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Action of FMRFamide on longitudinal muscle of the leech, *Hirudo medicinalis*

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Summary. 1. Nerve terminals associated with longitudinal muscle in the leech show FMRFamide-like immunoreactivity.

2. Structure-activity studies using FMRFamide analogs show that the C-terminal RFamide portion of the molecule is crucial for biological activity on leech longitudinal muscle.

3. The putative protease inhibitor FA (Phe–Ala) increases the peak tension produced by longitudinal muscle in response to superfused FMRFamide and the majority of its analogs, suggesting the presence of peripheral proteases capable of degrading RFamide peptides.

4. FMRFamide decreases the relaxation rate of neurally evoked contractions of longitudinal muscle. FA also decreases the relaxation rate of neurally evoked contractions.

5. Intact and isolated muscle cells respond to superfused FMRFamide with a conductance increase, that leads to depolarization and often with a delayed conductance decrease as the membrane potential is restored to resting levels.

6. The depolarizing response of isolated muscle cells to FMRFamide is dependent on external calcium.

Key words: FMRFamide – Longitudinal muscle – Leech

Introduction

FMRFamide (Phe–Met–Arg–Phe–NH_2), one of the first invertebrate peptide transmitters to be identified, was isolated from the clam, *Macrocallista nimbosa* (Price and Greenberg 1977a, b). Since then, FMRFamide has been found in the central nervous systems (CNS) of a large variety of mollusks (Agarwal and Greenberg 1969; Schaefer et al. 1985, Price et al. 1985; Price 1986), and closely related peptides have been isolated from the CNS of both a number of mollusks and other invertebrates (Price 1982; Voigt et al. 1983; Veenstra 1984; Trimmer et al. 1987; Price et al. 1985; Holman et al. 1986; Grimmelikhuijzen and Graff 1986; Spencer et al. 1987; Price et al. 1987; Marder et al. 1987; Ebberink et al. 1987; Schneider and Taghert 1988; Cowden et al. 1989), and vertebrates (Dockray et al. 1983; Yang et al. 1985). We now recognize that these peptides constitute a diverse family of RFamide peptides; all members share the same C-terminal RFamide sequence.

Immunocytochemical evidence (see e.g. O'Shea and Schaffer 1985) indicates that the RFamide family is even more widespread in the animal kingdom and extends to the leech, *Hirudo medicinalis* (Kuhlman et al. 1985a, b).

In the leech, FMRFamide-like immunoreactivity (FLI) is found in approximately 50 neurons in each segmental ganglion of the nerve cord (Kuhlman et al. 1985a, b). A large number of these FLI positive neurons were identified as excitatory motor neurons innervating longitudinal muscle (Norris and Calabrese 1987). These excitatory motor neurons had been previously shown to be cholinergic (Fuhner 1918; Bacq and Coppee 1937; Flacke and Yeoh 1968). They contain enzymes involved in the synthesis (choline acetyltransferase) and breakdown (acetylcholinesterase) of acetylcholine (ACh) (Sargent 1977; Wallace 1981a, b; Wallace and Gillon 1982), and curare blocks the effects of applied ACh and of motor neuron activity on longitudinal muscle (Kuffler 1978; Walker et al. 1970).

Longitudinal muscle contracts in response to superfused FMRFamide, and this response is unaffected by curare (Norris and Calabrese 1987). High pressure liquid chromatography (HPLC) analysis indicate that more than 85% of the immunoreactivity extractable from leech nerve cord and detected with RIA is indistinguishable from FMRFamide (Li and Calabrese 1987).

Here we extend the study of the action of FMRFamide on longitudinal muscle in the leech in three ways.

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1) We begin the characterization of the FMRFamide receptor on longitudinal muscle and the search for possible FMRFamide antagonists to this receptor by exploring the structure-activity relations of several FMRFamide analogs. 2) We describe an effect of FMRFamide on neurally evoked contractions of longitudinal muscle. 3) We characterize effects of FMRFamide on the membrane potential of isolated longitudinal muscle cells.

Methods

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

Physiological salines. All physiological salines used are listed in Table 1 and had a pH of 7.4 at room temperature.

Immunocytochemistry. Immunocytochemical staining with an anti-FMRFamide antiserum was performed on patches of longitudinal muscle from which the skin and other muscle layers had been dissected away. The tissue was stained using the procedure of Li and Calabrese (1985), with the following modifications; the muscle was fixed in Zamboni's fixative (2% parformaldehyde and 15% saturated picric acid solution in 0.1 mol l^{-1} phosphate buffer) and then treated for 1–2 h in collagenase (type 1A-Sigma) in normal saline (2000 units/ml). The primary antiserum used was 671C (Marder et al. 1987), and it was visualized by using a rhodamine-conjugated secondary antiserum.

Physiological methods. Three preparations, described below, the longitudinal muscle preparation, the DP neuromuscular preparation and dissociated longitudinal muscle cells in culture, were used to test the effect of FMRFamide on longitudinal muscle. Part or all of each preparation was superfused with a constant flow of aerated normal saline. The delivery system was arranged so that the superfusate could be rapidly switched from normal saline to experimental salines containing other ionic concentrations or different combinations and concentrations of peptides and other agents. Longitudinal muscle tension was monitored with a tension transducer (Grass FT 03 B) connected to one end of the body wall by a small hook and a piece of thread. Standard glass (Haer) microelectrodes filled with 4 mol l^{-1} potassium acetate and with a resistance of 30–40 MΩ were used for all intracellular recordings. Current was injected into neurons and muscle cells through the recording microelectrode using discontinuous current clamp (Axoclamp – DCC mode – Axon Instruments). While using DCC, electrode settling was monitored on a separate oscilloscope.

Table 1. Salines

	Normal salines	20 mmol l^{-1} Ca^{++}	40 mmol l^{-1} Mg^{++}	5 mmol l^{-1} Mn^{++}
NaCl	115.0	87.7	57.7	109.4
KCl	4.0	4.0	4.0	4.0
$CaCl_2$	1.8	20.2		0.1
$MgCl_2$			40.0	
$MnCl_2$				5.0
Glucose	10.0	10.0	10.0	13.3
Tris HCL	10.0	10.0	10.0	10.0

All values listed are in mmol l^{-1} and salines had a pH of 7.4 at room temperature

Longitudinal muscle response to superfused FMRFamide and FMRFamide analogs. Changes in longitudinal muscle tension evoked by FMRFamide and its analogs were monitored using the longitudinal muscle preparation (described in Norris and Calabrese 1987). For each preparation, 10^{-6} mol l^{-1} FMRFamide was applied at the beginning and end of the experiment and all test responses for that preparation were normalized to the average of the two responses to 10^{-6} mol l^{-1} FMRFamide. Normalization of results was necessary for comparisons among preparations, since preparations varied widely in the maximal tension they generated in response to FMRFamide as they do to the small molecule transmitter of excitatory motor neurons, acetylcholine (Norris and Calabrese 1987). The mean peak tension generated in response to 10^{-6} mol l^{-1} FMRFamide across 29 preparations used for quantitative data was 6.98 ± 5.46 g (standard deviation). Since several tests were often performed on a single preparation, we wished to ascertain that there was no consistent variation in the response to the initial FMRFamide standard and the final FMRFamide standard in these 29 preparations. The initial standard gave an average response of 6.60 ± 1.04 g while the final standard gave an average response of 7.63 ± 1.00 g. These results indicate that the preparation did not deteriorate during the course of our experiments; in fact they suggest increased responsiveness during the course of the experiment. This difference is not significant, however, as determined by a standard *t*-test ($t_s = 0.36 \ll t_{0.05[56]} = 2.004$). For all dose response curves only a single analog at various concentrations with and without the putative peptidase inhibitor FA (plus FMRFamide standards) was tested. Each point on the dose response curve is the average of measurements on 5 to 6 preparations.

The FMRFamide analogs tested were FMdRFamide, FMRdFamide RFamide, LPLRFamide, YMRFamide (Bachem), FMRF-OH, FLRFamide (CRB), pQDPFLRFamide, YGGFMRFamide (Peninsula), and LWMRFA (Research Plus). In addition, the peptides SCP_B (Met–Asn–Tyr–Leu–Ala–Phe–Pro–Arg–Met–NH_2) and proctolin (Arg–Tyr–Leu–Pro–Thr) (Bachem) were tested. Putative peptidase inhibitors tested were FA (Phe–Ala – 10 μg/ml), trypsin inhibitor II-T (1.25 mg/ml) and phenylmethylsulfonyl fluoride (1.5 mg/ml), (Sigma). FP (Phe–Pro) and BSA (both applied at 10 mg/ml) (Sigma) were also tested as possible protease inhibitors. Curare (d-tubocurarine – Sigma) was applied at 10^{-4} mol l^{-1}.

