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A COMPARISON OF THE EFFECTS OF FMRFamide-LIKE PEPTIDES ON LOCUST HEART AND SKELETAL MUSCLE

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Summary

1. The responses of the semi-isolated heart preparation of the locust to the peptide FMRFamide and a range of its analogues is described.

2. The nature of the response observed depended on the structure of the analogue and its concentration. In some cases the responses were cardioexcitatory, in some they were cardioinhibitory, and in others they were biphasic. The cardioexcitatory responses consisted of increases in both the amplitude and the frequency of spontaneous heart contractions. The nature of the latter effect depended on the form of the basal contractile activity pattern exhibited before the application of the peptide.

3. The pharmacological profile of the cardioexcitatory responses observed in the locust heart preparation was very similar to that observed for the potentiation of neurally evoked tension in the extensor tibiae muscle preparation of the locust. In addition, both the profiles show similarities with the responses of various molluscan hearts and non-cardiac muscle preparations to the same peptides.

4. The results are discussed in terms of possible physiological roles for FMRFamide-like peptides in the regulation of contractile activity of the locust heart.

Introduction

The tetrapeptide Phe-Met-Arg-Phe-amide (FMRFamide) was first isolated and characterized as a cardioacceleratory factor from the nervous system of the clam, *Marocallista nimbosa* (Price & Greenberg, 1977). However, extensive investigation of the actions of FMRFamide on the hearts from 50 species of bivalve molluscs indicates a great degree of variability in the responsiveness of molluscan hearts to this peptide (Painter & Greenberg, 1982). In some species the peptide is cardioacceleratory, in some it inhibits heart activity, and in others it exhibits concentration-dependent biphasic effects. Recent work indicates that FMRFamide is one member of a growing interphyletic family of peptides (Price *et al.* 1987) and evidence has been presented for the presence of multiple receptor types for members of this peptide family on molluscan neurones (Cottrell *et al.* 1984; Ruben *et al.* 1986; Cottrell & Davies, 1987), skeletal muscle (Cottrell *et al.*

Key words: peptides, insects, heart.

1983) and heart (Payza, 1987). FMRFamide has also been shown to modulate heart activity in the leech and may be released from both the HE motor neurones and the HA modulatory neurones which innervate the heart (Kuhlman *et al.* 1985*a*,*b*; Li & Calabrese, 1987).

In insects, a number of FMRFamide-related peptides have been isolated and sequenced, including leucomyosuppressin (pQDVDHVFLRFamide) from the cockroach, *Leucophaea* (Holman *et al.* 1986) and DPKQDFMRFamide from *Drosophila* (Nambu *et al.* 1987). In addition, there is immunocytochemical evidence for the presence of FMRFamide-like peptides in the nervous systems of a number of insect species (see Evans & Myers, 1986*a*). In the locust, detailed immunocytochemical evidence has been presented for the presence of FMRFamide-like material in specific subsets of neurones within the ventral nerve cord, some of which project to neurohaemal organs (Myers & Evans, 1985*a*,*b*), and also in the brain (Myers & Evans, 1987). In addition, the modulatory effects of FMRFamide-like peptides have been described on the extensor tibiae muscle of the locust hindleg (Walther *et al.* 1984; Evans & Myers, 1986*a*,*b*). However, the structures of the endogenous FMRFamide-like peptides in the locust nervous system have not yet been identified. The activity of insect visceral muscles, such as the heart, is also known to be modulated by a wide range of neuropeptides including proctolin (Miller, 1979), M1 and M2 (O'Shea *et al.* 1984) (also known as periplanetins CC-1 and CC-2, Scarborough *et al.* 1984) and the cardioacceleratory peptides identified and isolated from the nervous system of the tobacco hornworm, *Manduca sexta* (Tublitz & Truman, 1985*a*,*b*; Platt & Reynolds, 1985; Tublitz & Evans, 1986). Preliminary evidence has also been presented for the modulation of spontaneous contractile activity in the semi-isolated heart preparation of the locust by FMRFamide-like peptides (Myers, 1986; Evans *et al.* 1988).

In the present paper we describe, in detail, the different types of responses elicited by application of FMRFamide-like peptides to the semi-isolated heart preparation of the locust and the pharmacological specificity of these responses. In addition, we compare these responses to those obtained by the application of the same analogues to the extensor tibiae muscle preparation of the locust.

Materials and methods

Adult locusts (*Schistocerca gregaria*) of either sex, which had moulted 1–10 days previously, were obtained from a crowded laboratory culture fed on wheat seedlings. Small batches of animals were left for at least 30 min after removal from the main culture, to minimize any possible potentiation effects due to raised haemolymph octopamine levels (see Evans, 1981; Davenport & Evans, 1984*a*,*b*).

The semi-isolated locust heart preparation was set up essentially as outlined by Tublitz & Truman (1985*a*) for the heart of the tobacco hawkmoth, *Manduca sexta*, except that the cardiac musculature was left attached to a small strip of cuticle, as in the cockroach preparation (see Miller & Metcalf, 1968). The dorsal half of th

abdominal cuticle was removed by cutting through the body wall on both sides of the abdomen at the level of the spiracles. The preparation was then transferred to a Sylgard-coated Petri dish and pinned out ventral side uppermost under locust saline. It was then washed several times in fresh saline. The alary muscles on both sides of the heart in the posterior two abdominal segments were carefully severed and the small portion of the heart thus freed was ligatured with fine 6/0 suture silk (Ethicon). The preparation was then transferred to a simple perfusion chamber and pinned out leaving the free end of the silk to be attached to a force transducer to monitor the frequency and amplitude of spontaneous heart contractions almost isometrically. The position of the transducer was adjusted so that maximum deflections were obtained when the heart was in systole. The output of the transducer was recorded simultaneously on a chart recorder and a tape recorder.

The heart was superfused continuously (at a rate of 1 ml min^{-1}) with physiologically isotonic saline (pH 6·8) containing (in mmol l^{-1}) NaCl, 150; $CaCl_2$, 4; $KHCO_3$, 4; KH_2PO_4, 6; and sucrose, 90. Saline was applied to the anterior end of the preparation and flowed over the whole preparation. Excess fluid flowing off the posterior end of the preparation was removed by a suction pump so that the fluid in the chamber remained at a constant level. FMRFamide and related peptides were introduced into the superfusate as 5-min pulses. The first application of peptide was always preceded by a 30 min wash of the preparation in saline and further saline washes of at least 10 min were given between peptide pulses. Each preparation was only exposed to a single series of peptide concentrations and each peptide was tested on 3–7 animals. All experiments were carried out at room temperature. Measurements of heart rate and contraction amplitude were averaged over 1-min period, starting (a) 1 min before the beginning of the peptide pulse, (b) 1 min after the start of the peptide pulse, (c) 4 min after the start of the peptide pulse and (d) 1 min after the end of the peptide pulse. Maximum responses were also measured where they did not correspond to any of the above sample times. Results are expressed as percentage increases or decreases in the amplitude of contractions or their frequency with respect to the measurements obtained immediately before the application of the peptide.

The effect of FMRFamide-like peptide analogues was also measured on tension evoked in the extensor tibiae muscle of the hindleg of the locust by stimulating the slow extensor motor neurone as described previously (see Evans & Myers, 1986*b*).

All peptides were obtained from Peninsula Laboratories Inc. except for FLRFamide, FLRF and TNRNFLRFamide which were obtained from Cambridge Research Biochemicals. FMRYamide, RMRFamide, FPRFamide and FGRFamide were the kind gift of Dr D. A. Price. We would also like to thank Dr G. M. Holman for the gift of an initial sample of pQDVDHVFLRFamide.

