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THE MODULATORY ACTIONS OF FMRFamide AND RELATED PEPTIDES ON LOCUST SKELETAL MUSCLE

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SUMMARY

1. The modulatory actions of FMRFamide and related peptides on tension generated in the extensor-tibiae muscle of the locust hindleg by stimulation of the slow excitatory motor neurone (SETi) depend upon the frequency of stimulation of SETi. They have no effect on the tension induced by the fast motor neurone (FETi) or upon the myogenic rhythm present in this muscle.

2. At low frequencies of SETi stimulation (1 Hz and below) the predominant modulatory effects are increases in the amplitude, contraction rates and relaxation rates of twitch tension. At higher frequencies, where twitches summate but tetanus is incomplete (up to 20 Hz) these effects are superimposed upon an increase of maintained tension.

3. FMRFamide increases the amplitude and relaxation rate of slow twitch tension by different amounts in different regions of the extensor muscle. It is likely that the effects of FMRFamide are restricted to slow muscle fibres that are innervated by SETi but not FETi.

4. The modulatory actions of FMRFamide on SETi-induced tension are additive to, but do not potentiate, the modulatory actions of octopamine and proctolin in this muscle. The actions of FMRFamide show some similarities with the modulatory actions of octopamine in this preparation but they are mediated by an independent receptor system that does not change cyclic nucleotide levels. Other actions of FMRFamide are similar to the actions of proctolin.

INTRODUCTION

Immunocytochemical evidence has recently been presented for the presence of peptides related to the neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) in specific subsets of neurones within the nervous systems of insects including the Colorado potato beetle (Veenstra & Schooneveld, 1984), the locust (Myers & Evans, 1985*a*,*b*) and the cockroach (Verhaert, Grimmelikhuijzen & DeLoof, 1985). FMRFamide was originally isolated and identified from the nervous system of the clam *Macrocallista nimbosa* (Price & Greenberg, 1977). Since that time FMRFamide,

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and a family of structurally related peptides, have been shown to be present in nervous tissue from a wide range of invertebrate species, including the snail *Helix* (Price *et al.* 1985) and the horseshoe crab *Limulus* (Watson *et al.* 1984). Structurally related peptides have also been reported to be present in vertebrate nervous tissue (Dockray & Williams, 1983; Williams & Dockray, 1983; Dockray *et al.* 1983; O'Donohue *et al.* 1984).

In molluscs, FMRFamide is cardioexcitatory (see Price & Greenberg, 1980). In addition, it produces a variety of effects upon a range of non-cardiac muscles. For instance, it induces sustained contractions in molluscan smooth muscles such as the radula protractor muscle of the whelk *Buscycon contrarium* and the anterior byssus retractor muscle (ABRM) of the mussel *Mytilus edulis* (Price & Greenberg, 1977; Greenberg & Price, 1979; Painter, 1982). Further, in the tentacle retractor muscle of *Helix aspersa*, it produces a prolonged contracture on which phasic, rhythmical contractions may be superimposed (Cottrell, Greenberg & Price, 1983*a*; Cottrell, Schot & Dockray, 1983*b*). FMRFamide also has a variety of actions on gastropod neurones (Cottrell, 1983; Murphy, Lukowiak & Stell, 1985).

In the locust, the observations that FMRFamide-like immunoreactive neurones could be traced to identified skeletal muscles, and also that the immunoreactivity appeared concentrated in neurohaemal areas (Myers & Evans, 1985*a*,*b*) suggested that locust skeletal muscle would be an interesting target site in which to investigate the effects of FMRFamide and related peptides. Although we do not yet know the precise structures of the immunoreactive FMRFamide-like molecules in the locust nervous system, studies on the pharmacological profile of insect skeletal muscle responses will provide much useful information upon the nature and mode of action of the receptors mediating these effects. In addition such information will be useful in the development of appropriate bioassays for use during the isolation and purification of such peptides from the locust nervous system. The extensor-tibiae muscle of the hindleg of the locust was chosen for investigation since it is one of the most intensively studied insect skeletal muscles with a well-defined neuronal input from a limited number of neurones, all of which are physiologically identifiable (for reviews see Hoyle, 1983; Evans, 1985*a*; Evans & Myers, 1986). The present paper confirms and extends the results presented in a short communication (Walther, Schiebe & Voigt, 1984) published during the course of this work. It describes the actions of FMRFamide-like peptides on twitch, tetanic and maintained tension in this muscle and emphasizes the dependence of the effects upon the frequency of motor neurone stimulation. Regional differences in the responsiveness of the extensor-tibiae muscle to FMRFamide are described, and structure–activity studies give additional information on the structural requirements for a bioactive FMRFamide-like peptide in the locust.

MATERIALS AND METHODS

Adult locusts (*Schistocerca gregaria*) of either sex were obtained from crowded laboratory colonies fed on wheat seedlings. Small batches of animals were left for

1–2 h before use, after removal from the main culture, to minimize any initial potentiation effects due to elevated levels of octopamine in the haemolymph (see Evans, 1981; Davenport & Evans, 1984*a*,*b*).

Tension in the intact muscle and in the various regions of the extensor-tibiae muscle of the locust hindleg was measured almost isometrically with a tension transducer attached distally to the apodeme. Muscle blocks were isolated for tension recording by cutting the central apodeme on the proximal side of the bundle from which recordings were to be made, and by cutting the extensor nerve on the distal side of the block (Evans, 1985*b*). The slow extensor-tibiae (SETi) and the fast extensor-tibiae (FETi) motor neurones were excited by stimulating nerves 3b and 5, respectively, with a pair of silver hook electrodes. Patterned pulse trains were computer-generated and used to trigger the output of a stimulator (Evans & Siegler, 1982). An operational amplifier signal differentiator was used to measure continuously the rates of contraction and relaxation of neurally evoked tension in the different muscle regions (Buchan & Evans, 1980). Miniature end-plate potentials were recorded intracellularly from extensor-tibiae muscle fibres innervated by SETi, but not FETi, using microelectrodes, filled with 2 mol l^{-1} potassium acetate, which had d.c. resistances in saline of 15–30 MΩ.

Extensor-tibiae muscles were prepared for cyclic AMP measurements by incubation as described previously (Evans, 1984*a*). At the end of the incubation period, muscles were rapidly frozen, dissected away from their surrounding cuticle and divided up into appropriate regions for analysis. The muscle samples were homogenized in an ice-cold concentrated hydrochloric acid:absolute ethanol mixture (1:60 v/v) (Horn & McAfee, 1977). The cyclic AMP levels were assayed in the muscle extracts by a protein binding method (Brown, Ekins & Albano, 1972) using a commercial cyclic AMP assay kit (Amersham). Protein determinations were carried out according to Lowry, Roseburg, Farr & Randall (1951) using bovine serum albumin as standard.

FMRFamide and related peptides were superfused directly onto the surface of the muscle dissolved in a physiologically isotonic saline (pH 6·8) containing (in mmol l^{-1}) NaCl, 140; KCl, 10; $CaCl_2$, 4; $NaHCO_3$, 4; NaH_2PO_4, 6; and sucrose, 90. YGGFMRFamide, FMRFamide, LPLRFamide and RFamide were obtained from Peninsula Laboratories. All other peptides and drugs were obtained from Sigma except for phentolamine mesylate which was a gift from Ciba.

RESULTS

The actions of FMRFamide and related peptides on twitch tension

The contractions evoked by stimulation of the slow motor neurone (SETi) to the extensor-tibiae muscle of the locust hindleg are potentiated in amplitude by the presence of FMRFamide-related peptides in the muscle superfusate (Walther *et al.* 1984). The present study confirms this finding and extends it to show that the peptides of this family also increase the rates of both contraction and relaxation of slow motor neurone-evoked twitch tension in this muscle. Fig. 1 shows that a 30-s

pulse of 10^{-6} mol l^{-1} FMRFamide increases the amplitude, and rates of contraction and relaxation, of twitch tension when SETi is stimulated at 1 Hz. These effects outlast the presence of the peptide in the superfusate by several minutes. In the presence of a prolonged pulse of 10^{-6} mol l^{-1} FMRFamide, maximal effects on each of these parameters are reached after an exposure of 4–5 min (Fig. 1). FMRFamide, at concentrations up to 10^{-6} mol l^{-1}, produces no observable effect on twitch tension generated by stimulating the fast motor neurone (FETi) to this muscle at 1 Hz. In addition, it has no observable effect on the amplitude or frequency of the myogenic rhythm of contraction and relaxation observed in this muscle.

