFMRFamide. After a delay (in this case almost 2 min) the muscle began to oscillate, did so for about 1 min, and then stopped.

Dose dependence of peptide actions. When movement is used as the indicator of the physiological action of the peptides, the dose-response relationships for these peptides are not graded but show a sharp threshold for activation. Figure 5 shows the response of an isolated muscle to increasing concentrations of FMRFamide. As the concentration was increased, the latency to the onset of contractions decreased (Figs. 5, 6) but the amplitude and the maximal frequency of the oscillations recorded during the first 30 sec were the same at all concentrations (Fig. 7).

As peptide concentrations increased, the latency decreased exponentially, regardless of the order in which the different peptide concentrations were applied. Under our conditions we estimated the time constant of fluid exchange in the bath to be about 30 sec. Thus, the variation in latency with peptide concentration shown in Figure 6 is not merely a reflection of the time required for the bath to reach concentration, but represents a real variation in latency with peptide concentration.

When data from 16 preparations were pooled, FMRFamide concentrations less than or equal to 2×10^{-7} M elicited contractions in 0/16 times, while concentrations of 2.5×10^{-7} M or higher elicited contractions in 29/30 times. YGGFMRFamide elicited contractions at concentrations of 2×10^{-7} M or less in 0/27 times but evoked contractions at higher concentrations in 39/41 applications. *Helix* peptide (Price et al., 1985) evoked contractions in 2×10^{-7} M in 0/12 cases but elicited contractions in 21/22 cases in higher concentrations.

Several other peptides were less effective. FLRFamide produced contractions in 0/6 applications at 10^{-6} M or lower but

Figure 3. PD neurons are not FMRamide-like immunoreactive. *A*, Whole-mount view of the STG. Two PD somata were injected intracellularly with Lucifer yellow. The somata were overlapping slightly, in this plane of focus they appear as a single filled shape (large downward white arrow). Exiting from the ganglion (top right) into the dvn are the filled axons of the PD neurons (smaller downward white arrows). *B*, Higher magnification view continuing down the dvn. The filled axons of the PD neurons (downward white arrows) are seen to branch. Faint rhodamine signal (small upward white arrow), seen "breaking through," indicates that the Lucifer-filled axons are not in the same position as the rhodamine labeled immunoreactive fibers. *C*, Same plane of focus and position as *A*, but preparation viewed with the rhodamine filter set. Note the large immunoreactive cell (upward white arrow) is not in the position of the PD neurons (shown by the downward unfilled arrow). Note the dense immunoreactivity in the neuropil and in fibers in the stn (lower left in the pictures here). *D*, Same focal plane and position as *B*, viewed with rhodamine filters. Small upward arrows show the location of the FMRamide-like fibers in the dvn (the position of these is seen faintly in *B*, above). Calibration bars are 100 μm in all panels.

was effective in 4/5 trials at higher concentrations. Ineffective up to concentrations as high as 10^{-5} M were Met-enkephalin (0/5), LPLRFamide (0/3) and RFamide (0/4).

TNRNFLRFamide, purified and sequenced from *Homarus americanus* by Trimmer et al. (1987) had the lowest threshold

of all peptides tested [57% of the muscles tested ($n = 7$) produced rhythmic contractions at 10^{-8} M]. However, this excitatory effect decreased at higher concentrations, until at 10^{-6} M, 85.8% of the muscles failed to produce rhythmic contractions ($n = 7$).

Peptide-evoked rhythmic muscle contractions did not persist

Figure 4. Rhythmic muscle contractions activated by peptide. 2.2×10^{-7} M YGGFMRFamide (upward arrow) induced bout of rhythmic contractions after a delay of about 120 sec. Contractions lasted for about 1 min and then stopped.

Figure 5. The latency, amplitude, and frequency of FMRFamide-evoked oscillations as a function of peptide concentration. Movement recordings of single muscle bundle. At the downward arrow heads the indicated concentration of FMRFamide was applied to the bath. 2×10^{-7} M FMRFamide failed to elicit rhythmic contractions in the 280 sec shown here, or after 15 min at this concentration (not shown).

throughout long exposures of the muscle to peptide applied at either high or low concentrations. All of the FMRFamide-like peptides produced sequences of rhythmic contractions in which the duration of the sequence was proportional to the concentration of applied peptide. High peptide concentrations produced sequences of rhythmic activity as long as 300 sec.