Results

Types of responses of the semi-isolated heart to FMRFamide-like peptides

The effects of FMRFamide-like peptides on the semi-isolated heart preparation

of the locust varied with the different peptide analogues and their concentrations. FMRFamide itself elicited purely cardioexcitatory effects with increases in both contraction frequency and amplitude. However, the extent of the effects depended on the activity pattern exhibited by the heart prior to peptide application. This was particularly marked in terms of the peptide-induced changes in the frequency of the heart beat. Thus, hearts that were beating regularly prior to peptide application often showed little, or only slight, increases of frequency when exposed to FMRFamide. Irregularly beating hearts, or hearts showing 'bursting' activity, where the contractions occurred in groups interspersed by brief periods of little or no activity, demonstrated a more marked increase in frequency upon application of the peptide. The effects of 10^{-6} and 10^{-5} mol l^{-1} FMRFamide on such a bursting preparation can be seen in Fig. 1, where the response also persists for several minutes after the removal of the peptide from the superfusate. In general, frequency increased, either through a regularization and decrease of intercontraction periods, or through a more general effect whereby the beating pattern remained irregular but there was an overall heightening of activity. Hearts with bursting activity displayed a merging of each discrete burst into one continuous regular series of contractions in response to the cardioactive peptide.

The effects of the peptides on contraction amplitude were hardly affected by the pre-application heart activity. The response generally consisted of a dose-dependent increase in the contraction amplitude (Fig. 1). Occasionally, the baseline tension of the heart also increased during peptide application but this was difficult to quantify owing to its rare occurrence.

Other FMRFamide-like peptide analogues, such as the N-terminally extended

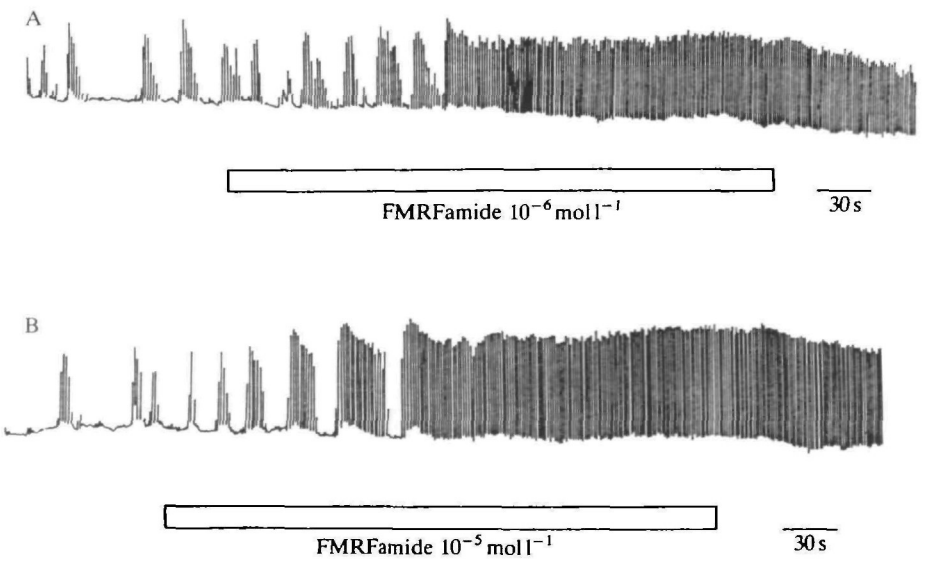

Fig. 1. The effects of 5-min pulses of (A) 10^{-6} mol l^{-1} and (B) 10^{-5} mol l^{-1} FMRFamide on spontaneous contractions in the semi-isolated heart preparation of the locust.

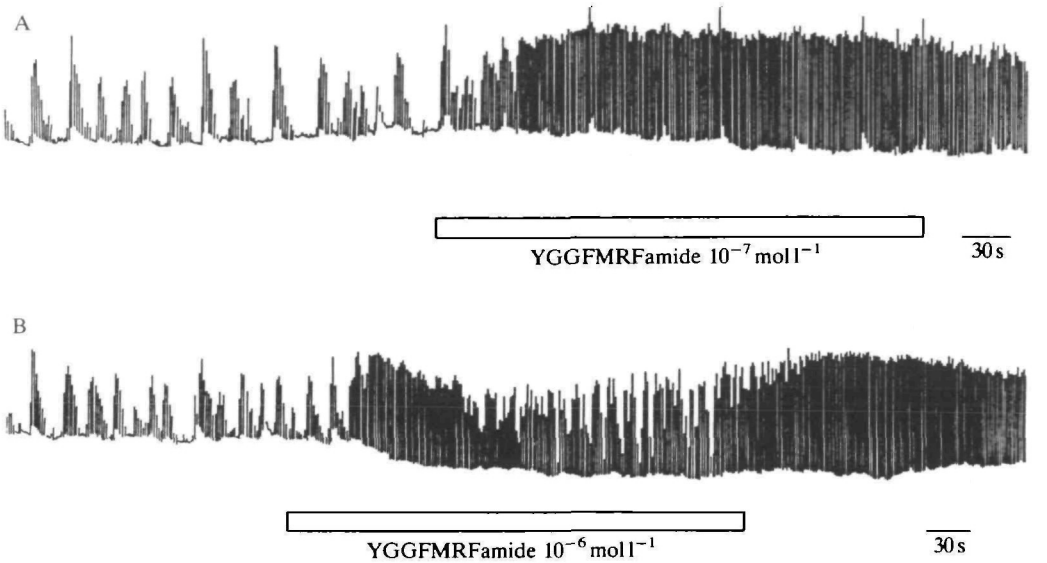

Fig. 2. The effects of 5-min pulses of (A) 10^{-7} mol l^{-1} and (B) 10^{-6} mol l^{-1} YGGFMRFamide on spontaneous contractions of the semi-isolated heart preparation of the locust. Note the biphasic effects at the higher concentration.

peptide YGGFMRFamide, exhibited the same cardioexcitatory effects as FMRFamide itself at low concentrations (up to 10^{-7} mol l^{-1}), but at higher concentrations exhibited biphasic cardioexcitatory and cardioinhibitory effects (Fig. 2). At these higher concentrations immediately after peptide application the heart transiently increased the amplitude and frequency of the contractions. This was followed by a period of decreased frequency and amplitude lasting as long as the peptide remained in the superfusate, the extent of the cardioinhibition being dose-dependent. Finally, when the peptide was removed from the superfusate, a post-application rebound increase in the amplitude and frequency occurred which was often larger and longer-lasting than the initial cardioexcitatory effect. In the case of some other peptides, such as RMRFamide, the degree of cardioinhibition induced upon the application of the peptide at concentrations of 10^{-6} mol l^{-1} and above was great enough to prevent any initial cardioexcitatory response, and excitation only became apparent during the post-application wash period (Fig. 3).

The only peptide tested that produced a purely cardioinhibitory response was the cockroach (*Leucophaea*) hindgut-inhibiting FMRFamide-like peptide, leucomyosuppressin, (pQDVDHVFLRFamide) (Holman *et al.* 1986) (see Fig. 4). This peptide exhibited a dose-dependent inhibition of both the frequency and the amplitude of the spontaneous heart contractions.