FMRFamide and neurally evoked contractions of longitudinal muscle. To test the effects of FMRFamide and FA on neurally evoked contractions the DP neuromuscular preparation was used. This preparation consisted of a thin strip of dorsal body wall, two segments in length, which was left innervated by a single ganglion through the DP nerve. The length of this nerve made it possible to separate the ganglion from the muscle by a vaseline dam. The ganglion was then placed in either 40 mmol l^{-1} Mg^{++} or 5 mmol l^{-1} Mn^{++} saline to block central synapses while the muscle was superfused by normal saline. Longitudinal muscle tension was monitored and changes in baseline tension caused by application of FMRFamide were subtracted from neurally evoked responses.

Individual motor neurons were stimulated either intracellularly by current injected through the recording microelectrode using DCC, in which case firing was monitored directly with the recording/stimulating microelectrode or extracellularly through a suction electrode placed on the dorsal posterior (DP) nerve, in which case firing was monitored with an intracellular microelectrode in the motor neuron. Cells 3 and L are the only identified excitatory motor neurons that innervate longitudinal muscle and have axons in the DP nerve (Ort et al. 1974). The axons of these motor neurons are both large and have similar thresholds for extracellular stimulation, but in any given preparation one or the other can be isolated for stimulation by adjusting stimulus strength. In experiments involving motor neuron stimulation, stimulus intensity was contin-

uously adjusted throughout the experiment to assure a constant firing rate of the motor neuron.

Electrical effects of FMRFamide on dissociated longitudinal muscle cells. In initial experiments, a patch of longitudinal muscle was dissected away from the skin and other muscle layers and single cells were impaled with microelectrodes. The muscle was superfused with either normal or 20 mM Ca^{++} saline. Due to the difficulties in obtaining high quality penetrations of these 'intact' cells, in most experiments enzymatically dissociated muscle cells were used.

Longitudinal muscle cells were dissociated using a modification of the procedure described by Maranto and Calabrese (1984b) for leech heart muscle cells. The gut, nephridia and other internal tissue were dissected away, leaving the longitudinal muscle exposed. A patch of body wall was pinned out in a sylgard coated Petri dish with the longitudinal muscle layer up. The preparation was covered with saline containing collagenase (type 1 A – Sigma) at 2 mg/ml and vigorously agitated (>70 revs/min) for 30 min on a shaker table. The saline was then replaced with saline containing 2 mg/ml of protease (type XIV – Sigma) and similarly agitated. As muscle cells were liberated by the agitation, aliquots of the saline containing suspended cells were removed, gently centrifuged to settle out the muscle cells and the saline was then returned to the preparation dish. The isolated muscle cells were resuspended in normal saline and centrifuged several times, to remove any remaining protease. The cells were then suspended in L-15 culture medium and refrigerated (4 °C) until used. Isolated cells survived and continued to respond to FMRFamide for several days when kept refrigerated.

To record from dissociated longitudinal muscle cells, the cells were placed in a clear plastic Petri dish (35 mm), which contained 1–2 ml of normal saline, and allowed to settle on the bottom. It was not necessary to affix the cells to the substrate in order to superfuse various salines over the cells or impale them with microelectrodes.

Results

Presence of FMRFamide-like immunoreactivity in nerve processes on longitudinal muscle

In a previous study (Norris and Calabrese 1987), we were unable to obtain staining of neuronal processes on longitudinal muscle fibers with a FMRFamide antiserum [321 (O'Donohue et al. 1984)]. We suggested that antibody penetration might have been a problem, since staining of neuronal processes on dorsoventral muscles, which are only a few muscle fibers thick and not enveloped in much connective tissue, was routine. Using a modified staining procedure in which muscle was fixed in Zamboni's fixative, treated with collagenase (1000 units/ml saline) and then processed as described in Li and Calabrese (1985), FMRFamide-like immunoreactive processes have now been detected on longitudinal muscle fibers. For these preparations we used a different antiserum from that used formerly. We have previously characterized this antiserum (671C) both immunocytochemically and in radioimmunoassay (Marder et al. 1987). Figure 1 shows immunoreactive nerve processes on a patch of dorsal longitudinal muscle. In addition to large processes which are beaded in appearance, extremely fine processes, which branch off the larger processes, can be seen running parallel to the muscle fibers (indicated by arrowheads). Preabsorption of antibody 671C with

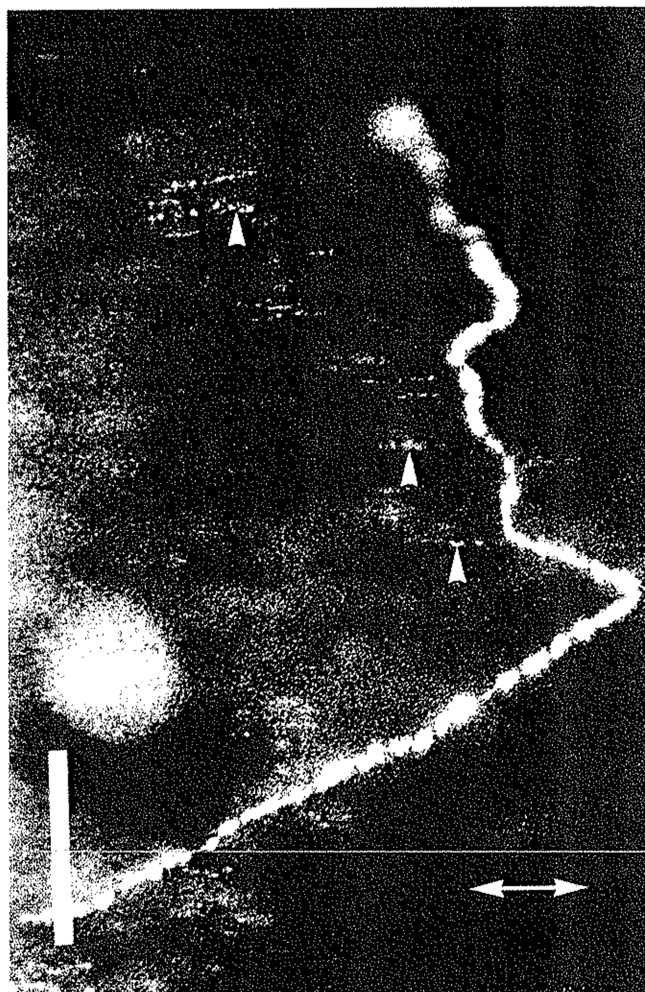


Fig. 1. Nerve fibers on longitudinal muscle contain a FMRFamide-like molecule. A large nerve process runs perpendicular to the long axis of the muscle fibers (arrow indicates long axis) and has numerous fine branches (some indicated by arrowheads) that run parallel to the muscle fibers. The focal plane was adjusted so the fine processes running along muscle fibers were in focus, making it impossible to see that the fine processes branched off the larger nerve process. Calibration bar = 0.1 mm

FMRFamide (10^{-6} mol l^{-1}) prevented staining in this and other leech tissues (Evans and Calabrese 1989).

Structure activity relations of FMRFamide analogs

To begin a characterization of FMRFamide receptors on longitudinal muscle, we performed structure-activity experiments in which a number of FMRFamide analogs were superfused over the longitudinal muscle preparation and the responses were compared to the response elicited by FMRFamide. The parameter chosen for determining differences in potency between FMRFamide and its analogs was peak tension. For FMRFamide and most analogs, other parameters of the response (time to contraction onset, rate of contraction and relaxation) showed considerable variability both between prepara-

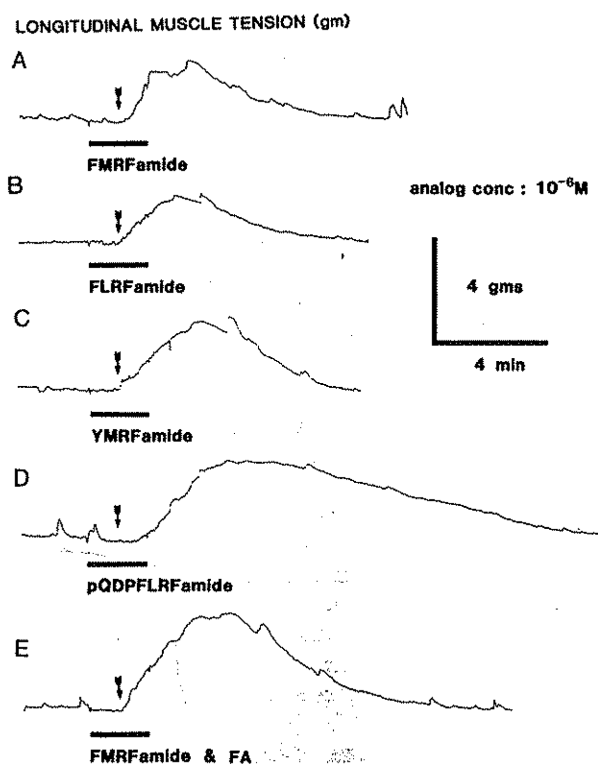


Fig. 2A–E. Response of longitudinal muscle to superfused FMRFamide **A**, FLRFamide **B**, YMRFamide **C**, pQDPFLRFamide **D** and FMRFamide in the presence of FA (10 mg/ml) **E**. FMRFamide, FLRFamide and YMRFamide all elicited similar responses, while pQDPFLRFamide and FMRFamide+FA elicited a stronger contraction. Bars indicate duration of peptide application. Arrows indicate time of contraction onset for the FMRFamide contraction. All peptides applied at 10^{-6} mol l^{-1}

tions and within a given preparation. For pQDPFLRFamide, however, the time to contraction onset was consistently longer than that for FMRFamide (Fig. 2).