Effects of peptides on membrane potential. The rhythmic contractions in the presence of the peptide were associated with rhythmic depolarizations (Fig. 8) that preceded and were time-locked to the contractions of the muscle. The addition of FMRFamide-like peptides to a nonactive preparation first caused a small depolarization of the muscle fiber without muscle contraction. After the fiber's membrane potential reached threshold, slow depolarizations, resembling pacemaker potentials, were followed by fast, spikelike events.

Figure 6. Plot of latency to onset of contractions as a function of peptide concentration. These data come from the same experiment as Figure 5.

The peptide-induced changes in membrane potential were unaffected by bath application of TTX (data not shown). The TTX insensitivity of the oscillations suggests that they do not depend on presynaptic action potentials and that a TTX-sensitive Na^+ current is not required for these oscillations. In contrast, peptide-induced membrane potential oscillations and movements were entirely suppressed by Co^{2+} and other Ca^{2+} blockers (data not shown), suggesting that an inward Ca^{2+} current is required for the depolarization and contraction.

To begin to characterize the mechanism by which the FMRFamide-like peptides excite the dilator muscles, we measured the effects of these peptides on the resting potential and on the apparent membrane input resistance. The resting potential depolarized by 5–10 mV in 2.5×10^{-7} M FMRFamide, YGGFMRFamide or *Helix* peptide (Fig. 8). Figure 9A shows a slow time base recording from a fiber in which hyperpolarizing current pulses were passed before, during, and after peptide application. Figure 9B shows expanded recordings at each of these times to illustrate the change of resistance at each segment of the slow record. Figure 9C shows a current–voltage curve for the fiber. These data indicate that the small depolarization of the resting membrane potential was associated with a 20%–30% resistance increase in the hyperpolarized region of the current–voltage curve (Fig. 9C). During the wash the repolarization of the membrane preceded the return of the resistance to control values. In the absence of peptide, it was possible to depolarize the muscle fiber as much as 30 mV (Fig. 9) without evoking substantial movement or eliciting action potentials. In the presence of peptide (Fig. 9), even small depolarizations were associated with large conductance increases and movements that made it impossible to depolarize the membrane past -45 mV.

Peptide oscillations are intrinsic to muscle. A classical test for an independent oscillator is its ability to be reset by a brief stimulus during its ongoing rhythm. Figure 10 illustrates that the cpv1 muscle behaves as a true oscillator when spontaneously

Figure 7. Plot of amplitude and frequency of oscillations in first 30 sec as a function of peptide concentration. From the same experiment as Figure 5.

contracting in the presence of peptide. In this experiment a brief intracellular injection of depolarizing current during an oscillatory sequence of rhythmic movement produced a premature muscle contraction. After the depolarizing pulse, the muscle fiber continued to oscillate at the same frequency as previous to the pulse, but the contractions were phase advanced (the arrows in Fig. 10 indicate the times at which the rhythmic contractions would have occurred in the absence of the pulse). This confirms that the spontaneous oscillations that occur in the presence of peptide are an inherent property of the muscle.

Figure 11 shows that FMRFamide-like peptides do not merely

depolarize the fibers into the range of membrane potentials at which the fibers can oscillate but also modulate the muscle fiber membrane properties. In control saline, when the fiber was depolarized by a pulse of intracellularly injected current, the membrane potential followed passively regardless of the membrane potential to which the fiber was depolarized. YGGFMRFamide was then applied to the muscle and the muscle fiber was repetitively depolarized by pulses of current of the same amplitude as that given during the control (Fig. 11*B*). At $t = 0$ the response was comparable to the control, because the peptide solution had just entered the bath but had not reached final concentration.

Figure 8. Rhythmic contractions in the dilator muscle are associated with rhythmic oscillations of membrane potential. Simultaneous movement (*Mvt*, top trace) and intracellular (bottom trace) recordings from a single cpv1 bundle. *Helix* peptide was added to the bath at the upward arrow. Note the slight depolarization of the fiber that precedes the onset of rhythmic contractions. The right-hand section of the recording is a faster chart speed to show that the oscillations in membrane potential lead the rhythmic contractions.

Figure 9. YGGFMRFamide-evoked depolarizations are associated with a decrease in input conductance of the muscle fiber. Two microelectrodes were placed in a muscle fiber. *A*, Slow time base recording. Top trace, membrane potential (*cpv1*). Resting potential indicated by arrowhead. Bottom trace, current monitor (*i*). Hyperpolarizing constant current pulses were passed into the fiber to monitor muscle fiber input impedance. 2.5×10^{-7} M YGGFMRFamide was added to the bath for the time indicated with the black bar. Slow time base recording shows the slow depolarization produced by the peptide. *B*, Fast-sweep recordings showing the membrane potential deflections produced by a constant current pulse in control saline (position indicated by open star in *A*), in the presence of 2.5×10^{-7} M peptide (position indicated by black star in *A*), and in the wash (position indicated by open star in black circle in *A*). Note the larger deflections of membrane potential and the slight depolarization of the baseline indicated by the displacement of the traces. *C*, Full voltage-current plot from the same muscle fiber showing that the input impedance of the fiber was increased in the voltage range of -100 mV to -60 mV by the peptide.