The FMRFamide-related molluscan heptapeptide, YGGFMRFamide, which was identified in the octopus (see Greenberg, Price & Lehman, 1985) also produces the same qualitative effects as FMRFamide (Fig. 2A,B). However, a prolonged pulse of YGGFMRFamide at 10^{-6} mol l^{-1} produces maximal effects in a much shorter time (2–3 min) than an equivalent pulse of FMRFamide. The effects of both FMRFamide and YGGFMRFamide are dose-dependent (Fig. 3). FMRFamide is almost an order of magnitude more potent than YGGFMRFamide, but both substances have the same maximal effects on the amplitude of slow twitch tension, its rate of relaxation (Fig. 3) and its rate of contraction (not shown). The threshold for an observable action of YGGFMRFamide on slow motor neurone-evoked tension occurs between 10^{-9} and 10^{-10} mol l^{-1}.

FMRFamide-related peptides have been reported to produce their effects on slow motor neurone-induced twitch tension by both presynaptic and postsynaptic effects (Walther *et al.* 1984). In the latter study, however, the presynaptic actions were only inferred from the fact that FMRFamide changed excitatory junctional potential

Fig. 1. The effect of various length pulses of 10^{-6} mol l^{-1} FMRFamide (black bars) on SETi-induced twitch tension in the extensor-tibiae muscle. SETi was stimulated at a frequency of 1 Hz. (A) The response of twitch tension; (B,C) the effects on contraction and relaxation rates, respectively.

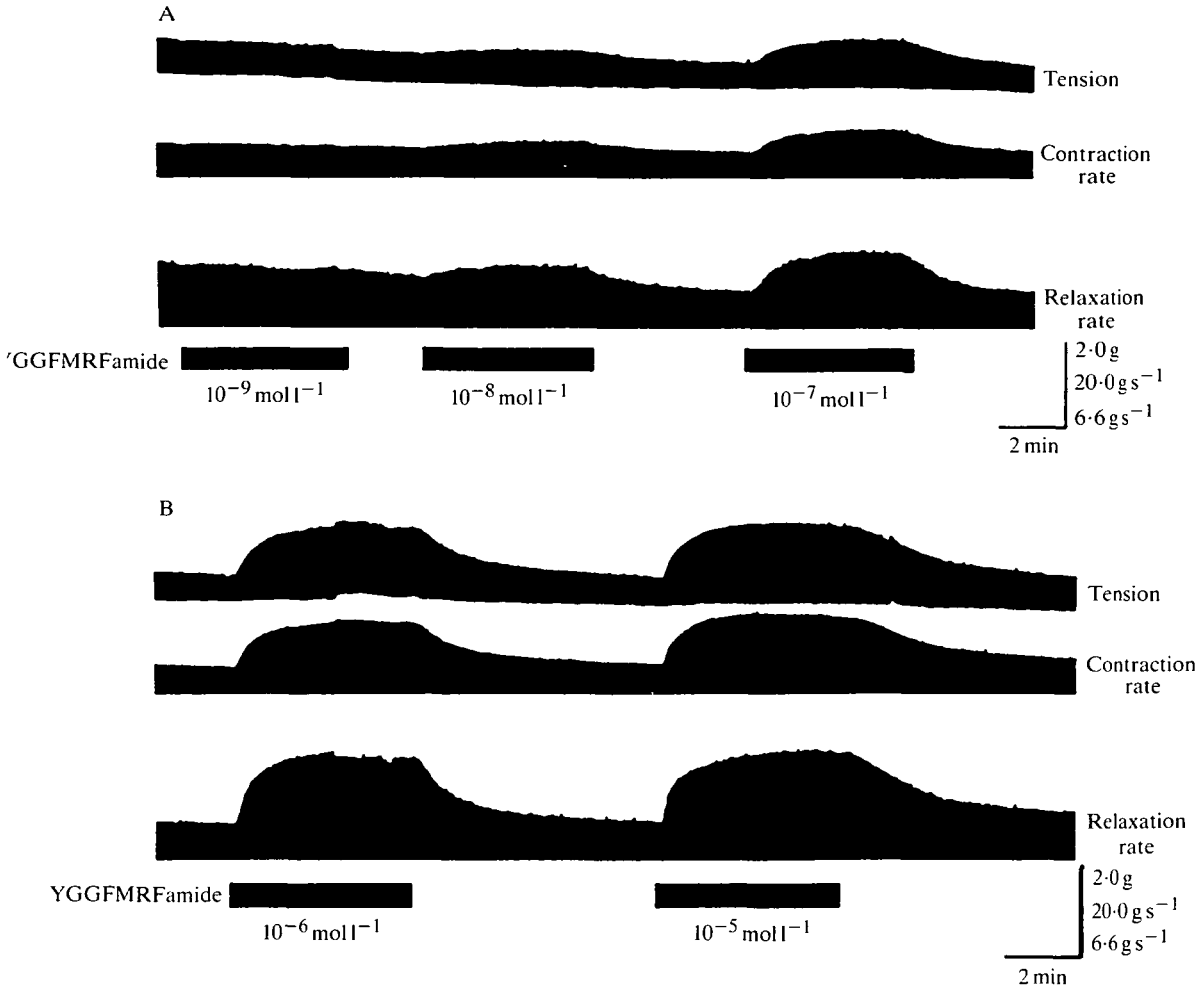

Fig. 2. The effect of 5-min pulses of YGGFMRFamide at various concentrations on SETi-induced twitch tension in the extensor-tibiae muscle. SETi was stimulated at a frequency of 1 Hz. (A) Effects of pulses of 10^{-9}, 10^{-8} and 10^{-7} mol l^{-1}; (B) effects of pulses of 10^{-6} and 10^{-5} mol l^{-1}.

amplitudes but not the time integrals of depolarizations caused by glutamate, the presumed rapid-acting transmitter of SETi. In the present study a direct measurement of the presynaptic action of FMRFamide has been obtained by studying its effects on the spontaneous release of transmitter from the slow motor neurone. Fig. 4 shows that a 3-min pulse of 10^{-6} mol l^{-1} FMRFamide increases the spontaneous frequency of miniature end-plate potentials (Fig. 4A) but does not change their amplitude distribution (Fig. 4B) in a muscle fibre innervated by SETi but not FETi. This provides direct evidence for a presynaptic action of FMRFamide on the terminals of the slow motor neurone. However, FMRFamide must also have postsynaptic actions in this preparation since it is capable of modulating the rate of relaxation of twitch tension.

Specificity of responses

The potentiating actions of FMRFamide show some similarities with the actions of the biogenic amine, octopamine, on slow motor neurone twitch tension in the locust

