PEPTIDE F₁, AN N-TERMINALLY EXTENDED ANALOG OF FMRFamide, ENHANCES CONTRACTILE ACTIVITY IN MULTIPLE TARGET TISSUES IN LOBSTER

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Summary

The physiological actions of lobster peptide F₁ (TNRNFLRFamide) have been examined on three different lobster nerve–muscle preparations (exoskeletal, cardiac and visceral). The peptide, which is found at high concentrations in a lobster neurosecretory gland, causes a long-lasting enhancement of contractility in each target tissue. On exoskeletal nerve–muscle preparations, peptide F₁ has the following actions: (1) it potentiates transmitter release from nerve terminals innervating exoskeletal muscle, leading to an increase in both spontaneous and nerve-evoked release of transmitter; (2) it acts directly on the muscle, in the absence of nerve activity, to induce tonic contractions; and (3) it shows a potent desensitization that

does not reverse with prolonged washing of the tissue. On each of the types of muscle examined, peptide F₁ is active at nanomolar concentrations and is 3–4 orders of magnitude more potent than FMRFamide. These findings suggest that peptide F₁ is a neurohormone with widespread myogenic actions throughout lobster peripheral tissues. The molecular mechanism(s) by which the peptide acts are not yet known, but do not appear to involve cyclic AMP or cyclic GMP.

Key words: TNRNFLRFamide, neuromodulation, crustacean neuromuscular junction, crustacean heart, lobster, *Homarus americanus*.

Introduction

More than 100 members of the invertebrate family of FMRFamide-related peptides (FaRPs) have been identified, all of which share the carboxy-terminal sequence –RFamide. The first member of this family sequenced, the tetrapeptide FMRFamide, was isolated from the molluscan central nervous system (CNS) by Price and Greenberg (1977). Since that time, short and long forms of these peptides have been isolated from the nervous systems of many invertebrate species, including echinoderms, annelids, nematodes, crustaceans and insects. In addition, genes encoding precursors of these peptides have been isolated and sequenced from several invertebrate species (for a review, see Greenberg and Price, 1992). Vertebrate members of this peptide family have also been isolated from chick and bovine brain (Yang and Majane, 1990) and they share sequence homology with the vertebrate family of opioid peptides, raising the possibility that vertebrate FaRPs might be active at opioid receptors. Indeed, when tested in specific vertebrate model systems, FaRPs can act either as agonists or as competitive antagonists at opiate receptors in different tissues (Raffa, 1991; Raffa and Jacoby, 1990). A genetic analysis of the FaRP precursor gene in *Aplysia* has led to the suggestion that the invertebrate and vertebrate FaRPs and the

opioid peptides may have resulted from the divergent evolution of common ancestral genes (Taussig and Scheller, 1986).

Physiologically, FaRPs mediate a wide and diverse set of actions on multiple target tissues in many different invertebrates. For example, members of the FaRP family modulate neural activity in rhythmically active circuits such as the stomatogastric ganglion of crustaceans (Hooper and Marder, 1984; Marder *et al.* 1987; Weimann *et al.* 1993) and alter the excitability of central neurons in molluscs (Ruben *et al.* 1984; Thompson and Ruben, 1988; Brezina *et al.* 1987a,b; Ichinose and McAdoo, 1988) and leeches (Simon *et al.* 1992). FaRPs potentiate neurally evoked contractions of skeletal muscle in locusts (Evans and Myers, 1986; Walther *et al.* 1984; Cuthbert and Evans, 1989), moths (Kingan *et al.* 1990) and crayfish (Mercier *et al.* 1990; Skerrett *et al.* 1995). They also regulate nerve–muscle interactions in the shrimp stomatogastric system, where the muscles are shifted from quiescence through an oscillatory state of rhythmic contractions, to a sensitized state in which the contractile effects of neural inputs are amplified (Meyrand and Marder, 1991). In addition, FaRPs also have direct excitatory and

inhibitory effects on muscles, notably on cardiac tissues in molluscs (Painter and Greenberg, 1982), chelicerates (Groome, 1993), crustaceans (Krajniak, 1991; Mercier and Russenes, 1992; Skerrett *et al.* 1995), insects (Cuthbert and Evans, 1989) and a leech (Norris and Calabrese, 1987, 1990; Thompson and Calabrese, 1992).