No consistent agonistic effects were observed for FMRF-OH, RFamide, FMdRFamide, FMRdFamide, LWMRFA, SCP_B or proctolin in 3 separate applications at 10^{-6} mol l^{-1}. FLRFamide, YMRFamide, pQDPFLRFamide, YGGFMRFamide and LPLRFamide, however, were all found to act as agonists at this concentration. The responses of one preparation to several analogs applied at 10^{-6} mol l^{-1} are shown in Fig. 2. Figure 3A, B shows the dose response curves for FMRFamide and effective agonists at concentrations from 10^{-8} to 10^{-5} mol l^{-1}. The response of longitudinal muscle to FLRFamide and YGGFMRFamide at all concentrations was roughly the same as the response to an equimolar concentration of FMRFamide. At the lower concentrations tested ($<10^{-6}$ mol l^{-1}), YMRFamide evoked responses which were also approximately equal to those evoked by FMRFamide, while at the higher concentrations the response was almost 150% of that evoked by FMRFamide. pQDPFLRFamide produced a response roughly 200% of that produced by FMRFamide at low concentrations, and more than 400% of that produced at higher concentrations. LPLRFamide produced no contraction at concentrations below 10^{-6} mol l^{-1} and a response roughly 10% of the response to FMRFamide at higher concentrations.

Effects of putative protease inhibitors

In initial experiments, 3 putative protease inhibitors, trypsin II-T, phenylalanyl-alanine (FA) and phenylmethylsulfonyl fluoride, were tested to determine if they increased the response of longitudinal muscle to 10^{-7} mol l^{-1} FMRFamide. Only FA had a consistent effect (Fig. 2E, Table 2).

FMRFamide and its analogs were then tested at 10^{-6} mol l^{-1} in the presence and absence of FA on at least 4 different preparations each. The results from these trials are shown in Fig. 4. FMRdFamide, FMdRFamide, FMRF, RFamide and LWMRFA did not give consistent responses in the absence of FA and were excluded from further analysis, although all but LWMRFA gave consistent responses in the presence of FA. A standard *t*-test was used to determine differences between the responses produced by each effective peptide in the presence and absence of FA ($P<0.05$). FA significantly increased the potency of FMRFamide and the potent agonists, YGGFMRFamide, FLRFamide and YMRFamide, but not of potent agonist pQDPFLRFamide or of the poor agonist LPLRFamide.

The dose response relations for the 6 effective analogs were measured in the presence of FA to determine if FA enhanced the responses of the peptides over the range of concentrations used in this study (Fig. 3C, D). The dose response relations of the FMRFamide and the potent agonists, YGGFMRFamide, FLRFamide and YMRFamide, were enhanced by FA (Fig. 3). The dose response relations of pQDPFLRFamide, a potent agonist, were very similar in the presence and absence of FA. FA had very little effect on the dose response relations of LPLRFamide, a poor agonist.

The effects of FA appear to be exerted through specific action on tissue proteases. FA alone had no effect on the longitudinal muscle preparation nor did it potentiate the response of longitudinal muscle to superfused ACh (data not shown). Another dipeptide Phe−Pro (FP) as well as the larger peptide bovine serum albumin (BSA) did not potentiate the response of the longitudinal muscle preparation to FMRFamide, nor did they cause measurable contraction, when applied at 10 mg/ml (Table 2).

FMRFamide antagonists

Several FMRFamide analogs, which had no effect on longitudinal muscle by themselves, were tested as possible FMRFamide antagonists. The results from these tests are summarized in Table 2. Only FRMdFamide acted as a weak antagonist. FMRdFamide at 10^{-6} mol l^{-1} decreased the response of longitudinal muscle to 10^{-7} mol l^{-1} FMRFamide by about 20%. In

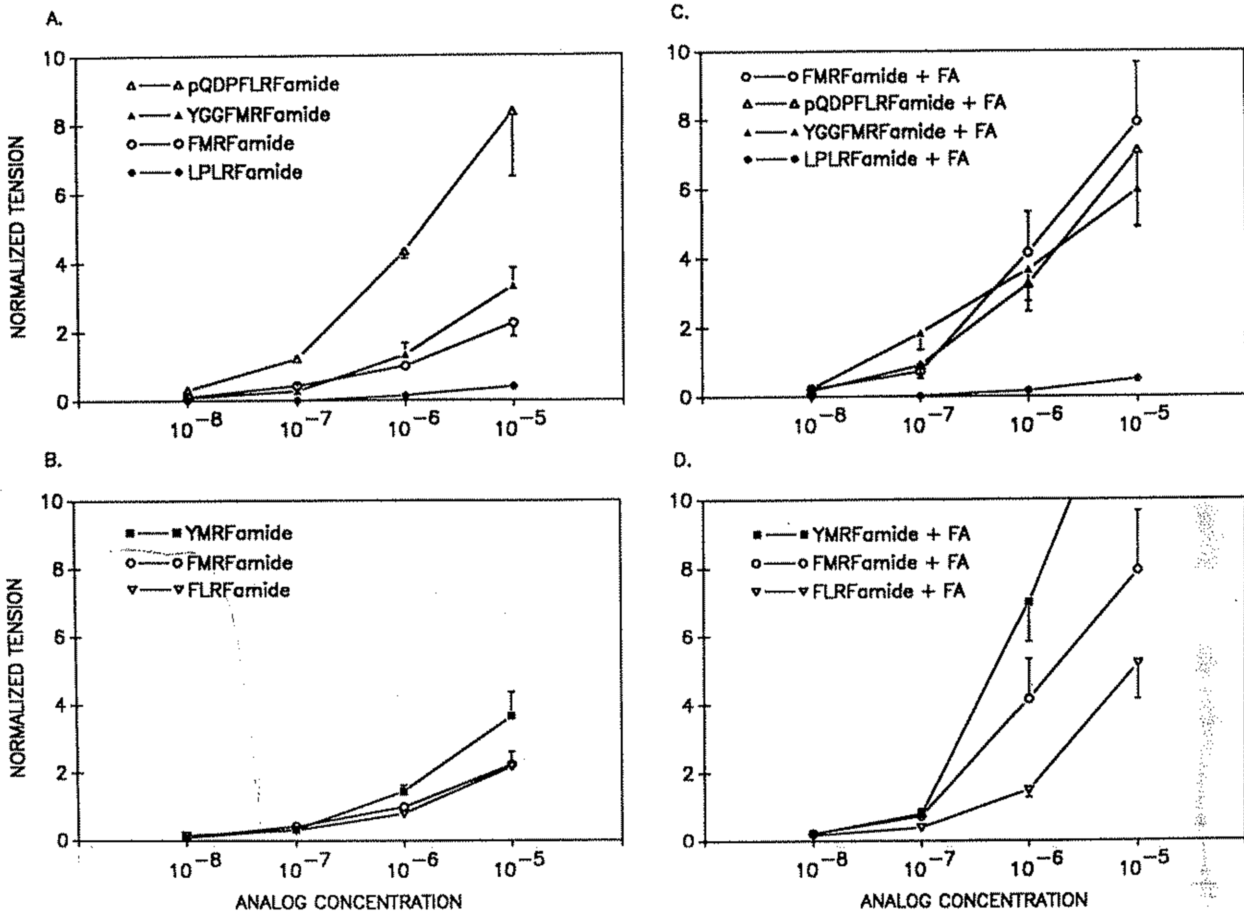


Fig. 3A–D. Dose response curves for FMRFamide and its agonists in the presence **C**, **D** or absence **A**, **B** of FA. All responses were normalized to the response to 10^{-6} mol l^{-1} FMRFamide to facilitate comparison. Each point is the average of 5 to 6 replications. Vertical lines: standard error. In panel **D**, one point on the YMRFamide+FA dose response curve is off scale (14.77 ± 2.91 at 10^{-5} mol l^{-1})

Table 2. Ability of FMRFamide analogs and protease inhibitors to affect the response of longitudinal muscle to FMRFamide (10^{-7} mol l^{-1})

FMdRFamide[a]	no effect
FMRdFamide[a]	20% reduction
RFamide[a]	no effect
LWMRFA[a]	50% potentiation
Proctolin[a]	no effect
FA (10 mg/ml)	400% potentiation
FP (10 mg/ml)	no effect
Trypsin type II-T (1.25 mg/ml)	no effect
Phenylmethylsulfonyl fluoride (1.5 mg/ml)	no effect

[a] These substances were applied as 10^{-6} mol l^{-1}, i.e. 10× the concentration of FMRFamide

3 out of 4 preparations, FMRdFamide at 10^{-6} mol l^{-1} caused no contraction by itself.