To examine the nature of the receptors mediating the cardioexcitatory and cardioinhibitory effects of FMRFamide-like peptides on the semi-isolated heart preparation of the locust, we constructed dose–response curves for a range of FMRFamide analogues. We measured their effects at the three time points

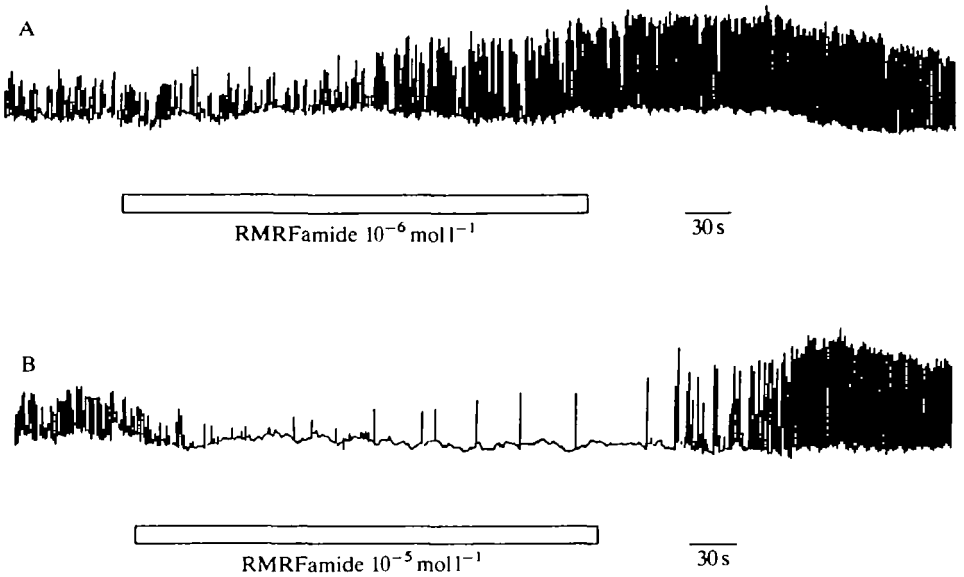

Fig. 3. The effects of 5-min pulses of (A) 10^{-6} mol l^{-1} and (B) 10^{-5} mol l^{-1} RMRFamide on spontaneous contractions in the semi-isolated heart preparation of the locust.

mentioned previously with respect to the introduction of a 5-min pulse of peptide into the superfusate. These periods were chosen to quantify any initial cardioacceleration upon peptide application, the extent of cardioexcitation or cardioinhibition during the peptide pulse and the extent of post-application cardioexcitatory effects in the absence of any cardioinhibition. A comparison of these effects for the N-terminally extended peptides tested at a concentration of 10^{-7} mol l^{-1}, and for the other peptides tested at a concentration of 10^{-6} mol l^{-1} is shown in Table 1.

Effects of tetrapeptide analogues of FMRFamide

The cardioexcitatory effects of FMRFamide itself on both the frequency and the amplitude of the heart beat were dose-dependent (Figs 1, 5) with thresholds for observable effects occurring between 10^{-7} and 10^{-6} mol l^{-1}.

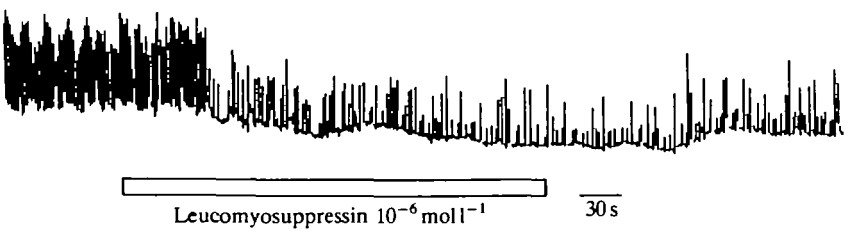

Fig. 4. The effects of a 5-min pulse of 10^{-6} mol l^{-1} leucomyosuppressin on spontaneous contractions in the semi-isolated heart preparation of the locust.

Table 1. *The specificity of action of FMRFamide-like peptides on the spontaneous contractions of the semi-isolated locust heart*

	% Increase in amplitude			% Increase in frequency			
Peptide	Initial	End	Post	Initial	End	Post	*N*
10^{-7} mol l^{-1}							
TNRNFLRFamide	118 ± 40	181 ± 40	185 ± 39	40 ± 8	63 ± 12	64 ± 12	5
GDPFLRFamide	99 ± 44	146 ± 33	142 ± 29	27 ± 13	35 ± 13	38 ± 14	5
SDPFLRFamide	75 ± 19	117 ± 24	113 ± 21	20 ± 14	31 ± 15	34 ± 18	5
YGGFMRFamide	70 ± 23	144 ± 56	90 ± 25	37 ± 15	−21 ± 23	40 ± 17	7
FLRFamide	13 ± 8	53 ± 20	63 ± 22	−2 ± 6	54 ± 20	59 ± 23	5
pQDPFLRFamide	9 ± 5	42 ± 11	46 ± 20	11 ± 12	34 ± 24	49 ± 37	7
FMRFamide	6 ± 1	18 ± 5	24 ± 9	9 ± 6	13 ± 5	21 ± 12	7
10^{-6} mol l^{-1}							
FLRFamide	16 ± 11	61 ± 28	145 ± 32	39 ± 27	86 ± 46	208 ± 203	5
FMRFamide	25 ± 7	83 ± 26	121 ± 37	49 ± 26	114 ± 63	125 ± 71	7
RMRFamide	−2 ± 8	−6 ± 12	67 ± 31	−19 ± 16	−37 ± 18	47 ± 25	5
FPRFamide	15 ± 13	36 ± 30	48 ± 30	14 ± 13	44 ± 42	57 ± 54	5
YGGFMRF	10 ± 8	38 ± 22	37 ± 30	−8 ± 7	−10 ± 8	−3 ± 11	4
YGGFM	11 ± 3	34 ± 13	32 ± 25	13 ± 4	5 ± 15	28 ± 21	5
FMRYamide	5 ± 6	30 ± 19	27 ± 12	30 ± 17	68 ± 43	80 ± 47	3
FMRF	−3 ± 5	0 ± 22	25 ± 17	21 ± 13	14 ± 19	43 ± 28	5
SCP_A	13 ± 4	42 ± 18	25 ± 13	6 ± 40	23 ± 15	22 ± 13	5
SCP_B	5 ± 2	13 ± 4	20 ± 7	3 ± 3	4 ± 1	7 ± 6	5
FGRFamide	1 ± 12	−6 ± 17	20 ± 10	5 ± 12	4 ± 35	7 ± 7	3
YFMRFamide	−12 ± 13	−12 ± 9	12 ± 13	−51 ± 11	−49 ± 8	13 ± 13	6
RFamide	−4 ± 8	12 ± 6	8 ± 6	3 ± 3	5 ± 5	4 ± 4	3
FLRF	0 ± 5	−1 ± 10	−2 ± 8	0 ± 0	−4 ± 6	−7 ± 8	3
pQGRFamide	−11 ± 3	−7 ± 6	−17 ± 6	3 ± 5	40 ± 43	56 ± 59	3
pQDVDHVFLRFamide	−57 ± 9	−63 ± 4	−43 ± 21	−66 ± 11	−64 ± 9	−63 ± 11	3

The data shown were obtained from dose–response curves constructed by applying 5-min pulses of increasing concentrations of the peptides.

Values for the N-terminally extended peptides are compared with those for FLRFamide and FMRFamide at 10^{-7} mol l^{-1} and those for the other peptides tested are compared at 10^{-6} mol l^{-1}.

Initial values were calculated during the second minute of the pulse. End values were calculated during the penultimate minute of the pulse. Post values were calculated during the 1 min period starting 1 min after the end of the pulse.

Each result is expressed as the mean ± standard error of the number of observations shown.

The peptides are ranked on the basis of their 'post' percentage increases in amplitude.