In the decapod crustacean *Homarus americanus*, two members of the FaRP family have been isolated and characterized. TNRNFLRFamide (peptide F₁) and SDRNFLRFamide (peptide F₂) were purified from the neurosecretory pericardial organs, a site of high concentration of these peptides in the lobster (Trimmer *et al.* 1987). Immunocytochemical studies suggest that F₁ and F₂, or other members of this family, are widely distributed in the lobster nervous system, being found in peripheral neurosecretory areas, within CNS neuropil regions and in a specific subset of neuronal cell bodies in the CNS (Kobierski *et al.* 1987). Peptide F₁ is released from pericardial organs by depolarization in the presence of calcium, is detected in the hemolymph and is bioactive on various muscle tissues at low concentrations, suggesting a possible neurohormonal role for this peptide (Trimmer *et al.* 1987; Kobierski *et al.* 1987; Goy *et al.* 1987a,b).

Peptides identical in sequence to F₁ have been isolated only from *Homarus americanus* and the crab *Cancer borealis* (Weimann *et al.* 1993), although a similar peptide is reported to be present in the spiny lobster *Panulirus interruptus* (Marder *et al.* 1987). A closely related peptide (NRNFLRFamide) has been purified from crayfish pericardial organs (Mercier *et al.* 1993). Interestingly, structure/activity studies in several species have shown that peptide F₁ is the most potent of all FaRPs tested in stimulating contractility in various neuromuscular preparations, including the heart in blue crabs (Krajniak, 1991), the heart and extensor tibialis skeletal muscle of locusts (Cuthbert and Evans, 1989), the stomatogastric muscles of shrimps (Meyrand and Marder, 1991) and the phasic skeletal muscles of crayfish (Mercier *et al.* 1990). In fact, in certain of the species in which endogenous FMRamide-related peptides have been isolated, F₁ is more potent than the purified native peptides.

In this paper, the physiological effects of peptide F₁ are tested on three different nerve-muscle preparations from the lobster *Homarus americanus*. In heart, oesophageal muscle and exoskeletal muscle preparations, peptide F₁ induces or augments contractility. In exoskeletal muscle preparations, pre- and post-junctional effects are seen and the peptide is about three orders of magnitude more potent than FMRamide. In all the preparations, peptide F₁ is active at nanomolar concentrations, consistent with its postulated neurohormonal role. A preliminary report has been published describing the results presented here (Goy *et al.* 1987b). In an accompanying paper, Skerrett *et al.* (1995) describe similar effects of the related crayfish peptides (NF₁, NRNFLRFamide; and DF₂, DRNFLRFamide) on crayfish target tissues.

Materials and methods

Animals and tissues

Lobsters (*Homarus americanus* Milne Edwards) of both sexes weighing approximately 0.5 kg were purchased locally and held in artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH) at 12–14 °C. All tissues were dissected in ice-cold lobster saline (462 mmol l⁻¹ NaCl, 16 mmol l⁻¹ KCl, 26 mmol l⁻¹ CaCl₂, 8 mmol l⁻¹ MgCl₂, 11 mmol l⁻¹ glucose, 10 mmol l⁻¹ Tris-maleate or Hepes, pH 7.4).

Exoskeletal muscle

The dactyl opener muscle and its associated excitatory and inhibitory nerves in the propopodite segment of the walking leg were exposed by removing overlying exoskeleton, muscle and sensory nerves, as described by Glusman and Kravitz (1982).

Heart

The heart was isolated by cutting dorsally through the thoracic carapace, removing a small section of exoskeleton with the heart attached and dissecting away surrounding tissues. The sternal artery was cannulated for perfusion, as described previously (Battelle and Kravitz, 1978).