At $t = 20$ sec, the pulse elicited small oscillations. At $t = 40$ sec, though the fiber was not spontaneously oscillating, the depolarization of the fiber produced full oscillations. Note that the muscle produced oscillations at a membrane potential that failed to evoke oscillations under control conditions. Between $t = 60$ sec and the next pulse in Figure 11*B* the muscle fiber started spontaneous rhythmic activity. When the muscle was depolarized during spontaneous activity (last pulse in Fig. 11*B*) the frequency of the oscillations increased during the pulse, as expected for an oscillator. Following the wash (Fig. 11*C*), the muscle reverted to its passive state.

Consequence of peptide-induced oscillations for neuromuscular functions

FMRFamide and similar peptides elicit bouts of contractions when applied to the *cpv1* muscle. It is therefore important to determine what consequences these effects will have for the muscle's response to rhythmic excitatory neural inputs. In neurogenic systems, muscles will depolarize and contract only in response to neurally released neurotransmitter. In this case the muscle is a "slave" of the innervating motor neurons. Alternatively, if the muscle is capable of producing triggered regen-

Figure 10. Resetting of the ongoing muscle rhythm by short depolarizing pulse of current injected into the muscle fiber. Simultaneous movement (*Mvt*) and intracellular recordings (*cpvt*) from a single isolated bundle of the dilator muscle. A 150 msec depolarizing pulse of current (*i*) was passed into the muscle fiber. Arrowheads indicate the expected time of contraction in the absence of the experimental perturbation. This record was made in the presence of 5×10^{-7} M *Helix* peptide.

erative plateaus or action potentials, even modest depolarizations that result from motor neuron activity can produce large amplitude contractions.

Figure 12 compares the contraction produced in a muscle in control saline with one in the presence of peptide when a PD motor neuron is depolarized intracellularly. In control saline a single burst of high frequency action potentials (Fig. 12*A*) evokes no measureable contraction, and repeated bursts evoke (Fig. 12*C*) only small contractions. However, in the presence of YGGFMRFamide, a single presynaptic burst (Fig. 12*B*) or trains (Fig. 12*D*) evoked contractions more than 100 times larger than those evoked in the absence of the peptide. The large amplification of contraction seen in peptide occurs because of the regenerative properties of the muscle demonstrated in Figure 11.

The PD neurons themselves can be silent, such as seen in Figure 11, or can be rhythmically active when central inputs to the STG are stimulated (Meyrand and Moulins, 1988b). True oscillators, such as the *cpvt* muscle in its spontaneously active state, can only be entrained in a narrow frequency range. Therefore, if the muscle and motor neurons are both rhythmically active, but at significantly different frequencies, it is unlikely that the rhythmically active motor neurons could drive the mus-

Figure 11. YGGFMRFamide modifies the response of the muscle fiber to injected current. Two microelectrodes were placed in a muscle fiber. In all records the top trace is membrane potential and the bottom trace is the current passed into the fiber with the second microelectrode. *A*, Depolarizing current pulse passively depolarized the muscle fiber membrane. *B*, at $t = 0$, 2.5×10^{-7} M YGGFMRFamide was added to the bath. The response of the muscle to the same size depolarizing current pulse is shown at $t = 0$, $t = 20$ sec, $t = 40$ sec, $t = 60$ sec, and $t = 80$ sec (the last depolarization was at $t = 80$ sec, but not so labeled on the figure because of space). *C*, The response to the same amplitude depolarizing current pulse shown after washing with control saline for 30 min.

Figure 12. Peptide enhancement of the muscle contraction produced by stimulation of a PD motor neuron. The PD motor neuron soma was depolarized intracellularly, and the movements of the cpv1 muscle were recorded. *A*, Single burst of action potentials in the motor neuron evoked no measurable contraction. *B*, Large amplitude contraction evoked by intracellular depolarization in peptide. *C*, Repeated bursts of action potentials in PD motor neuron evoked small contractions in muscle in control saline but evoked large amplitude contractions in peptide, *D*.

cle in a one-for-one manner through the entire frequency range of the pyloric rhythm. However, if the muscle were not spontaneously active but displayed regenerative properties when depolarized (as in Fig. 11*B*), the muscle would be able to generate large amplitude contractions (such as seen in Fig. 12*B*) in response to rhythmic motor neuronal activity, regardless of the pyloric rhythm frequency. This is illustrated in the experiments shown in Figure 13.