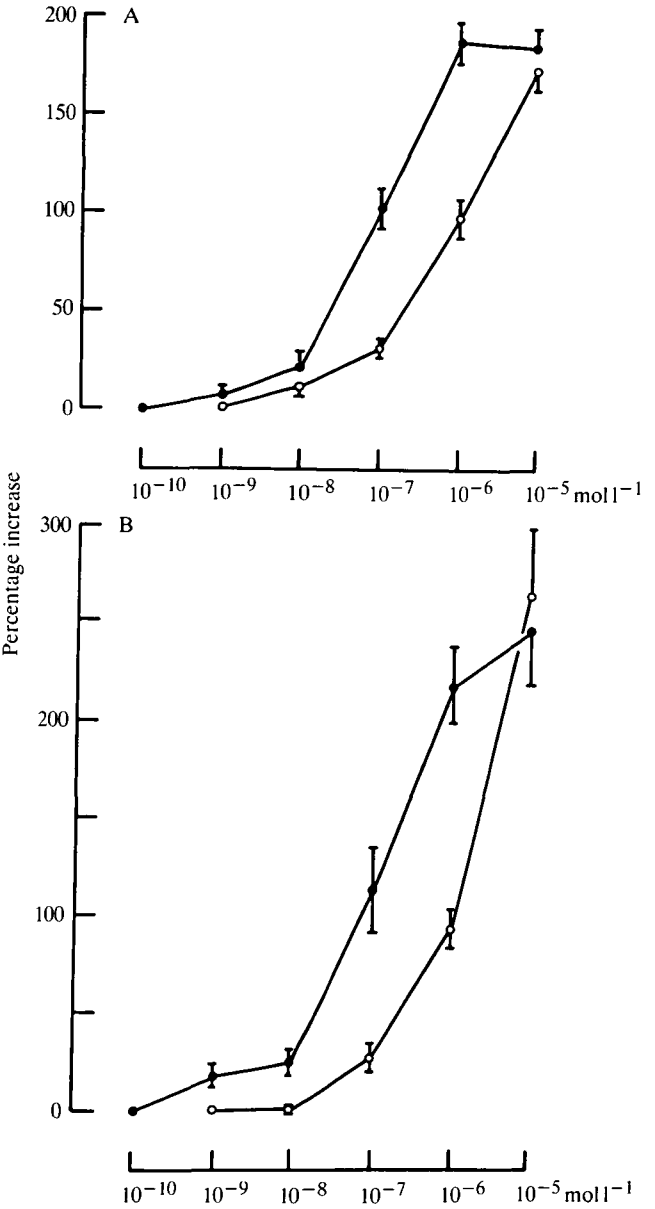

Fig. 3. Dose–response curves for the actions of FMRFamide (○) and YGGFMRFamide (●) on SETi-induced twitch tension. (A) Maximal effects on twitch amplitude; (B) maximal effects on the relaxation rate of twitch tension. SETi was fired at a frequency of 1 Hz and each of the peptides was introduced into the superfusate for a period of 5 min. Each point represents the mean of at least four determinations and the bars represent standard errors.

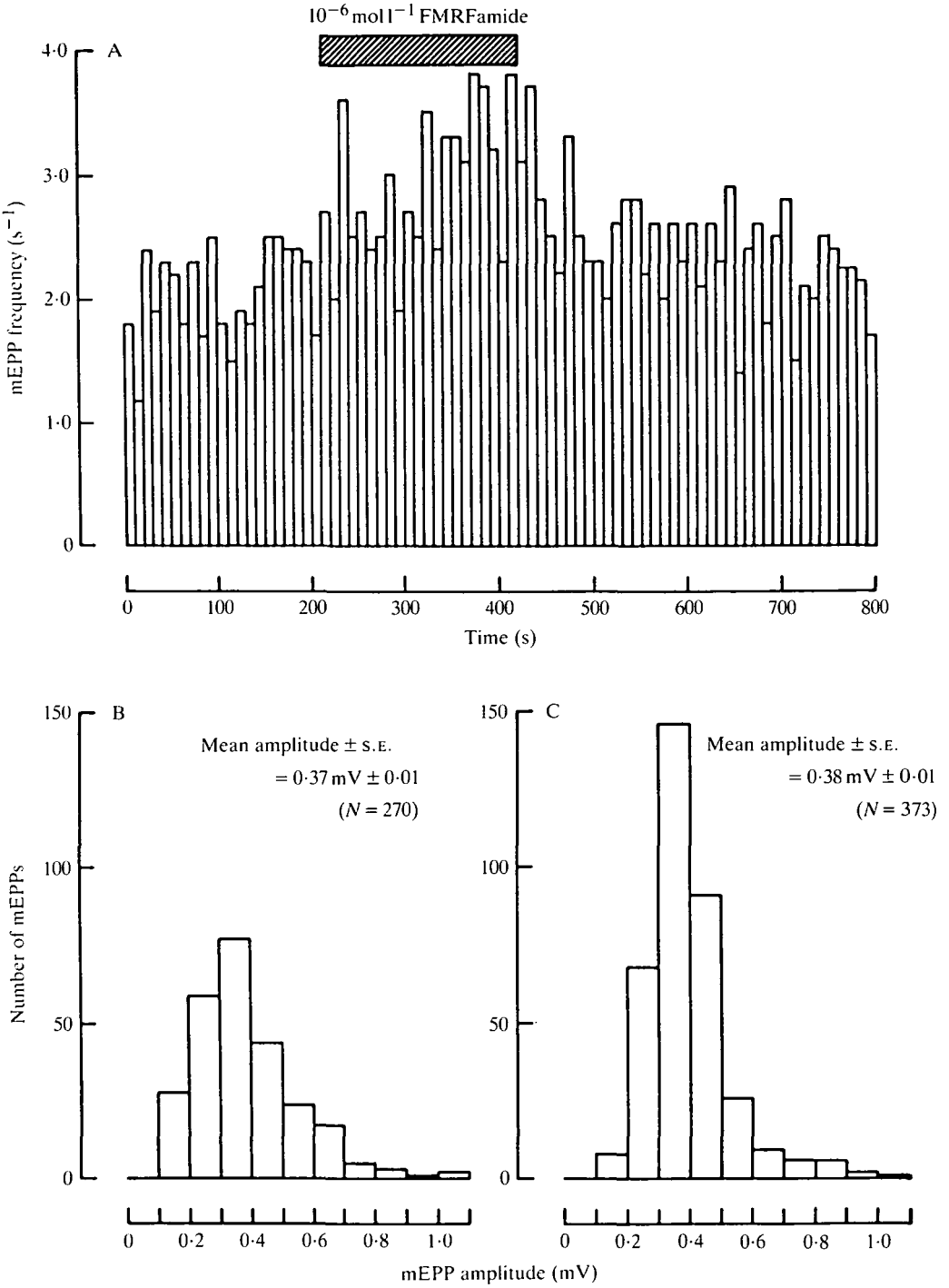

Fig. 4. Effect of 10^{-6} mol l^{-1} FMRFamide (hatched bar) on the spontaneous release of neurotransmitter from the terminals of the SETi motor neurone to a slow distal fibre of the extensor-tibiae muscle. (A) A plot of the frequency of miniature end-plate potentials (mEPPs) (mean frequency per second of 10 consecutive seconds) against time. (B,C) Amplitude histograms of mEPPs for periods of 3 min before and during the FMRFamide pulse, respectively.

extensor-tibiae muscle (Evans & O'Shea, 1977; O'Shea & Evans, 1979; Evans, 1981). Therefore, the muscle's response to FMRFamide was examined in the presence of 10^{-5} mol l^{-1} phentolamine, an effective blocking agent of octopamine receptors in this preparation (Evans, 1981). The responses to a 5-min pulse of 10^{-6} mol l^{-1} FMRFamide were not reduced in the presence of 10^{-5} mol l^{-1} phentolamine (not shown) indicating that they are mediated *via* a receptor system which is independent of the octopaminergic system in this preparation. Further evidence for this conclusion is provided by the observation that the responses to submaximal doses of FMRFamide (e.g. 10^{-7} mol l^{-1}), unlike those to submaximal doses of octopamine (see Evans, 1984*a*,*b*), are not potentiated in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) at a concentration of 10^{-7} mol l^{-1} (not shown). This suggests that FMRFamide and related peptides do not bring about their effects in this preparation *via* a mechanism that increases cyclic nucleotide levels. This suggestion is confirmed by direct measurements of cyclic AMP levels in the extensor-tibiae muscle which do not increase after 10-min exposures to FMRFamide in the presence of 10^{-4} mol l^{-1} IBMX at concentrations of FMRFamide between 10^{-11} to 10^{-4} mol l^{-1} (data not shown).

A preliminary screening of the actions of FMRFamide-related peptides on slow motor neurone twitch tension in the locust extensor muscle revealed that besides FMRFamide and YGGFMRFamide, the analogous peptides in which methionine was substituted by leucine, i.e. FLRFamide and YGGFLRFamide, were just as potent (Walther *et al.* 1984). The latter study concluded that the C-terminal sequence -Arg-Phe-NH_2 is essential for activity in the locust and that the preceding neutral amino acid (Met or Leu) may also be important, because γ_1-MSH, where the neutral amino acid is replaced by aspartate, is inactive. The results of specificity studies performed in the present study (see Table 1) confirm and extend these observations. The amidated dipeptide Arg-Phe-NH_2 is inactive at concentrations up to 10^{-6} mol l^{-1} and does not block the actions of FMRFamide. In addition, the

Table 1. *The specificity of action of FMRFamide-related peptides on the locust extensor-tibiae muscle*

Peptide	Increase in twitch amplitude (%)	(ED_{50}, mol l^{-1})	Increase in relaxation rate (%)	(ED_{50}, mol l^{-1})	*N*
YGGFMRFamide	185·7 ± 7·9	$8{\cdot}5\times10^{-8}$	218·2 ± 19·7	$1{\cdot}5\times10^{-7}$	3
FMRFamide	96·9 ± 7·2	$9{\cdot}7\times10^{-7}$	97·8 ± 14·2	$2{\cdot}0\times10^{-6}$	9
LPLRFamide	9·0 ± 2·3	5×10^{-5}	11·8 ± 4·2	$4{\cdot}0\times10^{-5}$	3
RFamide	0	—	0	—	3
BPP	0	—	0	—	3
APP	0	—	0	—	3
HPP	0	—	0	—	3
[Met]enkephalin	0	—	0	—	3

The peptides were tested as 5-min pulses at a concentration of 10^{-6} mol l^{-1}. Each result is expressed as the mean ± standard error of the number of observations shown (*N*). ED_{50} values were calculated from dose–response curves. The slow motor neurone was stimulated at 1 Hz.