C-terminally amidated tetrapeptide analogues of FMRFamide produced a variety of responses. FLRFamide produced a similar dose-dependent cardio-excitatory effect to FMRFamide itself but with a slightly lower threshold for observable effects occurring between 10^{-8} and 10^{-7} mol l^{-1}. However, at 10^{-5} mol l^{-1} its effect on the frequency of heart rate declined dramatically to zero (Fig. 5). Peptides where the methionine in the 2-position was substituted by

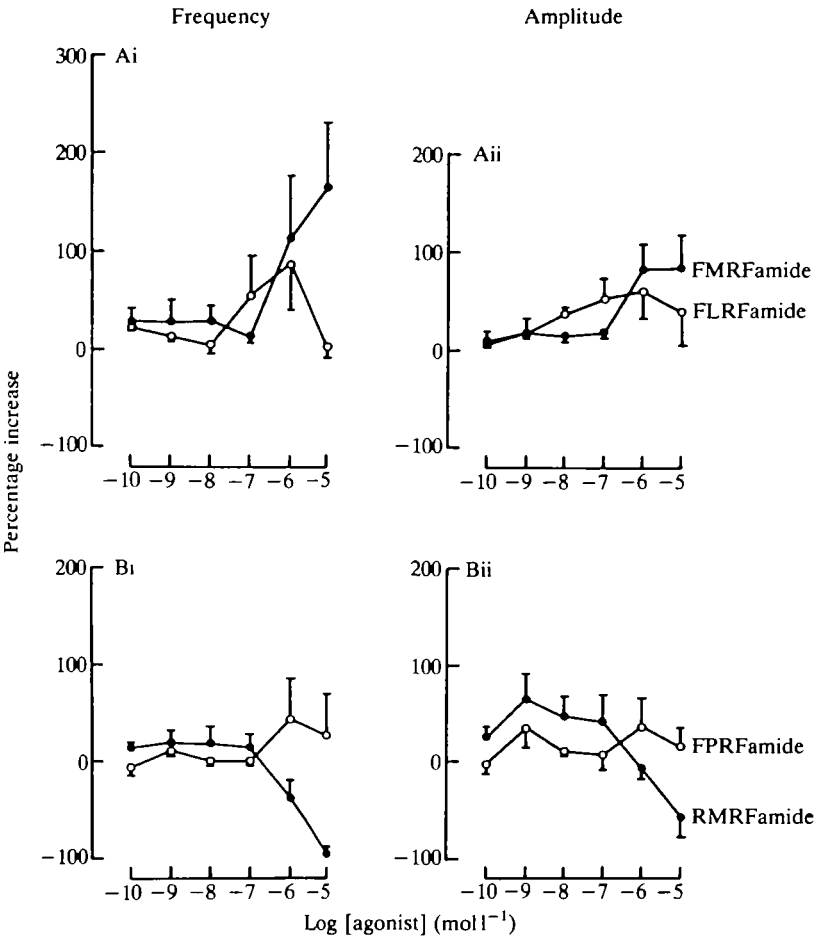

Fig. 5. Dose–response curves for the actions of (A) FMRFamide (●) and FLRFamide (○) and (B) FPRFamide (○) and RMRFamide (●) on (i) the contraction frequency and (ii) the contraction amplitude of the semi-isolated heart preparation of the locust measured during the penultimate minute of a 5-min exposure to the peptide. The error bars represent the standard error of the mean: $N = 7$ for FMRFamide and $N = 5$ for the other peptides.

proline (i.e. FPRFamide, Fig. 5) or by glycine (i.e. FGRFamide, see Table 1) were practically inactive at concentrations up to 10^{-5} mol l^{-1}, as was pQGRFamide (the anthozoan peptide) (Grimmelikhuijzen & Graff, 1986) in which phenylalanine in the 1-position was replaced by pyroglutamate (see Table 1). FMRYamide, where phenylalanine in the 4-position is replaced by tyrosine, only increased frequency and amplitude slightly at 10^{-6} mol l^{-1} during the course of the pulse. In contrast, a 10^{-5} mol l^{-1} pulse increased the amplitude of the heart contractions by over 100% during the post-application wash-out period (Fig. 6).

RMRFamide, however, was unusual amongst the amidated tetrapeptide ana

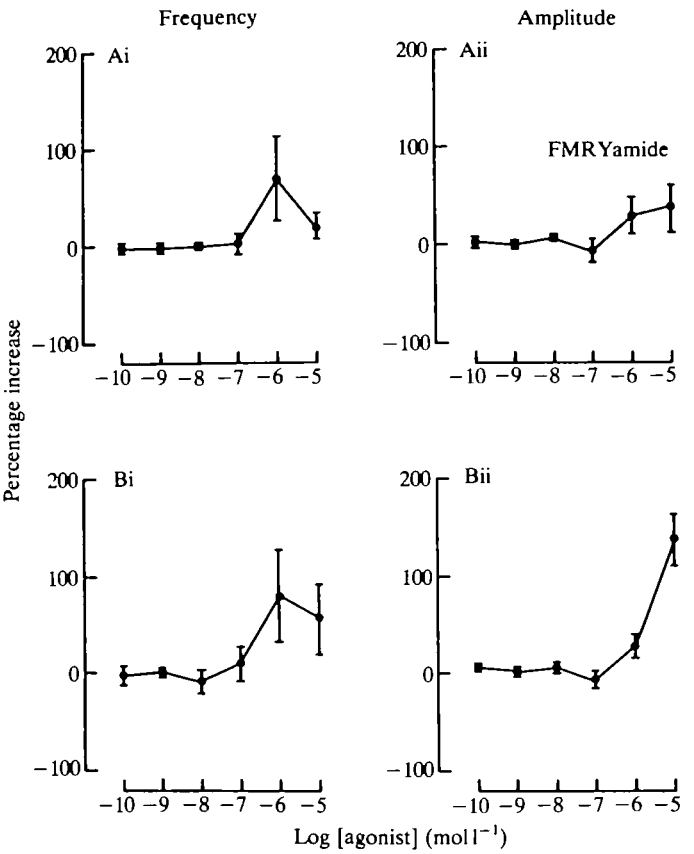

Fig. 6. Dose–response curves for the actions of FMRYamide on (i) the contraction frequency and (ii) the contraction amplitude of the semi-isolated heart preparation of the locust. The data in A were obtained during the penultimate minute of a 5-min exposure of the preparation to the peptide, and the data in B were obtained for a 1-min period starting 1 min after the end of the peptide pulse. The error bars represent the standard error of the mean and $N = 3$.

logues in that it exhibited biphasic cardioinhibitory and cardioexcitatory effects as mentioned above (Fig. 3). At low concentrations, in the region of 10^{-9} mol l^{-1}, cardioexcitation was manifested primarily as an increase in amplitude of the heart beat (Fig. 5B). However, this excitatory response normally appeared in the fourth or fifth minute of the peptide pulse and occasionally only occurred after the pulse had finished. Above 10^{-7} mol l^{-1} cardioinhibition occurred during the peptide pulse, frequently starting within 1 min of the peptide application. In Fig. 3, which shows the results with an irregularly beating heart, the onset of inhibition was slow with the 10^{-6} mol l^{-1} pulse but extremely rapid with the 10^{-5} mol l^{-1} pulse. However, during the latter pulse the amplitude of the contractions increased even during the period of frequency inhibition. In both examples the end of the peptide pulse was followed by a period of pronounced cardioexcitation.

Tetrapeptide analogues of FMRFamide lacking the C-terminal amidation (i.e. FMRF and FLRF) were essentially inactive on the locust heart, as was the dipeptide RFamide, at concentrations up to 10^{-5} mol l^{-1} (see Table 1).

Effects of N-terminally-extended analogues of FMRFamide

Amidated N-terminally-extended peptide analogues of FMRFamide were generally more potent than FMRFamide itself (see Table 1). The lobster peptide F_1 (TNRNFLRFamide) (Trimmer *et al.* 1987) was the most potent of all the peptides tested on the heart, being 1000 times more potent than FMRFamide. Its threshold for observable effects occurred between 10^{-10} and 10^{-9} mol l^{-1} (Fig. 7A), the increase in amplitude reaching 198 ± 59 % at 10^{-8} mol l^{-1} during the pulse and 228 ± 76 % 1 min after the pulse at the same concentration. From 10^{-8} to 10^{-6} mol l^{-1} there was no increase in response; however, at 10^{-5} mol l^{-1} the increases in frequency and amplitude declined to 9 ± 34 % and 42 ± 58 %, respectively (Fig. 7A).