Oesophagus

The oesophagus was isolated as a ring of muscle from the anterior part of the gut and cut from its posterior end to produce a flat sheet. The muscle was isolated together with the pair of circumoesophageal ganglia and the single suboesophageal ganglion attached.

Tension measurements and intracellular recording

Isolated preparations were superfused continuously with lobster saline at 10–13 °C (2–3 ml min⁻¹), to which hormones were added at the concentrations indicated. Based on the latency to muscle contraction in response to bath application of high-K⁺ saline, the exchange time of the bath by the perfusion system can be estimated as 1–2 min. Muscle tension was measured almost isometrically by fastening the apodeme of the muscle to a Grass FT03 force transducer with surgical thread. Quantitative tension measurements were made by calibrating the force transducer and measuring the force exerted at the peak of the contractile response in units of grams. For intracellular recordings, muscle fibers were penetrated with 3–5 MΩ glass microelectrodes filled with 2–4 mol l⁻¹ potassium acetate. Muscle fiber resting potentials ranged from –55 mV to –80 mV; recordings from fibers with resting potentials outside this range were discarded. Nerves were stimulated extracellularly, using suction electrodes. Membrane potential changes were recorded differentially between the microelectrode and the bath, grounded through a silver/silver chloride electrode. The force transducer and electrode outputs were amplified and recorded on tape, using an audio tape recorder (Hewlett-Packard 3968A Instrumentation Recorder) or a VCR (VR-100A Digital Recorder, Instrutech Corp). Tension recordings, excitatory (EJP) and inhibitory (IJP)

junctional potentials and miniature EJPs (MEJPs) were digitized and analyzed using a microcomputer with VR111 (Instrutech Corp.) and pClamp software (Axon Instruments).

Cyclic nucleotide measurements

For measurement of heart cyclic nucleotide levels, intact cardiac ganglia were obtained from the inner chambers of the hearts of several animals and the remaining heart muscle was cut into several pieces of approximately equal size. Exoskeletal muscles were obtained from walking legs of several animals as described above, except that the excitatory and inhibitory nerves were dissected from the surface of the muscles and discarded. When possible, dissected tissues were divided so that control and experimental groups contained samples from all animals. All experiments included a 2 h preincubation in lobster saline containing 0.5 mmol l^{-1} isobutyl methylxanthine (IBMX), followed by a 15 min exposure to test hormones in the same medium. At the end of the incubation period, each piece of tissue was frozen in methanol at -80°C and immediately homogenized in ice-cold 6% trichloroacetic acid. Samples were processed and analyzed for cyclic AMP or cyclic GMP content by radioimmunoassay (procedures and materials obtained from New England Nuclear and Biomedical Technologies, Inc.; see Goy *et al.* 1984, 1987a).

Trypsin digestion of peptide F_1

Peptide F_1 ($5 \times 10^{-4} \text{ mol l}^{-1}$) was incubated with trypsin (50 mg ml^{-1}) for 15 min at 21°C . The sample then was heated to 60°C for 1 min to inactivate the trypsin and diluted 1:2000 in lobster saline for application to an exoskeletal muscle preparation (final concentration of peptide: $2.5 \times 10^{-7} \text{ mol l}^{-1}$). As a control, trypsin (50 mg ml^{-1}) was heat-inactivated, diluted as above, and applied independently to the preparation. See legend to Fig. 2 for details.

Chemicals

Isobutylmethylxanthine (IBMX) was obtained from Aldrich, serotonin creatinine sulfate from Sigma and peptide F_1 (TNRNFLRFamide) and FMRFamide from Peninsula Laboratories. Some experiments were performed using peptide F_1 synthesized by Neosystems Laboratories (France). The results of experiments using peptide F_1 from either source were identical. All other chemicals were reagent grade, obtained from commercial suppliers.

Results

Peptide F_1 (TNRNFLRFamide) is an endogenous octapeptide found in, and released from, lobster pericardial organs. Because of the known neurohemal role served by pericardial organs, we examined the physiological effects of the peptide on three potential peripheral targets of this substance: exoskeletal muscle, heart and oesophagus.