Effect of FMRFamide on muscle tension generated by activity in motor neurons

To determine the effect of FMRFamide on neurally evoked contraction of longitudinal muscle, the DP neuromuscular preparation was used. Either motor neuron 3 or L was fired by extracellular stimulation at 10 Hz for 2 s once every 10 min. After 3 or 4 such stimuli, used to determine baseline responsiveness, FMRFamide (10^{-6} mol l^{-1}) was superfused over the muscle, but not the ganglion, for 30 min and then rinsed off. Figure 5 shows three tension records taken, (1) before FMRFamide was superfused, (2) 15 min after the beginning of FMRFamide superfusion and (3) 30 min after FMRFamide was washed from the bath. In this example using cell 3, as in all the preparations (n=12), the most consistently noticeable effect of FMRFamide was the decrease in the rate of relaxation of the muscle after the stimulations. Results from 7 preparations in which cell 3 was stimulated are combined in Fig. 6A. Peak tension, rate of contraction, rate of relaxation, and integral tension (the area under the tension curve) are all normalized to the response just prior to the application of FMRFamide and plotted. The duration of FMRFamide application is indicated beneath by a solid bar. While peak tension and rise rate only increased slightly in the presence of FMRFamide (15%), the rate of relaxation decreased as much as 70% in one preparation and de-

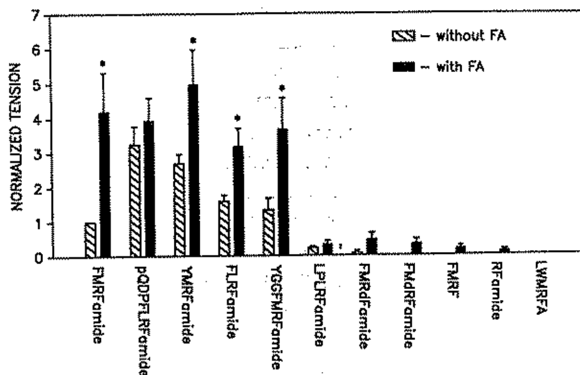


Fig. 4. Response of longitudinal muscle to FMRFamide and its analogs (10^{-6} mol l^{-1}) alone (slashed bars) and in the presence of FA (10 mg/ml) (solid bars). All responses are normalized to the response to 10^{-6} mol l^{-1} FMRFamide. Each bar is the average of 4 replications. Vertical lines: standard error. Asterisks: those peptides for which the response elicited in the presence of FA was significantly ($P<0.05$) greater than the response elicited in the absence of FA (standard t-test)

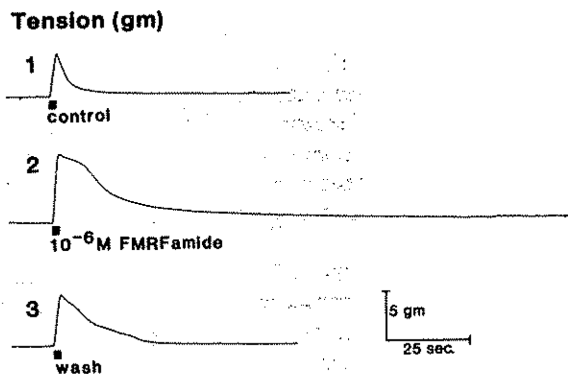


Fig. 5. FMRFamide decreases the relaxation rate of neurally evoked contractions in longitudinal muscle. Motor neuron 3 was stimulated extracellulary at 10 Hz for 2 s once every 10 min. After several stimulations, FMRFamide (10^{-6} mol l^{-1}) was superfused over the muscle for 30 min and then rinsed off. Tension records shown for stimulations before (1 – control), during (2 – 10^{-6} mol l^{-1}) FMRFamide and after (3 – wash) the muscle was exposed to FMRFamide. Solid bars: time of stimulation

creased an average of 40% across all preparations. This decrease in the rate of relaxation appears to be the major factor in the increase in the integral tension observed.

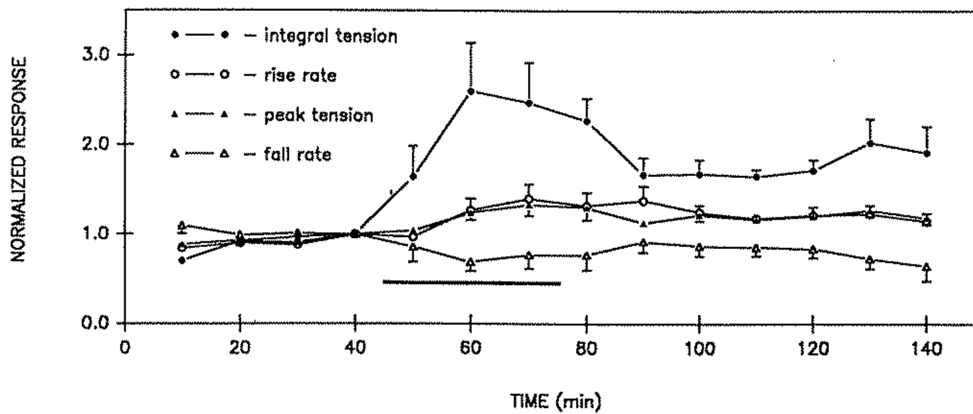
A control experiment was performed in which the stimulation regimen was repeated, but no FMRFamide was superfused. Responses from one control experiment, which correspond in time to the responses shown in Fig. 5, are shown in Fig. 7. Results from 4 control experiments in which FMRFamide was not applied are combined in Fig. 6B. During the time period from 55 to 85 min of the control experiment, which corresponds to the time when FMRFamide was superfused in the FMRFamide experiments, no change was seen in the response of longitudinal muscle to nerve stimulation. At about 100 min into the control experiment, however, there was a change in the longitudinal muscle response. This late developing neurally evoked change in the response resembles the effects seen when FMRFamide is superfused over the muscle. One possible explanation for this change is that it is evoked by nerve released FMRFamide. Inspection of Fig. 7A reveals a similar late developing change in the preparations where FMRFamide was applied. In Fig. 6C, each point from the control data (Fig. 6B) has been subtracted from the corresponding point of the experimental data (Fig. 6A) and one added to the difference, to isolate the effect of superfused FMRFamide in the absence of the late developing neural effect. Similar results were obtained when cell L was used in these experiments (data not shown).

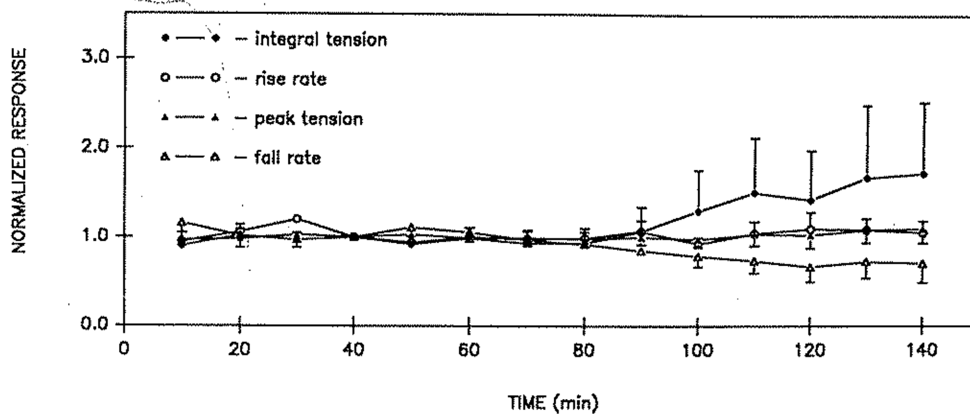
Given the ability of FA to enhance the response of longitudinal muscle to superfused FMRFamide, it seemed plausible that FA might have a noticeable effect on the contraction of longitudinal muscle in response to nerve stimulation, if neuromuscular transmission involved a peptidergic component. A motor neuron was stimulated for 1 s and then 20 s at 10 Hz by passing brief current pulses (<1 nA) through the recording microelectrode using DCC. The motor neuron was first stimulated with the muscle in normal saline to establish a control response. The muscle, but not the ganglion, was then superfused with saline containing FA (10 mg/ml) for 5 min and the neuron was again stimulated. The results from one such experiment using motor neuron L can be seen in Fig. 8. The response of the muscle to both short and long stimulations of the motor neuron was potentiated by FA; peak amplitude was increased and the rate of relaxation after a short stimulus was prolonged. As stated above, FA does not affect the response of longitudinal muscle to superfused ACh. This result reinforces the inference that there may be a component of neuromuscular transmission at longitudinal muscle of the leech which is noncholinergic and due possibly to a FMRFamide-like peptide.