BPP, APP and HPP = bovine, avian and human pancreatic polypeptides, respectively.

Table 2. *FMRFamide and octopamine potentiation of twitch tension in different regions of the extensor-tibiae muscle*

Muscle regions	FMRFamide		Octopamine	
	Increase in amplitude (%)	Increase in relaxation rate (%)	Increase in amplitude (%)	Increase in relaxation rate (%)
a	63·6 ± 8·0	43·0 ± 7·3	32·7	65·3
b	—	—	—	—
c	—	—	—	—
d	—	—	—	—
e	88·2 ± 9·7	25·6 ± 4·1	32·2	115·8
f	105·2 ± 14·1	70·9 ± 9·9	29·1	146·1
135*c*,*d*	—	—	—	—

The values indicate the mean maximal responses ± standard error of the mean ($N = 3$) to a 5-min pulse of 10^{-6} mol l^{-1} FMRFamide introduced into the superfusate. The slow motor neurone was stimulated at 1 Hz.

Regions *b* and *c* are not innervated by the slow motor neurone. The twitches in regions *d* and 135*c*,*d*, induced by the slow extensor-tibiae motor neurone, are very small and the effects of FMRFamide were not quantifiable.

The octopamine data is taken from Evans (1985*b*).

related peptide Leu-Pro-Leu-Arg-Phe-NH_2 (LPLRFamide in single letter notation) is almost an order of magnitude less potent than FMRFamide. This suggests that the presence of the phenylalanine residue at the beginning of the FMRFamide sequence and at position 4 of YGGFMRFamide may also be important for the action of these peptides on locust muscle.

Recent immunocytochemical studies have revealed the presence in the locust nervous system of cells immunoreactive to an antiserum raised against bovine pancreatic polypeptide (BPP), some of which are also positive for an antiserum raised against FMRFamide (Myers & Evans, 1985*a*,*b*). Thus the effect of a variety of pancreatic polypeptides was tested on slow motor neurone-induced twitch tension (Table 1). At concentrations up to 10^{-6} mol l^{-1}, however, 5-min pulses of bovine, human and avian pancreatic polypeptide had no effect on twitch tension produced by stimulating SETi at 1 Hz. In addition, they did not block the actions of FMRFamide on this preparation.

Regional variation in extensor muscle responses

The responses of the extensor-tibiae muscle to octopamine vary in different parts of the muscle in relation to the proportions of slow and intermediate muscle fibres present (Evans, 1985*b*). To investigate the effects of FMRFamide on different regions of this muscle, SETi-induced twitch tension was recorded from seven blocks of muscle fibres designated blocks *a*–*f* and 135*c*,*d* in a proximal to distal direction according to the nomenclature of Hoyle (1978) (Table 2). A 5-min pulse of 10^{-6} mol l^{-1} FMRFamide produced increases in slow twitch tension amplitude which varied from 63·6 % in region *a* to 105·2 % in region *f*. The relaxation rate of slow twitch tension also increased by different amounts in each of the regions of the

muscle innervated by SETi. Stimulation of SETi at 1 Hz also produced tension responses from blocks *d* and 135*c*,*d*, but these responses were small and the changes due to FMRFamide were difficult to quantify. Table 2 also includes data for regional variations in responsiveness to octopamine from Evans (1985*b*) for comparison.

Interactions with other modulators of locust extensor-tibiae muscle

The extensor-tibiae muscle of the locust is modulated by a range of other neuroeffectors including octopamine and the neuropeptide proctolin (Evans & O'Shea, 1977, 1978; May, Brown & Clements, 1979), so the ability of these substances to interact with the modulatory effects of FMRFamide-like peptides was assessed on this muscle. Fig. 5 shows that low doses of DL-octopamine (10^{-8} mol l^{-1}), which by themselves produce very little effect on the amplitude of slow twitch tension and its rate of contraction, do not block or potentiate the actions of a 5-min pulse of 10^{-7} mol l^{-1} FMRFamide. The effects of higher concentrations of DL-octopamine on these two parameters are additive to those of FMRFamide. At 10^{-8} mol l^{-1}, DL-octopamine causes an increase in the rate of relaxation of slow twitch tension to which the effect of the 5-min pulse of 10^{-7} mol l^{-1} FMRFamide is additive.

Proctolin, at a concentration of 10^{-9} mol l^{-1}, has a variety of effects on the extensor-tibiae muscle including an increase in the frequency of the myogenic rhythm and an increase in basal tension (May *et al.* 1979; Evans, 1982, 1984*c*). In addition, in some preparations it is possible to demonstrate an increase in the amplitude of slow motor neurone-evoked tension, together with increases in its

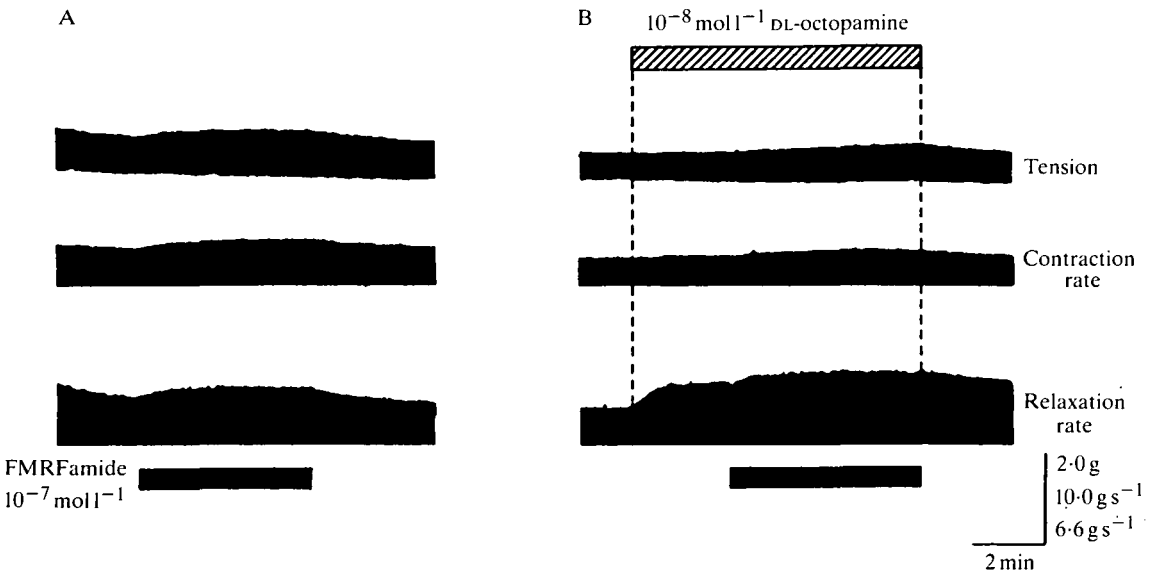

Fig. 5. The interaction of the modulatory effects of 10^{-8} mol l^{-1} DL-octopamine and 10^{-7} mol l^{-1} FMRFamide on SETi-induced twitch tension. (A) The small effects of a 5-min pulse of FMRFamide (black bar) given alone. (B) The additive effects of a 5-min pulse of FMRFamide (black bar) given in the presence of 10^{-8} mol l^{-1} DL-octopamine (hatched bar).

relaxation and contraction rates induced by proctolin. However, if a 5-min pulse of 10^{-6} mol l^{-1} FMRFamide is introduced into the muscle superfusate in the presence of 10^{-9} mol l^{-1} proctolin, the effects of FMRFamide are not blocked and are additive to the effects of proctolin on twitch tension amplitude, and its rates of contraction and relaxation (data not shown).