The left panel in Figure 13 shows a muscle in control saline. Under these conditions the muscle was inactive (top). When the innervating motor nerve was stimulated in a single burst a small movement occurred (Fig. 13*B*), and when the motor nerve was stimulated in repetitive bursts, the muscle generated a small movement in response to each motor neuron burst (Fig. 13*C*).

The middle vertical panel in Figure 13 shows a muscle that was spontaneously contracting in the presence of YGGFMRFamide. Figure 13*B* shows that a single burst of stimuli to the motor nerve was able to reset this spontaneous activity. However, Figure 13*C* shows that when the motor nerve was stimulated rhythmically, to mimic the pyloric rhythm, the muscle was not

able to follow the motor neuron stimulation at all frequencies, and the resultant muscle movements were a disorganized composite of nerve-evoked and spontaneous activity. Thus, here we have a failure of the motor neuron to entrain the muscle it innervates because of the strong expression of the regenerative properties of the muscle.

The right vertical panel was taken from a muscle still in peptide that had stopped spontaneously oscillating. Here, the amplitude of the muscle movement evoked by a single train of stimuli to the motor nerve was dramatically increased (compare left and right panels of Fig. 13*B*). Most importantly, when the motor nerve was stimulated repetitively to mimic the pyloric rhythm, the muscle generated large movements that followed one-for-one each burst of motor neuronal activity. Thus, the peptide-induced state in which the muscle is not spontaneously active, but in which the muscle can be induced to show oscillatory properties when depolarized, allows the muscle to generate large movements in response to rhythmic motor neuronal inputs that can follow the dynamic range of the presynaptic neuronal activity.

Figure 13. Functional consequences of the state of the muscle for neuromuscular control. *Left vertical panel*, muscle in a “nonoscillatory state” (empty white box) in the absence of peptide. *Center vertical panel*, muscle oscillating in the presence of peptide (solid oscillator symbol). *Right vertical panel*, muscle capable of producing single oscillations when stimulated or depolarized but not spontaneously rhythmic (dashed oscillator symbol). Muscle is found in this state in the presence of peptide, either directly before the onset of rhythmic contractions or after a bout of rhythmic contractions has stopped. *A*, movement recordings showing the behavior of the muscle in its 3 modes in the absence of nerve stimulation. *B*, Movement recordings showing the response of the muscle to a single train of stimuli to the lvn. *C*, Movement recordings showing the response of the muscle under these 3 conditions to repeating trains of stimuli that mimic physiological conditions in which the motor neurons are rhythmically active.

Discussion

Distribution and characterization of FMRFamide-like immunoreactivity

There are 2 interesting features of the distribution of FMRFamide-like peptides in the stomatogastric nervous system of the shrimp, *Palaemon serratus*. First, as in other crustaceans such as lobsters and crabs (Marder et al., 1987), there is intense staining throughout the neuropil of the STG. However, unlike the other species thus far studied, several STG neurons showed FMRFamide-like immunoreactivity. This is even more surprising because in the previously studied species none of the STG somata stain with antisera raised against a variety of amines and peptides (Marder, 1987; Marder and Nusbaum, 1989). We do not know which STG somata contain the FMRFamide-like peptide(s) but they are not the motor neurons to the dilator muscle (Fig. 3). Second, stained fibers were clearly seen in the motor nerves of the stomatogastric nervous system, including those that innervate the dilator muscle. This suggests that a FMRFamide-like peptide may be released in the vicinity of the dilator muscle. We do not yet know the source of the FMRFamide-like fibers, or how they are likely to be physiologically activated.

Both of the antisera used for these studies (231 of O'Donohue et al., 1984 and 671 of Marder et al., 1987) are reasonably specific for peptides of the FMRFamide-like family but will react with many, if not all, peptides that contain a terminal MRFamide or a LRFamide. Therefore, the sequence of the

FMRFamide-like peptide(s) found in the shrimp nervous system is an open question. Because other arthropod species show a number of different extended FMRFamide-like peptides (Trimmer et al., 1987; Nambu et al., 1988; Robb et al., 1989; Price and Greenberg, 1989), it is possible that the shrimp nervous system also produces several different FMRFamide-like peptides. Several other arthropod preparations contain extended FLRFamide peptides and are more sensitive to these than to extended FMRFamide-like peptides (Price and Greenberg, 1989). Thus, it is interesting that on the shrimp muscle preparation the extended FMRFamide-like peptides are strongly physiologically active.