In 5 DP neuromuscular preparations we attempted to block neurally evoked contractions in longitudinal muscle with 10^{-4} mol l^{-1} curare. In each case prolonged stimulation of the motor neuron caused a small but measurable contraction of the muscle (Fig. 9). Curare at this concentration blocks all longitudinal muscle contraction evoked by superfused ACh (Norris and Calabrese 1987). FA greatly prolonged the neurally evoked contraction in 10^{-4} mol l^{-1} curare in one such preparation tested (data not shown). These neurally evoked contractions observed in 10^{-4} mol l^{-1} curare could be due to our inability to block neuromuscular ACh receptors effectively or they may reflect a noncholinergic component of transmission.

Response of longitudinal muscle cells to FMRFamide

A further step in characterizing the response of longitudinal muscle to FMRFamide was to determine, by intracellular recording, the electrical response of muscle cells evoked by FMRFamide and to measure the underlying conductances change. Once the electrical response was determined, we attempted to uncover its ionic bases with ion substitution experiments.





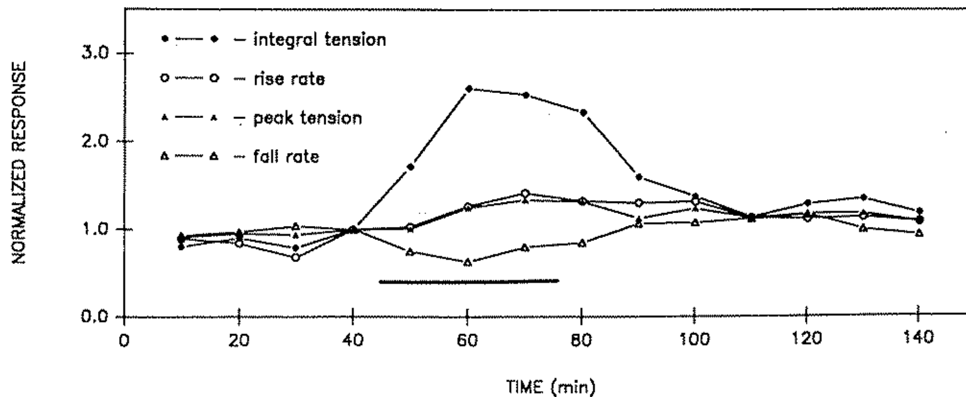


Fig. 6A–C. Effects of FMRFamide and repeated stimulation on the rate of contraction (rise rate), relaxation rate (fall rate), peak tension, and integral tension of neurally evoked contractions in longitudinal muscle. **A.** The experiment illustrated in Fig. 5 was repeated 7 times for cell 3 and the results combined. All responses normalized to the response immediately before FMRFamide was applied. Solid bar: duration of FMRFamide superfusion. **B.** Stimulation regimen of **A** was repeated 4 times without FMRFamide being superfused over the muscle (see Fig. 7). **C.** Each point of **B** was subtracted from the corresponding point of **A** and 1 was added to the difference, to isolate effects of superfused FMRFamide from long term stimulus effects

Response of intact longitudinal muscle cells to FMRFamide

In the first experiment, single cells within an intact patch of muscle were penetrated and recorded while FMRF-amide was superfused over the muscle. Because of the toughness of either the muscle's membrane or of the overlying extracellular matrix, it was necessary to use a stiff microelectrode to penetrate the cell. These electrodes made it difficult to hold the cell during contractions, therefore, most of the records from intact muscle cells were made in 20 mmol l^{-1} Ca^{++} saline, which seemed to stabilize recordings and suppressed contractions. Kuffler (1978) has reported a similar stabilizing effect of high Ca^{++} saline for leech longitudinal muscle.

Figure 10A shows an intracellular recording from an intact muscle cell made during an experiment in which FMRFamide was superfused over the muscle for a brief period. The muscle was constantly superfused with 20 mmol l^{-1} Ca^{++} saline and had a resting potential

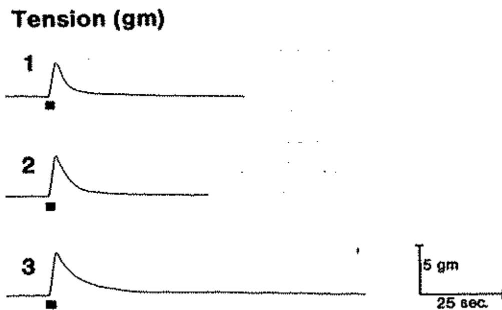


Fig. 7. Rate of relaxation of longitudinal muscle contraction in response to repeated stimulations of a motor neuron is slowly decreased with time. Motor neuron 3 was stimulated extracellularly at 10 Hz for 2 s once every 10 min as in Fig. 5, but no FMRFamide was superfused. The tension responses shown correspond in timing to responses shown in Fig. 5. Solid bars: time of stimulation

of −82 mV. 0.1 nA hyperpolarizing current pulses were injected through the recording electrode using DCC at 0.2 Hz to monitor input resistance. Initial input resistance averaged 60 MΩ in 4 such cells. As can be seen in Fig. 10A, superfused FMRFamide (10^{-6} mol l^{-1}) caused a depolarization and a concomitant decrease in input resistance. If this depolarization was sufficiently large, the muscle cells produced action potentials (data not shown). The depolarization occurred several seconds after FMRFamide reached the muscle, and the depolarization persisted for several seconds after FMRFamide was washed out. Following the depolarization and conductance increase, there was often a decrease in conductance, which persisted for several seconds after the superfusion of FMRFamide was terminated. Figure 10B shows a recording from an intact cell in normal saline. Due to the difficulty of penetrating cells and the movement of the cells upon penetration, it was only possible to record responses from 2 intact cells in normal saline. For both cells, there was a poor resting potential and input resistances were low (−29 mV and 2 MΩ for the cell in Fig. 10B). When FMRFamide was superfused over these two cells, there was a very slight depolarization (2–4 mV) and the accompanying conductance increase was small. However, the low input resistance of these cells appears to have emphasized the delayed conductance decrease evoked by FMRFamide.

Response of isolated longitudinal muscle cells to FMRFamide

Longitudinal muscle cells were enzymatically dissociated and kept in short term culture. Isolated cells were approximately 10–20 μm in diameter and 500–800 μm in length (Fig. 11). These cells were identified as muscle cells by their size, their form, the appearance of striations when the cells are viewed under Hoffmann interference contrast optics, their high resting potential and their ability to contract when depolarized with injected current. Other cells in our culture were round pigmented botryoidal cells.

The response of one isolated muscle cell to superfused FMRFamide (10^{-6} mol l^{-1}) in normal saline is shown in Fig. 12A. The response was similar in latency, amplitude and time course to that observed in intact muscle. When the same cell was placed in 20 mmol l^{-1} Ca^{++} saline and superfused with FMRFamide, the amplitude of depolarization was greater and the initial conductance increase was larger than in normal saline (Fig. 12B). If isolated muscle cells were left in 20 mmol l^{-1} Ca^{++} saline for an extended period of time (>20 min), the response to FMRFamide would become abbreviated and eventually vanish, even though there was no noticeable change in the muscle cell's resting potential or input resistance. The depolarization of an isolated muscle cell in normal saline in response to superfused FMRFamide often was large enough to elicit ac-