Frequency dependence of responses

A number of the modulatory effects of octopamine (Evans & Siegler, 1982) and proctolin (Evans, 1982) on basal and neurally evoked tension in the locust extensor-tibiae muscle are dependent on the frequency of motor neurone stimulation. To test the effects of FMRFamide, the SETi motor neurone was stimulated at several frequencies and at each a 5-min pulse of 10^{-6} mol l^{-1} FMRFamide was introduced into the muscle superfusate (Fig. 6). The result depended upon the frequency at which the motor neurone was being stimulated. At 1 Hz there was a pronounced increase in twitch amplitude but very little increase in basal tension. At 3 Hz there was a longer proportional increase in twitch amplitude which was superimposed upon an increase in basal tension. The increase in basal tension was maximal between 5 and 7 Hz.

As a further test of the effects of FMRFamide on muscle tension, SETi was stimulated every 60 s with 10-s trains of pulses that were increased in frequency stepwise from 3 to 50 Hz. The ability of the muscle to develop and maintain tension in normal saline was compared with that in the presence of 10^{-6} mol l^{-1} FMRFamide (Fig. 7). In normal saline, tension gradually increased throughout each 10-s stimulus and at the higher frequencies tested produced a smooth tetanic tension plateau. In the presence of FMRFamide the height of the individual tension transients visible up to 7 Hz was increased and this was superimposed upon an increased tension plateau. At frequencies of 10 Hz and above the height of the tetanic plateau was increased. The rate of rise and fall of tetanic tension did not appear to be altered in the presence of FMRFamide. At 20 Hz the threshold for an effect of FMRFamide on the height of the tetanic plateau occurred between 10^{-8} and 10^{-7} mol l^{-1} and the effects of 10^{-7} and 10^{-6} mol l^{-1} FMRFamide reached a maximum within 3 min of the start of a prolonged pulse (Fig. 8).

Effects of FMRFamide on tension during stimulation of SETi in stepping pattern

The behavioural significance of the effects of octopamine on the extensor-tibiae muscle have been assessed (Evans & Siegler, 1982) in experiments where SETi was stimulated using the pattern of SETi activity reported for a free-walking locust by Burns & Usherwood (1979). Fig. 9A shows the tension profile produced by a 10-step sequence of stimuli to SETi in normal saline, whilst Fig. 9B shows that produced after exposure of the muscle to 10^{-6} mol l^{-1} FMRFamide. In the presence of FMRFamide each of the steps produces more tension and the rates of increase and decrease of tension during the steps are increased. Up to the fifth step the tension maintained between the periods of stimulation is increased in the presence of FMRFamide but after this it is equal to that of the controls.

DISCUSSION

The effects of FMRFamide and related peptides on muscle tension

The effects of FMRFamide and related peptides on the amplitude (Walther *et al.* 1984), and the rates of contraction and relaxation of SETi-evoked twitch contractions in the locust extensor-tibiae muscle are very similar to those induced by octopamine (Evans & O'Shea, 1977; O'Shea & Evans, 1979; Buchan & Evans, 1980). Both the amine and peptides from this family increase the amplitude of the twitches

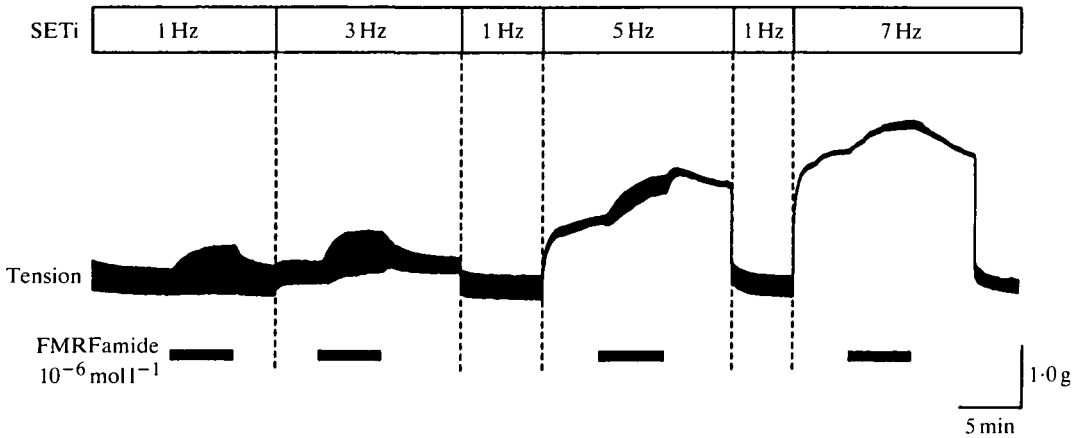

Fig. 6. The frequency dependence of FMRFamide effects on maintained tension. A continuous recording of the tension profile from a metathoracic extensor-tibiae muscle produced by firing SETi at different frequencies is shown. 5-min pulses of 10^{-6} mol l^{-1} FMRFamide (black bars) were introduced into the superfusate. The preparation was returned to 1 Hz stimulation between the 3- and 5-Hz stimulations and also between the 5- and 7-Hz stimulations.

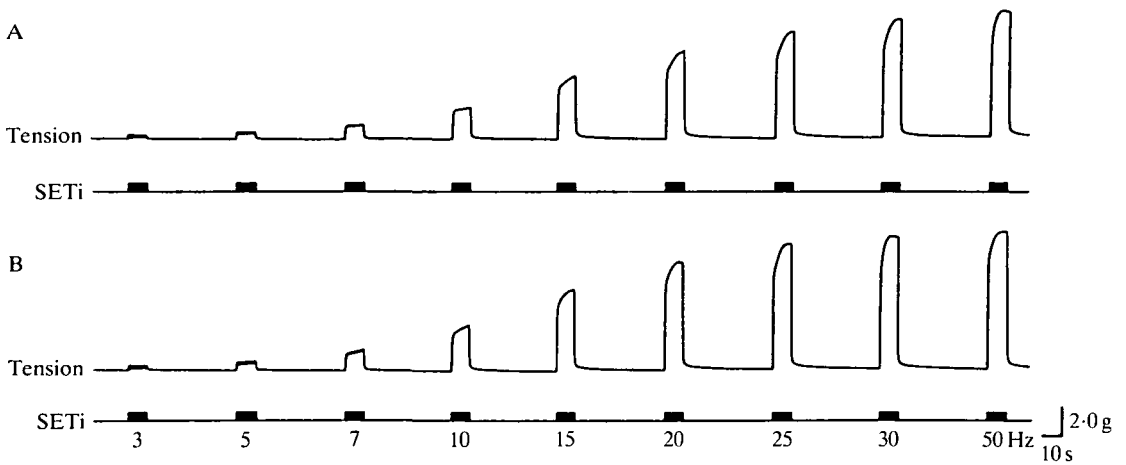

Fig. 7. The effects of 10^{-6} mol l^{-1} FMRFamide on tetanic tension produced in the extensor muscle by firing SETi for 10 s at different frequencies. (A) Control series in saline before FMRFamide application; (B) series in the presence of FMRFamide. FMRFamide increases the height of the tetanic plateau.

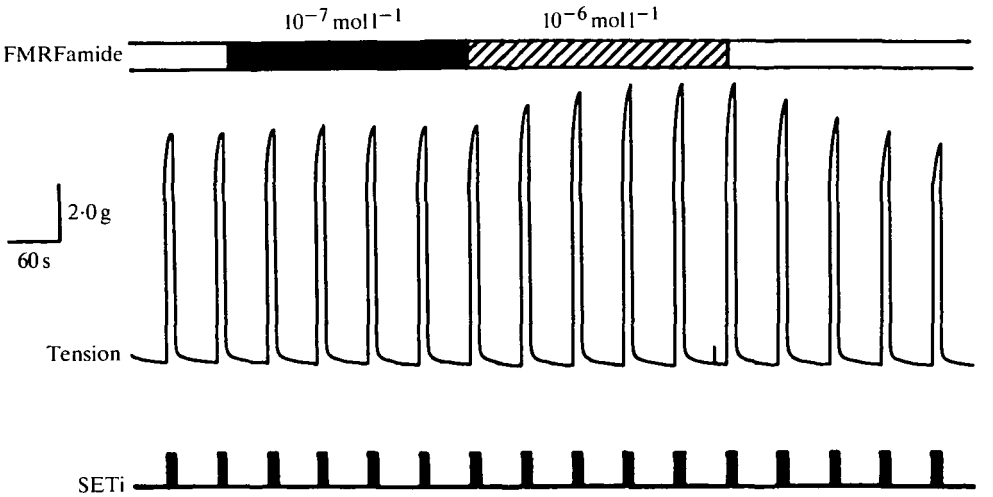

Fig. 8. Time course of the effect of FMRFamide on tetanic tension produced in the extensor muscle by firing SETi for 10 s at 20 Hz. Black bar shows time of exposure to 10^{-7} mol l^{-1} FMRFamide and hatched bar the time of exposure to 10^{-6} mol l^{-1} FMRF-amide.