In general the other N-terminally-extended peptides tested were cardioexcitatory at low concentrations and at higher concentrations showed some cardioinhibition or a complex combination of the two effects. Two peptides, SDPFLRFamide and GDPFLRFamide isolated from *Lymnaea* (Ebberink *et al.* 1987), were the next most potent peptides tested on the locust heart, being 100–1000 times more potent than FMRFamide. They had thresholds for cardioexcitatory effects between 10^{-10} and 10^{-9} mol l^{-1} and 10^{-9} and 10^{-8} mol l^{-1}, respectively, for their effects on the amplitude of contractions (Fig. 7B). At concentrations up to 10^{-6} mol l^{-1} the excitatory responses of SDPFLRFamide increased in a dose-dependent manner for increases in amplitude, with little increase in frequency occurring (Fig. 7B) . At 10^{-6} mol l^{-1} the frequency declined slightly, during and after the peptide pulse, but the amplitude increased by more than 100 % throughout. At 10^{-5} mol l^{-1} a biphasic effect on contraction amplitude was produced, with excitation followed by inhibition occurring during the peptide pulse and with excitation occurring again once the peptide had been washed from the preparation. The frequency was depressed throughout the pulse, decreasing by over 50 % by the fourth minute of the pulse (Fig. 7B). The responses to GDPFLRFamide were practically identical to those of SDPFLRFamide. At a concentration of 10^{-6} mol l^{-1} and above GDPFLRFamide produced biphasic effects during the period of peptide application, with an initial period of excitation being followed by a decrease in frequency and amplitude. A subsequent excitation occurred during the post-application wash. At 10^{-5} mol l^{-1}, it produced either an excitation followed by an inhibition and no recovery or simply an inhibition with no excitation in the post-application period (not shown).

The related *Helix* neuropeptide pQDPFLRFamide (Price *et al.* 1985) had a threshold for cardioexcitatory effects between 10^{-8} and 10^{-7} mol l^{-1}, although at higher concentrations, in the range $10^{-6} - 10^{-5}$ mol l^{-1}, it produced a decrease in the frequency of contractions associated with an increase in their amplitude (Fig. 8A) and an increase (126 ± 72 %, $N = 7$, at 10^{-6} mol l^{-1}) in amplitude in the

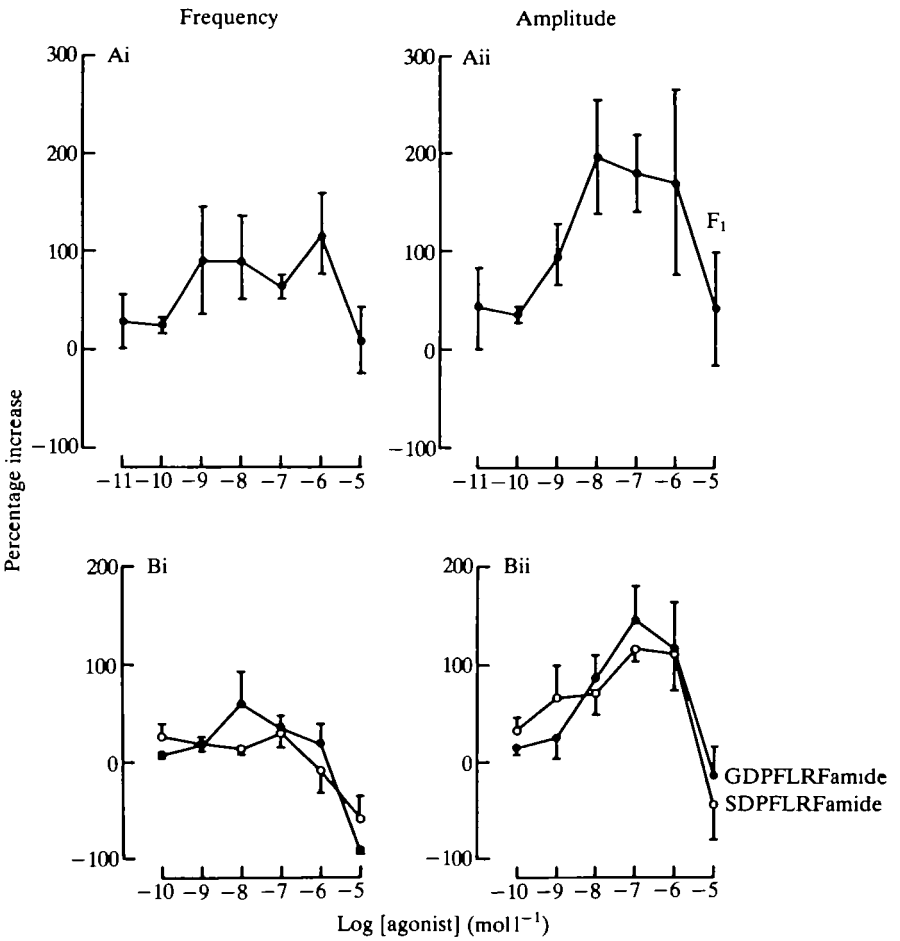

Fig. 7. Dose–response curves for the actions of (A) the lobster peptide, F_1 (TNRNFLRFamide) and (B) the *Lymnaea* peptides, GDPFLRFamide (●) and SDPFLRFamide (○) on (i) the contraction frequency and (ii) the contraction amplitude of the semi-isolated heart preparation of the locust measured during the penultimate minute of a 5-min exposure of the preparation to the peptide. The error bars represent the standard error of the mean and $N = 5$ in all cases.

post-application period. However, in this concentration range in several preparations it produced a complete inhibition of contractions (not shown).

YGGFMRFamide was 10–100 times more potent than FMRFamide with a threshold occurring between 10^{-9} and 10^{-8} mol l^{-1}. The peptide produced its effects rapidly on the heart, usually within 1 min, compared with those of FMRFamide which usually took at least 3 min to develop (compare Figs 1 and 2). At concentrations above 10^{-7} mol l^{-1} a biphasic response was produced with a large increase in amplitude ($155 \pm 31\%$, $N = 8$ at 10^{-6} mol l^{-1}) during the post-application period (Figs 2, 8A), whereas at 10^{-5} mol l^{-1} the heart contractions