Exoskeletal muscle

In the dactyl opener muscle, single EJPs and brief low-

Fig. 1. Peptide F_1 potentiates contractions and muscle tonus in an exoskeletal muscle preparation. (A) Muscle tension recorded from the dactyl opener muscle of the lobster walking leg. Upward vertical deflections in the record are twitch contractions evoked by firing brief trains of action potentials (30 Hz, 0.3 s) in the excitatory nerve at 10 s intervals. Peptide F_1 was applied as indicated by the horizontal bar. The slow upward shift in baseline tension represents the peptide-induced increase in muscle tone and can be observed both in the presence and in the absence of nerve-evoked twitch contractions (see Fig. 3). (B) A different dactyl opener preparation in which muscle tension was recorded during stimulation of the excitatory nerve (35 Hz, 0.3 s) at 10 s intervals. The peptide increases twitch contraction strength in this preparation without increasing muscle tone.

frequency ($<5 \text{ Hz}$) trains of EJPs are insufficient to produce changes in muscle tonus, whereas higher-frequency trains of facilitating EJPs evoke twitch contractions. When applied to the dactyl opener muscle, peptide F_1 elicits both an increase in muscle tone and an increase in the strength of nerve-evoked twitch contractions (Fig. 1A). These responses develop over a period of 5–10 min and can persist after washout of the peptide for up to 30 min. In some preparations, particularly at low doses, the increase in twitch contraction strength occurs without a change in muscle tone (Fig. 1B), indicating that the potentiation of twitch contractions is not secondary to the change in muscle tone.

To investigate the effects of the peptide on synaptic transmission and muscle membrane properties, recordings were made from individual fibers with intracellular microelectrodes. In the experiment shown in Fig. 2, stimuli were applied to both the excitatory and inhibitory nerves at a frequency too low to induce twitch contractions (single pulses at 0.2 Hz) and synaptic potentials were recorded. Peptide F_1 enhances the size of both EJPs and IJPs (Fig. 2, top trace) with a time course that parallels the increase in muscle tone (not shown). These effects reverse as the peptide is washed from the preparation.

To verify that the physiological effects observed were due to the intact octapeptide, the peptide was treated with trypsin before applying it to a neuromuscular preparation (Fig. 2). Trypsin treatment should cleave peptide F_1 at its arginine residues and render it biologically inactive. Consistent with this expectation, the effects of the peptide on synaptic potential

Fig. 2. Synaptic transmission is enhanced by peptide F₁. An intracellular recording from a muscle fiber is displayed at a very slow time scale in which individual synaptic responses cannot be resolved. (Boxed inset shows averaged representative responses of 50 traces taken during the periods denoted by the shaded bars at higher sweep speed.) Upward deflections from the baseline are EJPs, downward deflections are IJPs. The break in the record indicates a 1 h wash-out period during which no data were collected. Muscle resting potential was -68 mV. Bars below the traces indicate the presence of the following agents in the saline superfusing the preparation (from left to right): peptide F₁ (5×10^{-8} mol l⁻¹), heat-inactivated trypsin, peptide F₁ (2.5×10^{-7} mol l⁻¹) preincubated in trypsin and subsequently heat-treated to inactivate the trypsin (see Materials and methods), peptide F₁ (2.5×10^{-7} mol l⁻¹). Note that in this experiment the response to a second application of peptide F₁ is roughly 50% of the initial response, although the concentration of peptide has been increased fivefold.

amplitude were eliminated by pretreatment with trypsin (Fig. 2). This confirms that the intact N-terminally extended sequence is important for biological activity and rules out the possibility that the effects we see are due to a non-peptide contaminant in the synthetic peptide preparation.