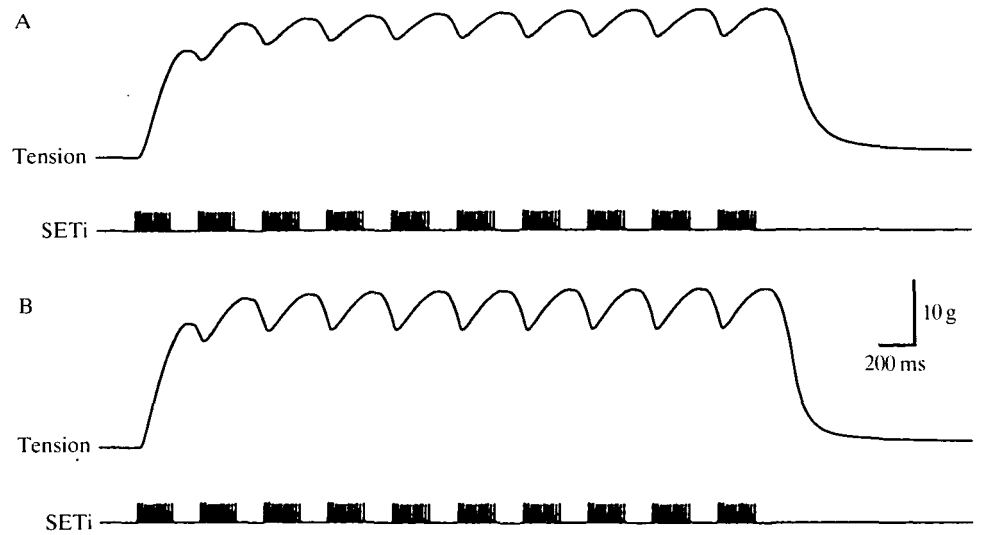

Fig. 9. Tension profiles in response to 10 cycles of SETi stimulation in a pattern derived from a walking animal (see text for details). The tension profile in the absence (A) and in the presence (B) of 10^{-6} mol l^{-1} FMRFamide in the muscle superfusate. There is a more rapid rise and fall of tension and an increase in the amplitude of tension transients in the presence of FMRFamide. In addition, more tension is maintained between the steps for the first four cycles.

and the rates of contraction and relaxation in a dose-dependent manner. In addition, the effects of both FMRFamide and octopamine outlast their presence in the superfusate by several minutes. There are, however, differences in the time courses of their actions. The effects of a prolonged pulse of 10^{-6} mol l^{-1} FMRFamide on the amplitude and the rates of contraction and relaxation of twitch tension do not reach a maximum until after 4–5 min of exposure. With shorter pulses of FMRFamide maximal effects are not achieved. Octopamine, on the other hand, can achieve almost maximal potentiation of all three parameters in less than 1 min, although the time course of this effect is longer with longer exposure. A 30-s pulse (10^{-6} mol l^{-1}) of DL-octopamine takes almost 1 min to achieve maximum effects, whereas a 6-min pulse takes 2–3 min to reach a maximum. O'Shea & Evans (1979) suggest that a desensitization of octopamine receptors may be occurring during prolonged exposures to octopamine. It is possible that the slower time course of the FMRFamide potentiation may be due to the fact that FMRFamide is probably not the endogenous mediator of these effects. The related N-terminally extended peptide, YGGFMRFamide, produces maximum effects in a much shorter time (2–3 min), which is more similar to the effects brought about by a prolonged pulse of octopamine (O'Shea & Evans, 1979). In addition, the threshold for an observable effect of YGGFMRFamide is 10–100 times lower than that for the FMRFamide itself. Another difference between the effects of FMRFamide-like peptides and octopamine on this preparation is that the dose–response curves for the effects of FMRFamide on twitch tension amplitude, relaxation rate and contraction rate can be superimposed, with roughly equal maximal effects of 200% increases occurring at 10^{-6} mol l^{-1}. In contrast, the effects of octopamine on relaxation rate are much bigger and have a lower threshold than its effects on twitch amplitude and contraction rate.

The effects of FMRFamide on slow twitch and tetanic tension in the extensor-tibiae muscle depend upon the frequency at which SETi is stimulated. At frequencies of stimulation above 1 Hz (3–10 Hz), where the twitches summate but tetanus is incomplete, FMRFamide causes a marked increase in basal tension in the muscle. In addition, it also increases the height of the plateau of smooth tetanic contractions. These effects are the exact opposite to those induced by octopamine, which causes a large reduction in basal tension over this frequency range (Evans & Siegler, 1982). It is, however, similar to the effect of the pentapeptide, proctolin, on this preparation (May *et al.* 1979; Evans, 1982). Data on the effects of proctolin on locust skeletal muscle are rather sparse, but proctolin also increases basal tension in a frequency-dependent manner in this preparation (Evans, 1982), although the time courses of the actions of the two peptides are different. Following a 30-s pulse of proctolin while stimulating SETi at 1, 5 or 7 Hz, tension is increased in each case slowly up to a maximum, where it is maintained for 30–60 s. The muscle then relaxes abruptly, in a sharp step, during the recovery phase (Evans, 1982). The increase in basal tension brought about by FMRFamide continues to rise for as long as the peptide is present (5 min was the longest time tested). On removal of FMRFamide from the superfusate, after a further brief increase, the tension gradually declines. As

well as the difference in the recovery rates, it is interesting to note that FMRFamide also increases the size of the individual twitch contractions on top of the increased basal tension. Proctolin does not usually produce such an effect, although in some preparations it has been observed to produce small increases in twitch tension amplitude, relaxation and contraction rates (P. D. Evans & C. R. Myers, unpublished observations). The mechanisms for the above actions of FMRFamide are unknown, but a possible explanation is that the effects of FMRFamide on the contraction and relaxation rates of the individual twitch contractions allow the muscle to continue to contract on top of an increased basal tension by decreasing the fusion of twitches which would otherwise result from the increased frequencies of stimulation. The effects of FMRFamide on SETi stepping patterns are also consistent with this explanation since FMRFamide increases the rates of rise and fall of tension in the individual steps, the amplitude of which increases. This is similar to the effect of octopamine on this pattern of stimulation (Evans & Siegler, 1982) but the basal tension maintained between the steps is not decreased by FMRFamide as it is by octopamine. A detailed behavioural analysis of the consequences of the actions of FMRFamide-like peptides will require information on the timing of their release and the degree of changes in their circulating haemolymph levels.

The actions of FMRFamide on locust skeletal muscle have some parallels and some differences with its observed actions on other invertebrate muscles. Thus in the locust, the ability of FMRFamide to increase maintained tension is similar to its effects on *Helix* tentacle retractor muscle (Cottrell *et al.* 1983*a*), on the anterior byssus retractor muscle of *Mytilus edulis* (Painter, 1982) on the isolated radula protractor muscle of *Busycon contrarium* (Greenberg & Price, 1979) and on leech heart (Kuhlman, Li & Calabrese, 1985). However, unlike its actions on the *Helix* tentacle retractor muscle and leech heart, FMRFamide does not induce or modulate the myogenic activity in the locust extensor-tibiae muscle, although it can do so in locust heart (C. M. Myers & P. D. Evans, in preparation).

Regional differences in responsiveness to FMRFamide-like peptides

The responses of the extensor-tibiae muscle to application of FMRFamide differ in different regions of the muscle, as has been found with the effects of octopamine on this muscle (Evans, 1985*b*). However, the variation in responsiveness to the peptide and the amine is different although their effects occur in the same blocks of muscle (i.e. blocks *a*, *e* and *f*) which contain the highest proportion of slow- and intermediate-type muscle fibres (Hoyle, 1978; Evans, 1985*b*). FMRFamide causes large increases in the amplitude of SETi-induced twitch contractions, with the largest increases occurring in region *f*. The effects on the relaxation rate are not as great as those on the amplitude, but again are most prominent in region *f*. The converse is true for octopamine. Octopamine-induced effects are greatest on the relaxation rates, with the largest increase again occurring in region *f*. Its effects on twitch amplitude are much smaller and are the same in all three regions measured. These results support the conclusion that the effects of FMRFamide are not mediated *via* an interaction with the octopaminergic receptor system.