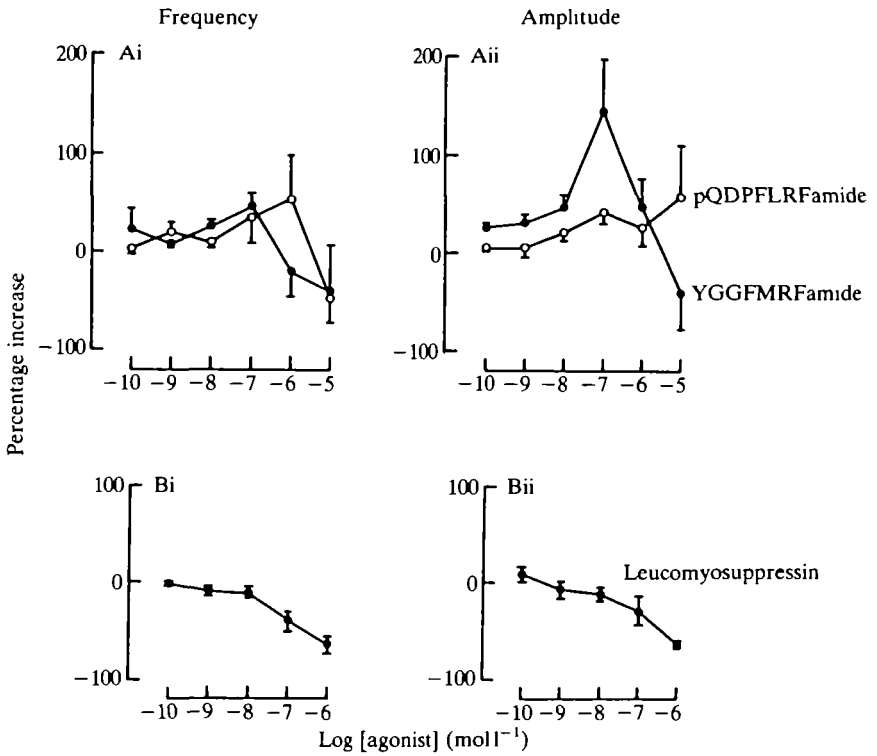

Fig. 8. Dose–response curves for the actions of (A) YGGFMRFamide (●) and pQDPFLRFamide (○) and (B) leucomyosuppressin (●) on (i) the contraction frequency and (ii) the contraction amplitude of the semi-isolated heart preparation of the locust measured during the penultimate minute of a 5-min exposure to the peptide. The error bars represent the standard error of the mean and $N = 7$ for YGGFMRFamide and pQDPFLRFamide and $N = 3$ for leucomyosuppressin.

were either inhibited completely with no recovery or showed a transient excitation followed by inhibition. Since YGGFMRFamide also contains the sequence of Met-enkephalin, YGGFM, it was of interest to see if the addition of the N-terminal extension was responsible for the cardioinhibitory effects, enabling the molecule to interact with opioid receptors at high concentrations. However, Met-enkephalin at concentrations up to 10^{-5} mol l^{-1} did not produce any significant cardioinhibitory effects on the locust heart. In fact at high concentrations, in the range $10^{-6} - 10^{-5}$ mol l^{-1}, it produced a slight cardioexcitatory effect, primarily in the post-application washout period (see Table 1). Additionally, Met-enkephalin (10^{-6} mol l^{-1}) produced no alteration in the cardioexcitatory effects of a 5-min pulse of 10^{-7} mol l^{-1} FMRFamide when the two peptides were applied to the locust heart simultaneously (not shown).

YFMRFamide produced excitatory effects with a threshold occurring around 10^{-9} mol l^{-1} and with maximal increases in frequency of heart beat (58 ± 47 %

occurring at 10^{-7} mol l^{-1} and maximal increases in amplitude (47 ± 22 %) occurring at 10^{-8} mol l^{-1}, both effects reaching a peak during the post-application wash period. However, at concentrations greater than 10^{-7} mol l^{-1} it produced inhibitory effects which even extended into the post-application phase at 10^{-5} mol l^{-1}.

Leucomyosuppressin was the only peptide to elicit a purely inhibitory response from the heart (see Fig. 4). The threshold for an observable inhibition was between 10^{-8} and 10^{-7} mol l^{-1} (Fig. 8B). Leucomyosuppressin was equally potent in decreasing frequency and amplitude of heart contractions.

Additionally, the non-C-terminally amidated N-terminally extended FMRFamide analogue, YGGFMRF, was found to be ineffective on the locust heart (see Table 1).

Other related peptides

Another class of molluscan peptides, the small cardioactive peptides or SCPs (Lloyd, 1982), also show a weak structural similarity to FMRFamide-like peptides (Morris *et al.* 1982; Lloyd *et al.* 1987). Immunocytochemical evidence has been presented for the presence of SCP-like molecules in the locust nervous system, and exogenously applied SCP_A and SCP_B are capable of activating the myogenic rhythm of the locust extensor tibiae muscle in a dose-dependent manner (Evans & Myers, 1986*a*; P. D. Evans, unpublished data). However, when applied to the semi-isolated locust heart preparation, neither SCP_A nor SCP_B were particularly effective cardioacceleratory agents at concentrations below 10^{-6} mol l^{-1}. At 10^{-5} mol l^{-1}, in contrast, both SCP_A and SCP_B increased contraction amplitudes by over 270 % and 120 %, respectively, whilst only producing much smaller increases in the frequency of the heart contractions (Fig. 9). The low potency of these molluscan peptides on the locust heart may reflect the fact that they differ structurally from the endogenous locust SCP-like neuropeptides.

Effects of FMRFamide analogues on the extensor tibiae muscle

To facilitate a comparison between the cardioexcitatory effects of FMRFamide analogues on the locust heart and those produced by the same analogues on neurally induced contractions of the locust extensor tibiae muscle, we have surveyed the effects on the latter preparation of all the analogues used in the present study whose effects have not previously been reported (see Evans & Myers, 1986*a*,*b*). Table 2 shows the increases in the amplitude and relaxation rate of slow motor neurone-induced twitch tension in the extensor tibiae muscle produced by the application of 5-min pulses of the various peptides at a concentration of 10^{-6} mol l^{-1}. The results show a considerable similarity in the response profiles of the analogues with their cardioexcitatory effects described above.

N-terminally extended analogues of FMRFamide were the most effective compounds tested. The crustacean octapeptide, F_1, was again the most potent potentiator of both slow motor neurone twitch tension amplitude and relaxation rate. The threshold for an observable action of this peptide occurred between

10^{-11} and 10^{-12} mol l^{-1}. Its maximal effect on the increase in twitch tension amplitude of 183·3 ± 3·4 % ($N = 3$) occurred at 10^{-7} mol l^{-1}, with a smaller effect being observed at 10^{-6} mol l^{-1} (see Table 2). Maximal effects were also obtained with three of the heptapeptide analogues tested, namely YGGFMRFamide (see Evans & Myers, 1986*a*,*b*) and the two *Lymnaea* peptides, SDPFLRFamide and GDPFLRFamide. These peptides were less potent than the crustacean peptide, F_1, and had thresholds for an observable action between 10^{-9} and 10^{-10} mol l^{-1}. The C-terminally blocked heptapeptide, pQDPFLRFamide, isolated from *Helix*, gave maximal responses at 10^{-5} mol l^{-1}, but was less potent than the other heptapeptides tested, having a threshold for an observable effect occurring between 10^{-8} and 10^{-9} mol l^{-1}. YFMRFamide was equipotent with pQDPFLRFamide.

The tetrapeptide analogues of FMRFamide were not as potent as their N-terminally extended counterparts described above. FMRFamide itself was the only tetrapeptide to produce maximal effects, a concentration of 10^{-5} mol l^{-1} being required. FLRFamide and FMRYamide produced submaximal effects at 10^{-5} mol l^{-1}, whereas FPRFamide, RMRFamide, FGRFamide and pQGRFamide (anthoRFamide) produced no detectable effects up to a concentration of 10^{-6} mol l^{-1}. In addition, no detectable effects were observed with either N-terminally extended analogues or tetrapeptides lacking the C-terminal amide group (see Table 2). Furthermore, the other molluscan neuropeptides, SCP_A and SCP_B, which show a weak structural homology with FMRFamide, did