Modulation of muscles and neuromuscular junctions

In many preparations the ability of muscles to generate action potentials or plateau potentials is under modulatory control (Lingle, 1981; Glusman and Kravitz, 1982; Calabrese and Marranto, 1984; Evans and Myers, 1986a; Meyrand and Moulines, 1986; Calabrese, 1989). Moreover, in many preparations modulatory substances, among them the FMRFamide-like peptides, are likely to have multiple sites of action, including presynaptic terminals as well as the muscles themselves (Beltz and Kravitz, 1986; Evans and Myers, 1986a,b; Calabrese, 1989; Mercier et al., 1989). Although it is not the focus of this study, FMRFamide-like peptides potentiate the amplitude of the synaptic potentials evoked by motor neuron stimulation in the dilator muscle (P. Meyrand and E. Marder, unpublished observations).

Multiple modulators

The dilator muscle responds similarly to both dopamine (Meyrand and Moulins, 1986) and the FMRFamide-like peptides. Assuming that both of these substances reach the dilator muscle, either through direct neuronal pathways or hormonally, this poses 2 questions: (1) whether these 2 substances are physiologically released at different times, and (2) whether they act by the same or different mechanisms.

The cpv1 muscle is not unusual in receiving multiple modulatory inputs. For example, the well-studied Accessory Radula Closer (ARC) muscle in *Aplysia* is modulated by a variety of amines and peptides (Weiss et al., 1978; Lloyd et al., 1984; Cropper et al., 1987a,b). The myogenic rhythm of the locust hindleg is modulated by a number of amines and peptides (Evans and Myers, 1986a,b). Thus, multiple modulatory inputs to a muscle may turn out to be the rule rather than the exception, though the physiological consequences of this are unclear (Calabrese, 1989).

Physiological consequences of the conditional oscillatory properties of the dilator muscle

When spontaneously contracting, the cpv1 muscle displays all the characteristics of an endogenous oscillator (Meyrand and Moulins, 1986): (1) the frequency of the oscillation is voltage dependent (Fig. 11B), (2) the response to a brief stimulus is all-or-none, (3) the ongoing rhythm can be reset by a brief stimulus to the muscle itself (Fig. 10), and (4) the endogenous rhythm can be entrained by cyclic stimulation, when the stimulus is close in frequency to the intrinsic frequency of the muscle (Meyrand and Moulins, 1986). However, unlike the vertebrate heart or some myogenic insect skeletal muscles (Hoyle, 1978), the cpv1 muscle shows 3 states of activity: (1) spontaneous oscillatory activity, (2) passive follower behavior, or (3) a triggered slow spike or single oscillation. The ability of the muscle to display these 3 different states means that the response of the muscle to a given pattern of motor neuronal stimulation will be remarkably different. Indeed, each of these states may be suited to a different physiological context.

In its quiescent "control" mode, the muscle contracts vigorously only when the motor neurons fire in dense, high frequency bursts; otherwise only small movements result. In the spontaneously oscillatory mode, the muscle can provide rhythmic movements even in the absence of a neuronal input. Since both dopamine and FMRFamide-like peptides can induce this spontaneous activity, it is possible that hormonally released modulators can provide a non-neural activation of the muscle. In its third state, the muscle produces vigorous movements in response to even modest levels of motor neuron activity. In this state the regenerative properties of the muscle act as an "amplifier" for the synaptically released neurotransmitter, because even a small depolarization is sufficient to trigger a full large depolarization and contraction of the muscle. Furthermore, in this state the muscle can follow faithfully almost any pattern of motor neuron discharge, which is not always possible when the muscle is spontaneously oscillatory (Fig. 13).

In the laboratory the application of dopamine or FMRFamide-like peptides triggers spontaneous oscillations that last for hundreds of seconds or minutes, and then stop, to be succeeded by the state in which the muscle is sensitive to small depolarizations. It is possible that in the animal these modulatory substances are released more slowly so that the muscle does not

actually go through a full oscillatory mode, but makes a smooth transition between our "control" mode and the "amplifier" mode. However, without knowing the kinetics and site of modulator release, this is difficult to determine. A challenge for the future will be to understand how and when the FMRFamide-like peptides are released in the animal and how these are used behaviorally.

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