FMRFamide has no effect on twitch tension induced by stimulation of the fast motor neurone (FETi) in the extensor-tibiae muscle, unlike octopamine which causes a large increase in the relaxation rate but has little effect on the rate of contraction or the amplitude of the twitches (Evans, 1981). This suggests that the actions of FMRFamide-like peptides in the extensor muscle may be confined to the slow muscle fibres which are innervated by SETi alone, receptors for these peptides being absent from the intermediate muscle fibres that are innervated by both SETi and FETi.

Presynaptic versus *postsynaptic actions*

FMRFamide-related peptides produce their effects on slow motor neurone-induced twitch tension in the locust extensor-tibiae muscle by a combination of presynaptic and postsynaptic effects. Contractions, but not the depolarization, induced by the application of pulses of a high-$[K^+]$ saline to the muscle are potentiated by FMRFamide suggesting a postsynaptic effect on the excitation–contraction coupling mechanism (Walther *et al.* 1984). Further, the observation that FMRFamide also increases the rate of relaxation of twitch tension provides more evidence for a postsynaptic site of action. Evidence for a presynaptic site of action is provided by the observation that FMRFamide increases the average amplitude of excitatory junctional potentials but not the time integrals of depolarizations caused by superfusion of glutamate, the presumed rapid-acting transmitter of SETi (Walther *et al.* 1984). In addition, a direct measurement of the presynaptic action of FMRFamide has been obtained in the present study where it has been shown to increase the frequency, but not the amplitude distribution, of spontaneous miniature end-plate potentials in muscle fibres innervated by SETi but not FETi. The relative contributions of the presynaptic and postsynaptic components to the overall FMRFamide-induced increases in twitch tension amplitude and the rate of contraction are unknown at present.

The mechanisms of the pre- and postsynaptic actions of FMRFamide are also unknown at present. Blocking experiments using phentolamine, an effective blocking agent of the actions of octopamine in this preparation (Evans, 1981), indicate that FMRFamide does not mediate any of its effects by the activation of octopamine receptors. A similar conclusion has been reached for the potentiating actions of FMRFamide on acetylcholine-evoked and electrically stimulated contractions in the anterior byssus retractor muscle of the mussel (Muneoka & Matsuura, 1985). In the locust muscle, the actions of FMRFamide do not appear to be mediated *via* increases in cyclic nucleotide levels. This again suggests that FMRFamide and octopamine are working on separate receptor systems in this preparation. In all non-cardiac muscles from molluscs so far examined, except for the gill muscles of *Aplysia* (Weiss *et al.* 1984), FMRFamide does not appear to mediate its excitatory effects *via* mechanisms that use cyclic nucleotides. In contrast, in some molluscan neurones, FMRFamide can induce changes in potassium and calcium conductances, some of which are

mediated by cyclic nucleotides in some cells but not in others (Colombaioni, Paupardin-Tritsch, Vidal & Gerschenfeld, 1985).

Nature of endogenous FMRFamide-like modulator of extensor muscle

The nature of the endogenous FMRFamide-like peptide that modulates slow motor neurone twitch tension in the locust extensor-tibiae muscle has not yet been elucidated. However, the different potencies of the FMRFamide-related peptides described in this study and the preliminary communication of Walther *et al.* (1984) have given some clues as to the structural requirements for the endogenous peptide. The studies of Walther *et al.* (1984) have indicated that the carboxyl-terminal sequence -Arg-Phe-NH_2 is essential for activity. They also indicate that activity is maintained in this preparation if the methionine preceding these two residues is replaced by another neutral amino acid (such as leucine, as in FLRFamide) but not by an acidic amino acid (such as aspartate, as in γ_1-melanocyte stimulating hormone). The present work confirms these observations and in addition shows that the C-terminal dipeptide Arg-Phe-NH_2 is not active. This suggests that this is not the only structural requirement and that the N-terminal may also play an important role. This possibility is reinforced by the fact that YGGFMRFamide is so much more potent than FMRFamide itself on this preparation. The related peptide, LPLRFamide, first isolated from chicken brain (Dockray *et al.* 1983), is an order of magnitude less potent than FMRFamide. This lends support to the above argument, since the proline residue may alter the structure of the N-terminal quite substantially. Proline has a pronounced 'kink' in its structure which may make an important contribution to the secondary structure of a polypeptide chain; for example, in avian pancreatic polypeptide, the proline residues form a partial helix, such that the proline rings contribute towards hydrophobic interactions in the tertiary structure of the molecule (Blundell & Wood, 1982). In addition to revealing FMRFamide-like peptides in the locust nervous system, immunocytochemical studies indicate the presence of pancreatic polypeptide-like (PP-like) peptides in the brain, corpora cardiaca, abdominal ganglia and neurohaemal organs (Myers & Evans, 1985*a*,*b*). However, none of the closely related peptides, avian PP, bovine PP or human PP, had any effect on the extensor-tibiae muscle at 10^{-6} mol l^{-1}. These peptides all terminate in the sequence -Arg-Tyr-NH_2, and thus it seems likely that the receptors in this muscle are quite specific for the C-terminal sequence -Arg-Phe-NH_2. It should be noted, however, that the residue which precedes arginine in the pancreatic polypeptides is proline, which as discussed above is important in the three-dimensional structure of the peptide and will thus affect its biological activity. Immunocytochemical evidence (see Evans & Myers, 1986) has also revealed that some of the neurones containing FMRFamide-like peptides in the locust are also immunoreactive with a monoclonal antibody raised against another peptide first isolated from the molluscan nervous system, namely small cardioactive peptide (SCP_B) (Morris *et al.* 1982). SCP_B antagonizes the actions of FMRFamide on various molluscan preparations (Murphy *et al.* 1985; P. E. Lloyd, personal communication) but does not alter any of the modulatory actions of FMRFamide

described in the present paper. SCP_B does not modulate neuromuscular contraction directly in the locust extensor muscle but it does produce a dose-dependent accelerator effect upon the myogenic rhythm (Evans & Myers, 1986).

Future studies on the biological role of FMRFamide-like peptides in locust skeletal muscle require the isolation and sequencing of the endogenous peptides from the locust. The extensor-tibiae muscle of the locust hindleg will undoubtedly be of much use as a bioassay during these isolation procedures.

REFERENCES

BLUNDELL, T. & WOOD, S. (1982). The conformation, flexibility and dynamics of polypeptide hormones. *A. Rev. Biochem.* **51**, 123–154.

BROWN, B. L., EKINS, R. P. & ALBANO, J. D. M. (1972). Saturation assay for cyclic AMP using endogenous binding protein. *Adv. cyclic Nucleotide Res.* **2**, 25–40.

BUCHAN, P. B. & EVANS, P. D. (1980). Use of an operational amplifier signal differentiator reveals that octopamine increases the rate of development of neurally evoked tension in insect muscle. *J. exp. Biol.* **85**, 349–352.

BURNS, M. D. & USHERWOOD, P. N. R. (1979). The control of walking in Orthoptera. II. Motor neurone activity in normal free-walking animals. *J. exp. Biol.* **79**, 69–98.

COLOMBAIONI, L., PAUPARDIN-TRITSCH, D., VIDAL, P. P. & GERSCHENFELD, H. M. (1985). The neuropeptide FMRFamide decreases both the Ca^{2+} conductance and a cyclic 3′,5′-adenosine monophosphate-dependent K^+ conductance in identified molluscan neurons. *J. Neurosci.* **5**, 2533–2538.

COTTRELL, G. A. (1983). Actions of FMRFamide and related peptides on snail neurones. In *Molluscan Neuroendocrinology* (ed. J. Lever & H. H. Boer), pp. 213–221. New York: North-Holland.

COTTRELL, G. A., GREENBERG, M. J. & PRICE, D. A. (1983*a*). Differential effects of the molluscan neuropeptide FMRFamide and the related met-enkephalin derivative YGGFMRFamide on the *Helix* tentacle retractor muscle. *Comp. Biochem. Physiol.* **75C**, 373–375.