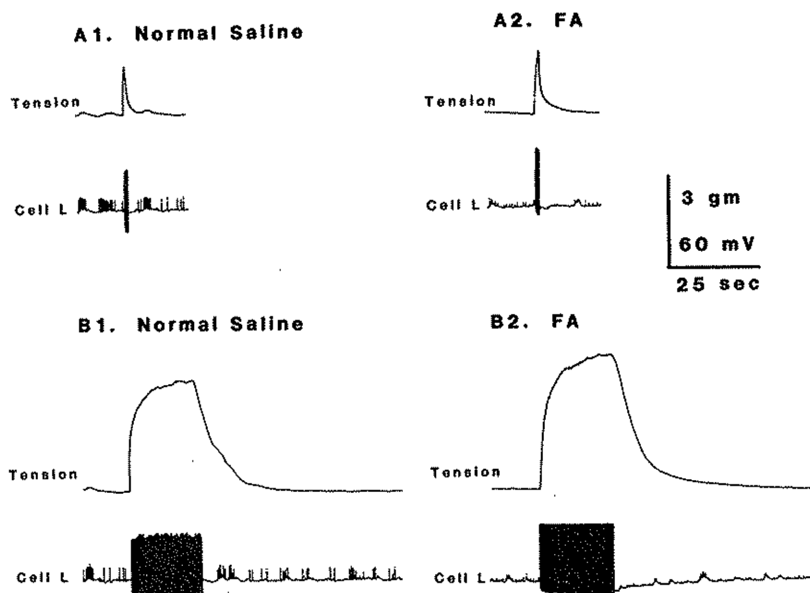


Fig. 8A, B. FA increases amplitude of short and long contractions and decreases relaxation rate of contractions in longitudinal muscle evoked by motor neuron stimulation. A dorsal patch of leech body wall was left innervated by a single ganglion. The ganglion was separated from the muscle by a vaseline dam and bathed in 5 mmol l^{-1} Mn^{++} saline to block central synapses. Cell L was stimulated intracellularly through the recording electrode using DCC at 10 Hz for 1 (**A1**) or 20 (**B1**) s and tension was recorded from the patch of longitudinal muscle. FA (10 mg/ml) was then applied to the muscle and cell, L was again stimulated (**A2, B2**)

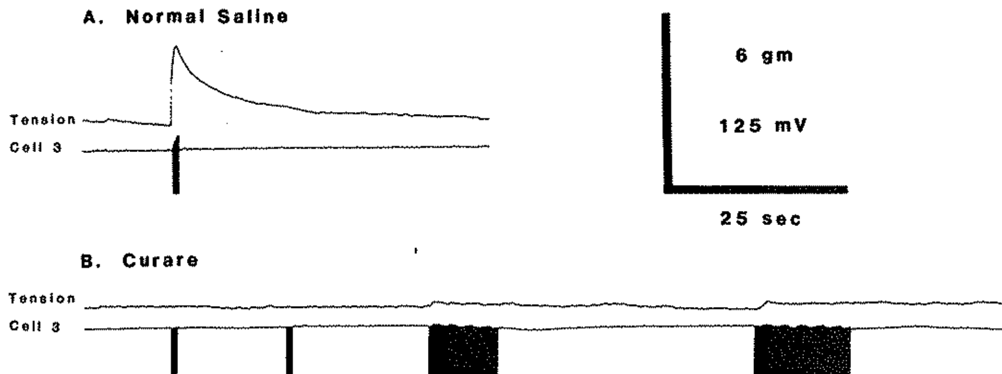


Fig. 9 A, B. Longitudinal motor neurons can still elicit contraction when cholinergic transmission is blocked with curare. A dorsal patch of leech body wall was left innervated by a single ganglion. The ganglion was separated from the muscle by a vaseline dam and bathed in 5 mmol l^{-1} Mn^{++} saline to block central synapses. Motor neuron 3 was stimulated intracellularly through the recording electrode using DCC at 10 Hz for 1 or 20 s and tension was recorded from the muscle patch. **A.** Response of body wall to short stimulations of cell 3. **B.** When the body wall was placed in 10^{-4} mol l^{-1} curare, it no longer responded to 1 s stimulation of cell 3 but responded weakly to longer stimulation

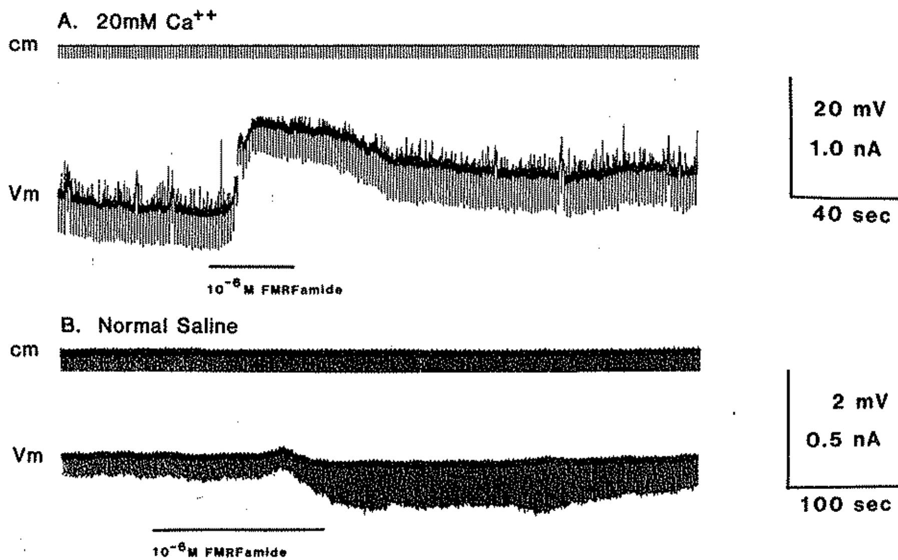


Fig. 10 A, B. Longitudinal muscle cells depolarize in the presence of FMRFamide. Single muscle cells in an intact muscle were recorded intracellularly and hyperpolarizing current pulses were injected through the recording electrode using DCC to measure input resistance. Muscles were superfused with either 20 mmol l^{-1} Ca^{++} **A** or normal saline **B**. Solid bars: duration of FMRFamide application. Spontaneous junctional potentials are seen in **A**. Vm – membrane potential of the muscle cell. cm – current monitor. [FMRFamide] = 10^{-6} mol l^{-1}

tion potentials and persisted as long as FMRFamide was superfused over the muscle (Fig. 13). Occasionally an isolated cell would respond to FMRFamide with a series of rhythmic depolarizations. One such cell is shown in Fig. 14. In the example shown, action potentials are seen on top of each wave of depolarization.

Extracellular Ca^{++} and the FMRFamide response

The more robust responses of isolated cells in 20 mmol l^{-1} Ca^{++} saline compared to those in normal saline suggests that Ca^{++} is involved in the FMRFamide response. The initial conductance increase that appears to underlie the depolarizing response to FMRFamide may be caused by the opening of Ca^{++} channels. To test this possibility, isolated cells were exposed to 10^{-6} mol l^{-1} FMRFamide in normal saline to record an initial response and then superfused with saline containing 5 mmol l^{-1} Mn^{++} (0.1 mmol l^{-1} Ca^{++}) to block Ca^{++} channels. After a time in 5 mmol l^{-1} Mn^{++} saline, the cells were superfused with 5 mmol l^{-1} Mn^{++} saline containing 10^{-6} mol l^{-1} FMRFamide. The cells were then rinsed with normal saline for at least 30 min and tested for recovery of the FMRFamide response. The records of one such experiment are shown in Fig. 15. Figure 15A shows the initial response of an isolated cell to FMRFamide. After a 20 min exposure to saline containing 5 mmol l^{-1} Mn^{++}, the cell no longer responded to FMRFamide (Fig. 15B). After the cell was superfused with normal saline for 30 min, the FMRFamide response recovered (Fig. 15C). Blockade of the FMRFamide response took 10 to 15 min to develop and at least 30 min to reverse. In a few preparations, when an isolated cell was exposed to FMRFamide in the presence of Mn^{++}, a hyperpolarization with an associated conductance increase was observed (data not shown).

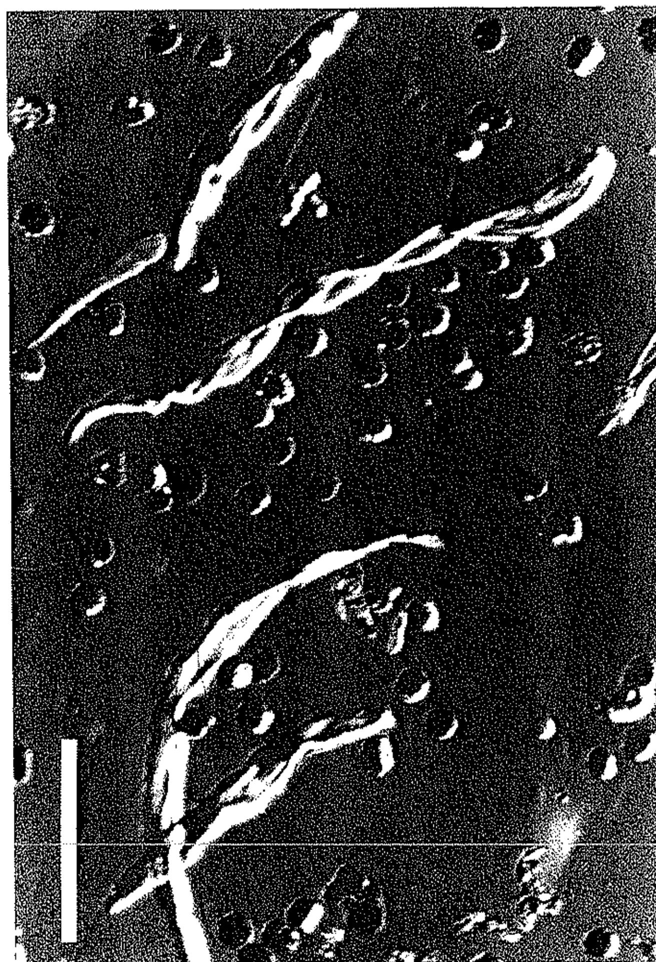


Fig. 11. Light micrograph of isolated longitudinal muscle cells photographed using Hoffmann interference optics. The small dark round objects are botryoidal cells that contaminate some cultures. Scale bar = 0.1 mm