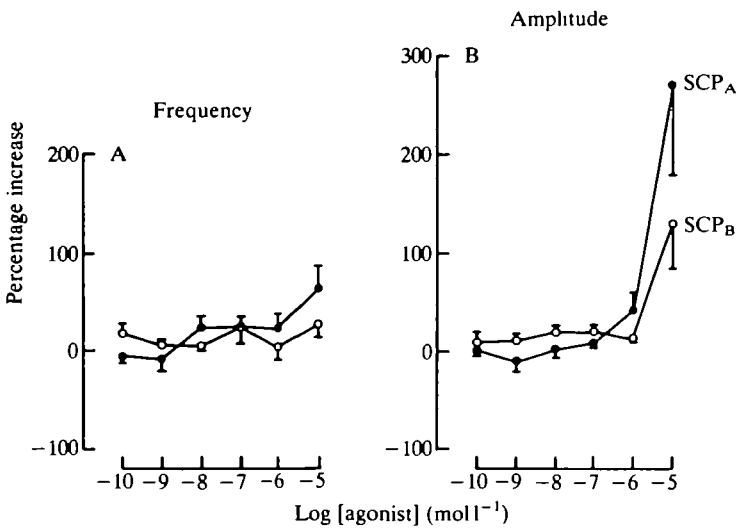

Fig. 9. Dose–response curves for the actions of SCP_A (●) and SCP_B (○) on (A) the contraction frequency and (B) the contraction amplitude of the semi-isolated heart preparation of the locust measured during the penultimate minute of a 5-min exposure to the peptide. The error bars represent the standard error of the mean and $N = 5$.

not have any observable potentiating effects on slow motor neurone-induced twitch tension up to a concentration of 10^{-5} mol l^{-1}. However, both peptides were potent activators of the myogenic rhythm found in the extensor tibiae muscle with thresholds for an increase in rhythm frequency occurring between 10^{-8} and 10^{-9} mol l^{-1} (see Evans & Myers, 1986*a*; P. D. Evans, unpublished data).

Leucomyosuppressin was unusual since it reduced both the amplitude and the rate of relaxation of slow motor neurone-induced twitch tension at concentrations of 10^{-6} mol l^{-1} and above. At lower concentrations, between 10^{-7} and 10^{-8} mol l^{-1}, it produced a small increase in these parameters of 10 – 20 %, and at concentrations of 10^{-9} mol l^{-1} and below no effects were observed. Leucomyosup-

Table 2. *The specificity of FMRFamide-like peptides on the locust extensor tibiae muscle*

Peptide	Increase in twitch amplitude (%)	Increase in twitch amplitude ED_{50} (mol l^{-1})	Increase in relaxation rate of twitch tension (%)	Increase in relaxation rate of twitch tension ED_{50} (mol l^{-1})	*N*
TNRNFLRFamide	160·5 ± 4.4	$2{\cdot}0 \times 10^{-9}$	218·9 ± 4·7	$4{\cdot}0 \pm 10^{-9}$	3
GDPFLRFamide	170·0 ± 13·2	$7{\cdot}4 \times 10^{-8}$	188·0 ± 24·7	$5{\cdot}0 \pm 10^{-8}$	3
YGGFMRFamide	185·7 ± 7·9	$8{\cdot}5 \times 10^{-8}$	218·2 ± 19·7	$1{\cdot}5 \pm 10^{-7}$	3
SDPFLRFamide	188·3 ± 6·3	$1{\cdot}2 \times 10^{-7}$	220·5 ± 35·2	$8{\cdot}2 \pm 10^{-8}$	3
YFMRFamide	107·2 ± 32·0	$6{\cdot}0 \times 10^{-7}$	127·0 ± 33·3	$6{\cdot}8 \pm 10^{-7}$	3
pQDPFLRFamide	109·5 ± 21·1	$6{\cdot}2 \times 10^{-7}$	130·4 ± 26·6	$5{\cdot}6 \pm 10^{-7}$	3
FMRFamide*	96·9 ± 7·2	$9{\cdot}7 \times 10^{-7}$	97·8 ± 14·2	$2{\cdot}0 \pm 10^{-6}$	9
FLRFamide	65·7 ± 1·1	–	77·5 ± 5·6	–	3
FMRYamide	28·8 ± 11·1	–	22·7 ± 13·6	–	3
LPLRFamide*	9·0 ± 2·3	$5{\cdot}0 \times 10^{-5}$	11·8 ± 4·2	$4{\cdot}0 \pm 10^{-5}$	3
FPRFamide	0	–	0	–	3
RMRFamide	0	–	0	–	3
FGRFamide	0	–	0	–	3
pQGRFamide	0	–	0	–	3
RFamide*	0	–	0	–	3
YGGFMRF	0	–	0	–	3
FMRF	0	–	0	–	3
FLRF	0	–	0	–	3
YGGFM*	0	–	0	–	3
SCP_A	0	–	0	–	3
SCP_B	0	–	0	–	3
BPP*	0	–	0	–	3
APP*	0	–	0	–	3
HPP*	0	–	0	–	3
pQDVDHVFLRFamide	−11·8 ± 6·7	–	−8·3 ± 4·8	–	3

5-min pulses of peptides were applied to the preparation at a concentration of 10^{-6} mol l^{-1}.
Each result is expressed as the mean ± standard error of the number of observations shown.
ED_{50} values were calculated from dose–response curves.
The slow motor neurone was stimulated at 1 Hz.
* indicates values taken from Evans & Myers (1986*b*).
BPP, APP and HPP, bovine, avian and human pancreatic polypeptides, respectively.
The peptides are ranked on the basis of their ED_{50} for increases in twitch amplitude.

pressin also inhibited the myogenic rhythm found in the extensor tibiae muscle, with a threshold occurring between 10^{-8} and 10^{-9} mol l^{-1} (data not shown).

Discussion

The actions of FMRFamide-like peptide analogues on the semi-isolated heart preparation of the locust are complex. They can be cardioexcitatory, cardioinhibitory or biphasic depending on the structures of the analogues and their concentrations. In addition, the cardioexcitatory effects, which are manifested as increases in the frequency and amplitude of heart contractions, are influenced by the form of the beating activity exhibited by the heart prior to the peptide application. This is particularly true for frequency. The simplest explanation for the presence of both cardioexcitatory and cardioinhibitory effects elicited by the different structural analogues could be that there are multiple receptor types for FMRFamide-like peptides present in the heart preparation, each with a different mode of action. More complex explanations are possible but discussion of these awaits the development of specific antagonists for the receptors mediating the actions of FMRFamide-like peptides in this preparation.

The cardioexcitatory effects of the FMRFamide-like peptides are likely to be mediated by a second messenger system since they are long-lasting and do not disappear until several minutes after the end of the peptide pulse. We do not know the nature of this second messenger, but have ascertained that FMRFamide, at concentrations up to 10^{-5} mol l^{-1}, does not change either the cyclic AMP or the cyclic GMP levels in this preparation (P. D. Evans & B. A. Cuthbert, unpublished data). In addition, exogenously applied arachidonic acid (at estimated concentrations up to $3{\cdot}3\times10^{-4}$ mol l^{-1}) did not appear to mimic the excitatory actions of FMRFamide-like peptides on locust hearts (B. A. Cuthbert & P. D. Evans, unpublished results). Similarly, arachidonic acid (at the same concentration) did not mimic the actions of FMRFamide on the extensor-tibiae muscle preparation of the locust. Further, in this preparation the actions of FMRFamide (10^{-6} mol l^{-1}) were not blocked in the presence of 4-bromophenacyl bromide (10^{-5} mol l^{-1}) which inhibits the enzymes responsible for the breakdown of phospholipid to arachidonic acid, by nordihydroguaiaretic acid (10^{-6} mol l^{-1}) which blocks the action of lipoxygenases or by indomethacin (10^{-5} mol l^{-1}) which blocks the production of prostaglandins by cyclo-oxygenase (Evans *et al.* 1988). These results contrast with the actions of FMRFamide on *Aplysia* sensory neurones where it has been suggested that lipoxygenase metabolites of arachidonic acid mediate the inhibitory responses to FMRFamide (Piomelli *et al.* 1987).