COTTRELL, G. A., SCHOT, L. P. C. & DOCKRAY, G. J. (1983*b*). Identification and probable role of a single neurone containing the neuropeptide *Helix* FMRFamide. *Nature, Lond.* **304**, 638–640.

DAVENPORT, A. P. & EVANS, P. D. (1984*a*). Stress-induced changes in the octopamine levels of insect haemolymph. *Insect Biochem.* **14**, 135–143.

DAVENPORT, A. P. & EVANS, P. D. (1984*b*). Changes in haemolymph octopamine levels associated with food deprivation in the locust, *Schistocerca gregaria*. *Physiol. Entomol.* **9**, 269–274.

DOCKRAY, G. J., REEVE, J. R., SHIRELY, J., GAYTON, R. J. & BARNARD, C. S. (1983). A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature, Lond.* **305**, 328–330.

DOCKRAY, G. J. & WILLIAMS, R. G. (1983). FMRFamide-like immunoreactivity in rat brain: Development of a radioimmunoassay and its application in studies of distribution and chromatographic properties. *Brain Res.* **266**, 295–303.

EVANS, P. D. (1981). Multiple receptor types for octopamine in the locust. *J. Physiol., Lond.* **318**, 99–122.

EVANS, P. D. (1982). Properties of modulatory octopamine receptors in the locust. *Ciba Fdn Symp.* **88**, 48–69.

EVANS, P. D. (1984*a*). A modulatory octopaminergic neurone increases cyclic nucleotide levels in locust skeletal muscle. *J. Physiol., Lond.* **348**, 307–324.

EVANS, P. D. (1984*b*). The role of cyclic nucleotides and calcium in the mediation of the modulatory effects of octopamine on locust skeletal muscle. *J. Physiol., Lond.* **348**, 325–340.

EVANS, P. D. (1984*c*). Studies on the mode of action of octopamine, 5-hydroxytryptamine and proctolin on a myogenic rhythm in the locust. *J. exp. Biol.* **110**, 231–251.

EVANS, P. D. (1985*a*). Octopamine. In *Comprehensive Insect Biochemistry, Physiology and Pharmacology* (ed. G. A. Kerkut & L. Gilbert), pp. 499–530. Oxford: Pergamon Press.

EVANS, P. D. (1985*b*). Regional differences in responsiveness to octopamine within a locust skeletal muscle. *J. Physiol., Lond.* **366**, 331–341.

EVANS, P. D. & MYERS, C. M. (1986). Peptidergic and aminergic modulation of insect skeletal muscle. *J. exp. Biol.* **124**, 143–176.

EVANS, P. D. & O'SHEA, M. (1977). An octopaminergic neurone modulates neuromuscular transmission in the locust. *Nature, Lond.* **270**, 257–259.

EVANS, P. D. & O'SHEA, M. (1978). The identification of an octopaminergic neurone and the modulation of a myogenic rhythm in the locust. *J. exp. Biol.* **73**, 235–260.

EVANS, P. D. & SIEGLER, M. V. S. (1982). Octopamine mediated relaxation of maintained and catch tension in locust skeletal muscle. *J. Physiol., Lond.* **324**, 93–112.

GREENBERG, M. J. & PRICE, D. A. (1979). FMRFamide, a cardioexcitatory neuropeptide in molluscs: An agent in search of a mission. *Am. Zool.* **19**, 163–174.

GREENBERG, M. J., PRICE, D. A. & LEHMAN, H. K. (1985). FMRFamide-like peptides of molluscs and vertebrates: Distribution and evidence of function. *Proc. int. Symp. Neurosecretion, Tokyo*, pp. 79–85.

HORN, J. P. & MCAFEE, D. A. (1977). Modulation of cyclic nucleotide levels in peripheral nerve without effect on resting or compound action potentials. *J. Physiol., Lond.* **269**, 753–766.

HOYLE, G. (1978). Distributions of nerve and muscle fibre types in locust jumping muscle. *J. exp. Biol.* **73**, 205–233.

HOYLE, G. (1983). *Muscles and Their Neural Control.* New York: Wiley-Interscience.

KUHLMAN, J., LI, C. & CALABRESE, L. (1985). FMRFamide-like substances in the leech. II. Bioactivity on the heartbeat system. *J. Neurosci.* **5**, 2310–2317.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MAY, T. E., BROWN, B. E. & CLEMENTS, A. N. (1979). Experimental studies upon a bundle of tonic fibres in the locust extensor tibialis muscle. *J. Insect Physiol.* **25**, 169–181.

MORRIS, H. R., PANICO, M., KARPLUS, A., LLOYD, P. E. & RINIKER, B. (1982). Elucidation by FAB-MS of the structure of a new cardioactive peptide from *Aplysia*. *Nature, Lond.* **300**, 643–645.

MUNEOKA, Y. & MATSUURA, M. (1985). Effects of the molluscan neuropeptide FMRFamide and the related opoid peptide YGGFMRFamide on *Mytilus* muscle. *Comp. Biochem. Physiol.* **81C**, 61–70.

MURPHY, A. D., LUKOWIAK, K. & STELL, W. K. (1985). Peptidergic modulation of patterned motor activity in identified neurons of *Helisoma*. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7140–7144.

MYERS, C. M. & EVANS, P. D. (1985*a*). The distribution of bovine pancreatic polypeptide/FMRFamide-like immunoreactivity in the ventral nervous system of the locust. *J. comp. Neurol.* **234**, 1–16.

MYERS, C. M. & EVANS, P. D. (1985*b*). A FMRFamide antiserum differentiates between populations of antigens in the ventral nervous system of the locust, *Schistocerca gregaria*. *Cell Tissue Res.* **242**, 109–114.

O'DONOHUE, T. L., BISHOP, J. F., CHRONWALL, B. M., GROOME, J. & WATSON, W. H. (1984). Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system. *Peptides* **5**, 563–568.

O'SHEA, M. & EVANS, P. D. (1979). Potentiation of neuromuscular transmission by an octopaminergic neurone in the locust. *J. exp. Biol.* **79**, 169–190.

PAINTER, S. D. (1982). FMRFamide catch contractions of a molluscan smooth muscle: Pharmacology, ionic dependence and cyclic nucleotides. *J. comp. Physiol.* **148**, 491–501.

PRICE, D. A., COTTRELL, G. A., DOBLE, K. E., GREENBERG, M. J., JORENBY, W., LEHMAN, H. K. & RIEHM, J. P. (1985). A novel FMRFamide-related peptide in *Helix*: pQDPFLRFamide. *Biol. Bull. mar. biol. Lab., Woods Hole* **169**, 256–266.

PRICE, D. A. & GREENBERG, M. J. (1977). Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**, 670–671.

PRICE, D. A. & GREENBERG, M. J. (1980). Pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. *Gen. Pharmac.* **11**, 237–241.

VEENSTRA, J. A. & SCHOONEVELD, H. (1984). Immunocytochemical localization of neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. *Cell Tissue Res.* **235**, 303–308.

VERHAERT, P., GRIMMELIKHUIJZEN, C. J. P. & DELOOF, A. (1985). Distinct localization of FMRFamide- and bovine pancreatic polypeptide-like material in the brain, retrocerebral complex and suboesophageal ganglion of the cockroach, *Periplaneta americana* L. *Brain Res.* **348**, 331–338.

WALTHER, C., SCHIEBE, M. & VOIGT, K. H. (1984). Synaptic and non-synaptic effects of molluscan cardioexcitatory neuropeptides on locust skeletal muscle. *Neurosci. Letters* **45**, 99–104.

WATSON, W. H., GROOME, J. R., CHRONWALL, B. M., BISHOP, J. & O'DONOHUE, T. L. (1984). Presence and distribution of immunoreactive and bioactive FMRFamide-like peptides in the nervous system of the horseshoe crab, *Limulus polyphemus*. *Peptides* **5**, 585–592.

WEISS, S., GOLDBERG, J. I., CHOHAN, K. S., STELL, W. K., DRUMMOND, G. I. & LUKOWIAK, K. (1984). Evidence for FMRFamide as a neurotransmitter in the gill of *Aplysia*. *J. Neurosci.* **4**, 1994–2000.

WILLIAMS, R. G. & DOCKRAY, G. J. (1983). Immunohistochemical studies of FMRFamide-like immunoreactivity in rat brain. *Brain Res.* **276**, 213–229.