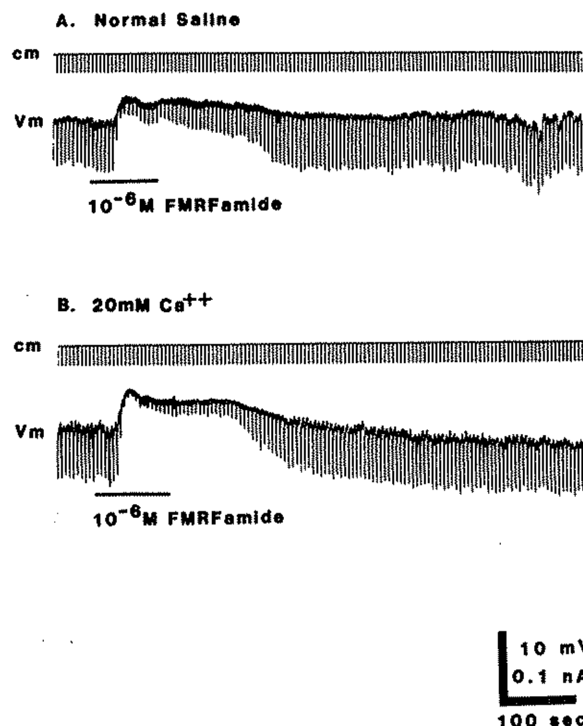


Fig. 12 A, B. Isolated longitudinal muscle cells depolarize in response to superfused FMRFamide. Isolated muscle cells were recorded intracellularly and hyperpolarizing current pulses were injected through the recording electrode using DCC to measure input resistance. Muscles were superfused with either normal saline A or 20 mmol l^{-1} Ca^{++} B. Solid bars: duration of FMRFamide application. Vm – membrane potential of the muscle cell. cm – current monitor. [FMRFamide] = 10^{-6} mol l^{-1}

Discussion

Structure-activity relations of FMRFamide analogs for longitudinal muscle

In the discussion of the structure-activity relation of FMRFamide analogs for longitudinal muscle YMRFamide, FLRFamide, and LPLRFamide can be classed as N-terminals analogs, FMRF-OH, FMRdFamide, FMdRFamide and LWMRFA as C-terminal analogs, pQDPFLRFamide and YGGFMRFamide as N-terminal extensions and RFamide as an N-terminal deletion.

All 3 N-terminal analogs were found to be FMRFamide agonists although LPLRFamide was less effective than the other two. The reduced effectiveness of LPLRFamide might be due to the Pro^2 residue, which puts a 'kink' in the peptide backbone.

The C-terminal analogs tested indicate the terminal amide is necessary for activity; FMRF-OH and LWMRFA have no agonist action. The lack of agonist action seen with application of FMdRFamide or FMRdFamide suggests the orientation of the amino acid side chains in the C-terminal portion of the peptide is also important for binding or activity.

Of the two N-terminal extension analogs, YGGFMRFamide elicits responses similar to those of FMRFamide, while pQDPFLRFamide was substantially more potent at all concentrations than FMRFamide. The increased potency of pQDPFLRFamide is especially interesting when compared to the normal potency of FLRFamide. The pQDP sequence may not serve to increase receptor binding or activation, but rather to decrease the rate of degradation or removal of the pQDPFLRFamide peptide (Price et al. 1985). Aminopeptidases capable of degrading neuropeptides may be present in the muscle tissue, and the pQ residue could serve to protect the peptide from degradation by these enzymes. The possibility still remains, however, that it is pQDPFLRFamide or a similar peptide, and not FMRFamide, that is the endogenous ligand of leech longitudinal muscle FMRFamide receptors.

The N-terminal deletion RFamide was without activity as it is in other preparations (e.g. Painter et al. 1982).

The similarities between the structure-activity relationships for RFamide peptide receptors in leech longitudinal muscle and those in molluscan muscle are striking. As was found previously in molluscs (Painter et al. 1982), the RFamide portion of the molecule seems to be crucial for activity. However, differences do exist in

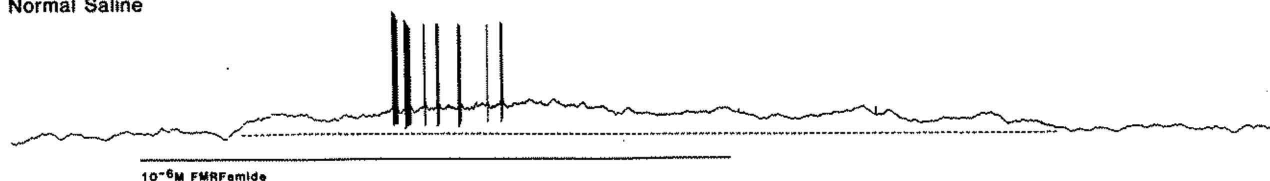


Fig. 13. An isolated muscle cell was recorded intracellularly, and superfused with 10^{-6} mol l^{-1} FMRFamide for 10 min. Resting potential = −68 mV. Solid bar: duration of FMRFamide application

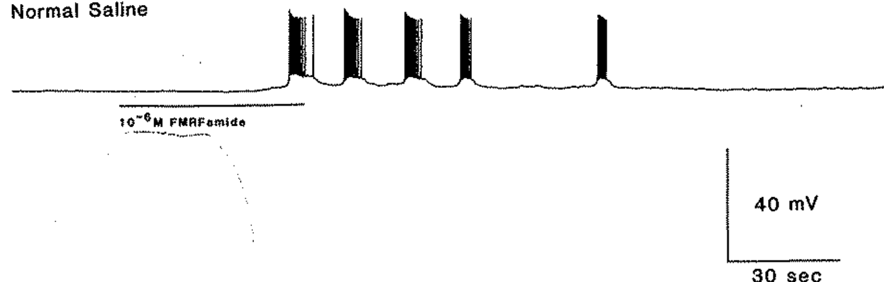
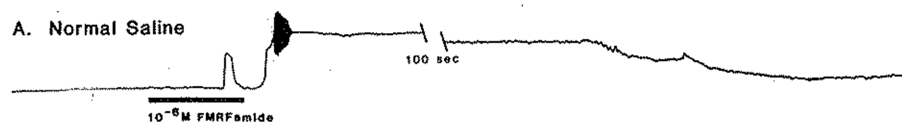
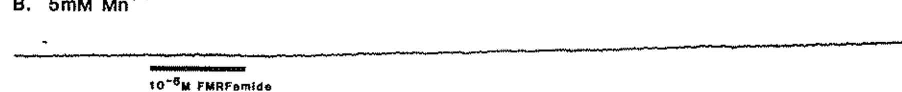


Fig. 14. Isolated longitudinal muscle cells occasionally displayed a polarization rhythm in response to superfused FMRFamide. Isolated muscle cells were recorded intracellularly and superfused with normal saline. Solid bar: duration of FMRFamide application. [FMRFamide] = 10^{-6} mol l^{-1}





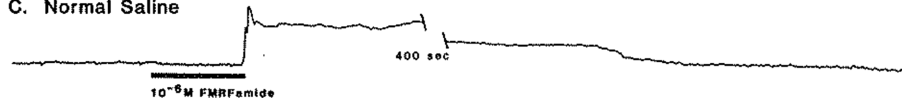


Fig. 15A–C. Extracellular Mn^{++} blocks the response of isolated longitudinal muscle cells to FMRFamide. Isolated muscle cells were recorded intracellularly and superfused with normal saline or 5 mmol l^{-1} Mn^{++} saline. **A.** Response of isolated muscle cell to superfused FMRFamide in normal saline. **B.** Response of isolated muscle cell to superfused FMRFamide is blocked after 20 min in 5 mmol l^{-1} Mn^{++} saline. **C.** The response of isolated muscle to superfused FMRFamide recovers after 60 min in normal saline. Recordings in **A** and **C** shortened by 100 and 400 s respectively. Solid bars: duration of FMRFamide application. [FMRFamide] = 10^{-6} mol l^{-1}

the action of pQDPFLRFamide and FLRFamide. Previous tests of pQDPFLRFamide have been conducted on preparations of the radula protractor muscle of *Busycon contrarium* (Price et al. 1985), the heart of *Helix aspersa* (Price et al. 1985) and the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* (Muneoka and Saitoh 1986). In *Mytilus*, pQDPFLRFamide produces no effect; in *Busycon* it is roughly equipotent to FMRFamide, while in *Helix* it is about one hundred times more potent than FMRFamide. FLRFamide has been tested in *Mytilus* ARBM, where it produced no effect (Muneoka and Saitoh 1986).