The cardioinhibitory effects, in contrast to the cardioexcitatory effects, are generally only observed during the presence of the peptide in the tissue superfusate and decay rapidly at the end of the pulse. This makes it easier to compare the cardioexcitatory effects of FMRFamide analogues than their cardioinhibitory effects, since measurements taken during the post-application saline wash are unlikely to be confused by the presence of the two competing effects.

In general, N-terminally extended, C-terminally amidated, FMRFamide analogues are the most potent cardioexcitatory peptides tested on the semi-isolated heart preparation of the locust. Removal of the C-terminal amide group results in a drastic loss in activity (compare YGGFMRFamide with YGGFMRF, FMRFamide with FMRF, and FLRFamide with FLRF). Substitution of the methionine in position-2 of FMRFamide with a leucine does not reduce its cardioexcitatory potency (compare FMRFamide with FLRFamide), but substituting it with a glycine or a proline substantially reduces its potency (compare FMRFamide with FGRFamide and FPRFamide). Substitution of the phenylalanine at position-1 for arginine (compare FMRFamide with RMRFamide) or substitution of the phenylalanine at position-4 with tyrosine (compare FMRFamide with FMRYamide) also substantially reduces the potency of the molecule. In addition, the dipeptide RFamide is not long enough to produce a cardioexcitatory effect. The above pharmacological profile is extremely similar to that obtained for the modulation of neuromuscular transmission in the extensor tibiae muscle of the locust (see Table 2 and Evans & Myers, 1986*a*,*b*). In both cases the N-terminally extended analogues of FMRFamide are the most effective analogues tested and removal of the C-terminal amide group leads to a total loss of activity. Some slight differences do, however, occur since the tetrapeptides FPRFamide and RMRFamide together with the SCPs, which are all totally inactive on the extensor tibiae muscle, produce slight cardioexcitatory effects on some heart preparations. In addition, YFMRFamide, which is almost equipotent with FMRFamide on the locust extensor tibiae muscle, is much less effective than FMRFamide on the locust heart preparation.

The presence of simultaneous cardioexcitation and cardioinhibition during the application period of some of the peptide analogues makes it impossible to make any meaningful comments on the pharmacological profile of the cardioinhibitory response in the absence of specific inhibitors of the cardioexcitatory response, except to point out that, in general, it appears that higher concentrations of the peptides are required to induce cardioinhibition than cardioexcitation. Leucomyosuppressin was the only FMRFamide analogue to produce purely cardioinhibitory effects. In addition, it also reduced the amplitude and rate of relaxation of slow motor neurone-induced twitch tension in the extensor tibiae muscle, as well as inhibiting the myogenic contractions found in a bundle of specialized muscle fibres in this muscle. This peptide was originally isolated and sequenced from purified head extracts of the cockroach, *Leucophaea maderae*, as a peptide factor that inhibited spontaneous contractions of the isolated hindgut (Holman *et al.* 1986), so it may be an inhibitory neuropeptide that can reduce the size of both spontaneous and neurally evoked contractions in a variety of insect muscles. Its ability to produce a weak potentiation of neurally evoked contractions, at low concentrations, in the extensor tibiae muscle, suggests it may also have a weak affinity for the FMRFamide-like receptors responsible for the potentiation of twitch tension in this muscle, but that at higher concentrations this effect is masked by its much stronger inhibitory effects.

FMRFamide-like peptides also have cardioexcitatory effects on the crustacean heart (Krajniak & Greenberg, 1988). A structure–activity study of the effects of FMRFamide-related peptides on the heart of the blue crab, *Callinectes sapidus*, indicated that the two known crustacean peptides, TNRNFLRFamide and SDRNFLRFamide, had the lowest thresholds (10^{-9}–10^{-8} mol l^{-1}) whereas the heptapeptides, SDPFLRFamide and pQDPFLRFamide were 1000-fold less potent. Interestingly, leucomyosuppressin had a very high threshold (10^{-5} mol l^{-1}) on the crab heart and it was not reported to produce any cardioinhibitory effects.

The pharmacological profile outlined above, for the actions of FMRFamide-like peptides on the locust heart, has many similarities with those observed for the actions of the same analogues on some molluscan preparations, such as the hearts of *Mercenaria*, *Helix*, *Rapana, Macrocallista* and *Lampsilis* (Price & Greenberg, 1980; Painter *et al.* 1982; Kobayashi & Muneoka, 1986; Payza, 1987), the radula protractor muscle of *Busycon* and the anterior byssus retractor muscle of *Geukensia* (Painter *et al.* 1982). In all cases the C-terminal amide and arginine in the 3-position are essential for activity and the structure of the C-terminal portion of the molecule appears to be more important than that of the N-terminal portion. In addition, the locust heart, like that of *Helix* (Payza, 1987), is more susceptible to N-terminally extended analogues. Thus, low concentrations of the heptapeptides pQDPFLRFamide, SDPFLRFamide and GDPFLRFamide are more effective than FMRFamide itself in causing cardioexcitation, although they exhibit biphasic effects at higher concentrations. Of the other molluscan bioassays that have been examined in detail, only the heart of *Macrocallista* and the anterior byssus retractor muscle of *Geukensia* show slight preferences for N-terminally extended analogues (Painter *et al.* 1982; and see Payza, 1987). The differences in the speeds of action and in the effects produced by the tetrapeptide analogues of FMRFamide and the N-terminally extended analogues, such as YGGFMRFamide and the various heptapeptides, in different molluscan preparations, have led authors to propose that a variety of different classes of receptor site for FMRFamide-like molecules may exist on molluscan hearts (Payza, 1987) and molluscan neurones (Cottrell *et al.* 1984; Cottrell & Davies, 1987). These may mediate their actions by modulation of at least four different ion channel types in molluscan neurones (Ruben *et al.* 1986; Cottrell & Davies, 1987). It seems very likely that a similar diversity of receptors for FMRFamide-like peptides will exist in the locust heart. The cardioexcitatory effects of the N-terminally extended analogues can be initiated more rapidly than those produced by the tetrapeptide derivatives and the former peptides also induce cardioinhibition at higher concentrations. Investigations into the exact number of receptor types present, and their individual modes of action, will have to wait until specific antagonists become available for the different receptor subtypes.

The site of action of the FMRFamide-like peptides superfused onto the semi-isolated heart preparation of the locust is not known. They could act directly on the myocardium or act indirectly on the nervous elements associated with the heart. Thus, they could release material from the endings of the neurone

projecting to the heart from the ventral nerve cord *via* the lateral segmental nerves or from the endings of the intrinsic neurosecretory neurones found in the lateral cardiac nerve cords of the locust, which are still present in the semi-isolated heart preparation. Further experiments are in progress to determine their site of action in this preparation.

An assessment of the physiological significance of the observed cardioregulation of the locust heart by FMRFamide-like peptides requires that the tissue be shown to be exposed to the endogenous FMRFamide-like peptides at physiological concentrations. At present we do not know the sequences of all the FMRFamide-like peptides present in the locust nervous system. We do know, however, that on high pressure liquid chromatography we can identify at least three major and several minor peaks of FMRFamide-like immunoreactivity (S. Robb & P. D. Evans, in preparation). One of the major peaks from the nervous system can also be identified in haemolymph samples, suggesting that it may be released as a neurohormone. We have detected this peak in haemolymph samples up to a concentration of 10^{-8} mol l^{-1}, using FMRFamide as the radioimmunoassay standard (S. Robb & P. D. Evans, in preparation). Thus the observed effects described in this paper for the most active peptides tested could well occur in the physiological range for the endogenous peptides. In addition, at least one of the other major peaks of immunoreactivity found in the locust nervous system is also present in the locust heart (S. Robb & P. D. Evans, in preparation). Thus the activity of the locust heart could be regulated by FMRFamide-like peptides, both released into the haemolymph as neurohormones and released more locally as local neuromodulators or neurotransmitters from neuronal elements within the heart itself.

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