Structure-activity relations have also been explored for RFamide peptides on the extensor tibiae muscle of locusts. FMRFamide increases contraction amplitude and decreases the relaxation rate of neurally evoked twitches in this muscle (Walther et al. 1984; Evans and Meyers 1986). Here YGGFMRFamide and YGGFLRFamide (N-terminal extensions) are 100 times more potent than FMRFamide or FLRFamide, otherwise the structure activity profile is much like that for leech longitudinal muscle (Walther et al. 1984; Evans and Meyers 1986).

The differences in the structure-activity relations found support the hypothesis that there is more than one type of FMRFamide receptor across phyla, while the similarities suggest that these receptors may be related.

Several of the FMRFamide analogs which did not cause a contraction of longitudinal muscle were tested as FMRFamide antagonists. Only one analog, FMRdFamide had any antagonistic effect, and it only resulted in a 20% reduction of the response of longitudinal muscle to 10^{-7} mol l^{-1} FMRFamide when applied at 10^{-6} mol l^{-1}. Muneoka and Saitoh (1986) showed that the analogs FMdRFamide and FMRdFamide were antagonistic to FMRFamide on the anterior byssus retractor muscle (ABRM) of *Mytilus*.

Effect of putative protease inhibitors on bath applied FMRFamide and its analogs

Results from experiments where FMRFamide or its analogs were coapplied with the putative protease inhibitor (FA) provide evidence that proteases are present in the muscle tissue, and that the potency of pQDPFLRFamide is a result of its protection from proteolytic degradation. When FMRFamide is applied in the presence of the putative protease inhibitor FA, its potency is increased, whereas the potency of pQDPFLRFamide is not enhanced by FA. Moreover, the dose response curve for FMRFamide in the presence of FA is similar to the curve for pQDPFLRFamide. (The potency of LPLRFamide is also not enhanced by FA, but it is such a poor agonist that accurate estimates of potency are difficult with our experimental paradigm.)

Using this hypothesis, the mechanism for this protection can be evaluated in light of other putative inhibitors coapplied with FMRFamide. The ineffectiveness of trypsin II-T and phenylmethylsulfonlyfluoride suggests that the protease is neither trypsin-like nor has an essential serine residue. FA could be effective because any peptide at high concentrations would inhibit the protease. This possibility seems unlikely, however, since when FMRFamide was applied in the presence of bovine serum albumin (10 mg/ml) or another dipeptide (FP-10 mg/ml), no potentiation was observed. FA is reported to block mammalian enkephalinase which cleaves enkephalin (YGGFM) at the Gly^3-Phe^4 bond (Rocques et al. 1980; Schwartz et al. 1981; McKelvey and Blumberg 1986). Rocques et al. (1980) believe that enkephalinase is a dicarboxypeptidase, while McKelvey and Blumberg (1986) suggest it cleaves at the N side of Phe-x where x is a non-polar amino acid. While the leech body wall protease may not function by either of these mechanisms, it is likely to be an aminopeptidase, since the pGLu residue appears to protect pQDPFLRFamide from degradation and FA, which is effective for unprotected analogs, does not increase pQDPFLRFamide's potency. The effectiveness of FA as an inhibitor does suggest that the protease is a dipeptidase like enkephalinase, although there is no direct evidence on this point. In the future, it will be advantageous to test peptidase inhibitors such as thirophin and captropril, which inhibit enkephalinase and angiotensin converting enzyme (ACE), respectively, or bestatin and purmycin, which are aminopeptidase inhibitors with different specificities (Chou et al. 1984; Lentzen et al. 1984; McKelvey and Blumberg 1986; Roques et al. 1980).

Effect of FMRFamide on neurally evoked contraction of longitudinal muscle

The major effect of superfused FMRFamide on neurally evoked contractions is a decrease in the rate of muscle relaxation and therefore a concomitant increase in the integral tension produced during a contraction (total amount of tension generated over time). This effect was observed within 5 min of FMRFamide application and seemed to reach a peak within 15 min. The effect of FMRFamide washed out with a similar time course.

After roughly 100 min of stimulating motor neurons every 10 min, even in the absence of FMRFamide application, the evoked contraction begins to change in a manner that resembles the change caused by superfused FMRFamide. We speculate that this effect may be caused by accumulated RFamide peptide released from the motor neuron.

Possible non-cholinergic transmission by longitudinal motor neurons

If there is a noncholinergic component of neuromuscular transmission by longitudinal motor neurons due to a RFamide peptide, then FA might be expected to have a noticeable effect on neuromuscular transmission. FA increased the peak tension and decreased the rate of relaxation of neurally evoked contractions in longitudinal muscle. Moreover, we were unable to block all neurally evoked contractions with 10^{-4} mol l^{-1} curare. While these experiments are not conclusive, they do suggest the possibility of a RFamide peptide as a transmitter in longitudinal motor neurons of the medicinal leech.

Physiological effect of FMRFamide on longitudinal muscle

Although we have just begun to characterize the mechanism of FMRFamide's action on leech longitudinal muscle, two clear results have been obtained. First, FMRFamide produces a conductance increase in the muscle that results in a depolarization, and is usually followed by a conductance decrease. Second, the depolarizing response of longitudinal muscle to FMRFamide is dependent on extracellular Ca^{++}.

The observation that the response of isolated muscle cells to FMRFamide is blocked in salines where Ca^{++} is replaced with Mn^{++} suggests that the response to FMRFamide is triggered by an influx of Ca^{++}. Further, the depolarization and associated conductance increase observed in response to FMRFamide are both larger in cells bathed for a brief period in elevated calcium saline. This observation suggests that FMRFamide triggers a conductance increase for calcium and the resulting

Ca^{++} influx (Standen 1981) and/or depolarization causes other ion channels to open. If isolated cells are left for an extended period in elevated Ca^{++}, the FMRFamide response is eliminated, possibly due to internal Ca^{++} inactivating Ca^{++} channels (Kramer and Zucker 1985).

FMRFamide's postsynaptic effects on muscle have been studied in a number of mollusks. In general, FMRFamide causes a contraction, although it may be inhibitory or have mixed effects (Painter et al. 1974; Austin et al. 1982; Doble and Greenberg 1982; Painter and Greenberg 1982; Cottrell et al. 1983). In the ABRM of *Mytilus*, FMRFamide causes a slight depolarization, elicits a catch contracture and releases catch contracture elicited by ACh (Painter 1982a; Muneoka and Matsumura 1985; Muneoka and Saitoh 1986). Very little is known about the ionic mechanisms involved in FMRFamide's effect on molluscan muscles. In the ABRM of *Mytilus* and *Geukensis*, FMRFamide's action is dependent on extracellular Ca^{++}, and there is some evidence that FMRFamide causes release of Ca^{++} from internal stores and that this effect may be mediated by cGMP (Painter 1982a; Raffa and Bianchi 1986). In other molluscan muscles, FMRFamide has been shown to cause an increase in intracellular cAMP levels (Higgins et al. 1978; Painter 1982b; Weiss et al. 1984).

Conclusion

Our experiments involving isolated cells make it possible to correlate the effects of FMRFamide on muscle contraction with FMRFamide evoked potential changes. Superfused FMRFamide causes a depolarization in longitudinal muscle cells that lasts for the duration of peptide application. This depolarization probably causes the persistant contraction of longitudinal muscle observed in response to superfused FMRFamide (Norris and Calabrese 1987). Similarly, the polarization rhythm sometimes evoked by FMRFamide appears to underlie the rhythmic myogenic contractions occasionally evoked by FMRFamide (Norris and Calabrese 1987). The delayed conductance decrease caused by FMRFamide could account for the FMRFamide induced decrease in the rate of muscle relaxation of neurally evoked contractions observed here.

It is also possible that FMRFamide acts presynaptically or directly on excitation-contraction coupling to exert its effects on neurally evoked contraction.

The question still remains, however, as to why FMRFamide is present in neurons that also use ACh as a transmitter. Two possible answers suggest themselves, first, continuous release of small amounts of FMRFamide, brought about by slow, tonic firing of the motor neurons, could set and maintain a steady contraction. Second, extended release of FMRFamide, during extended periods of intense motor activity such as during prolonged swimming, could potentiate the response of muscle to motor activity to offset desensitization of ACh receptors on the muscle or depletion of the ACh transmitter pool in motor neurons.

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