The intracellular recording of Fig. 2 also illustrates an unusual feature of the actions of this peptide: the direct effects of the peptide on synaptic transmission are fully reversible, but a single application of the peptide strongly desensitizes the preparation to subsequent applications. In three experiments in which EJP size was monitored, the facilitation produced by a second exposure to 5×10^{-8} mol l⁻¹ peptide F₁ was approximately $54.0 \pm 23.3\%$ of that produced by the initial exposure; a third response was 22.0% of the initial size ($N=1$).

This desensitization was also apparent in experiments in which muscle tone was measured. An extreme example of the desensitization of the muscle contractile response to peptide F₁ is shown in the tension recording in Fig. 3, in which a second application of peptide was completely ineffective at stimulating an increase in muscle tone. In four experiments in which muscle tension was measured, a second application of 5×10^{-8} mol l⁻¹ peptide stimulated a contractile response averaging $42.9 \pm 10.3\%$ of the initial response; a third response was $26.0 \pm 5.5\%$ of the initial size ($N=2$). Washing preparations for as long as 4 h between exposures was insufficient to permit recovery. Owing to the long-lasting nature of the desensitization and the intrinsic variability between preparations, it has not been possible to establish a quantitative correlation between the concentration or duration of the initial exposure to peptide and the extent of desensitization. A similar desensitization of the synaptic potential and muscle tension response has not been observed for serotonin, octopamine and

Fig. 3. Peptide F₁ increases muscle tone. Tension was recorded from the dactyl opener muscle in the absence of nerve stimulation. Horizontal bars indicate bath applications of 5×10^{-7} mol l⁻¹ peptide F₁. Note that the second peptide application is ineffective at inducing muscle contraction.

proctolin, the other neurohormones that modulate this neuromuscular system (Kravitz, 1986).

No consistent effects on membrane potential were seen in muscle preparations treated with peptide F₁ (20 experiments). While occasional slow changes in membrane potential of small amplitude were observed (less than 2 mV), these were in hyperpolarizing or depolarizing directions with equal frequency. It is likely, therefore, that they result from movement of the preparation, or from electrode drift over the course of the experiment, and are not a consequence of treatment with the peptide. In one preparation in which resting potentials were measured before and after peptide treatment (2.5×10^{-8} mol l⁻¹ F₁ for 13 min), the resting potentials of muscle fibers recorded under control conditions (-71.2 ± 6.7 mV; $N=11$) did not differ from those recorded in the presence of the peptide (-72.7 ± 5.7 mV; $N=7$).

To test whether the peptide alters muscle membrane resistance, periodic hyperpolarizing current pulse injections

Fig. 4. Peptide *F*₁ increases EJP size without increasing muscle input resistance. The excitatory motoneuron innervating the muscle was stimulated at 0.1 Hz. Following each stimulus (with a 300 ms delay), a hyperpolarizing current pulse was delivered to the muscle fiber through the recording electrode. The histogram shows the sizes of EJPs (open bars) and voltage responses (filled bars) to current injection. Each bar is the mean value (+ S.E.M.) for 20 sequential responses; the standard errors for the current injection measurements are too small to be observed on this scale. The inset shows average traces of EJPs and voltage responses to current injections. The open circles were recorded in control saline; the filled circles were recorded after 10 min of exposure to the peptide ($N=5$ for both). The horizontal box indicates the period during which peptide *F*₁ ($5 \times 10^{-8} \text{ mol l}^{-1}$) was present in the superfusion saline.

were delivered through the intracellular microelectrode while membrane potential and muscle tension were monitored. The analysis of an experiment in which nerve-evoked EJPs and muscle input resistance were measured every 5 s as the preparation was superfused with peptide is shown in Fig. 4. Muscle input resistance (filled bars, Fig. 4) did not change during the period in which peptide *F*₁ induced increases in EJP size (open bars, Fig. 4) and muscle tone (not shown). The inset illustrates averaged EJP and current pulses recorded in control saline and after 10 min of exposure to the peptide. Peptide *F*₁ increased the amplitude of the EJP by 41 %, while the voltage response elicited by current injection was not changed. Identical results were obtained in three other experiments in which muscle input resistance was measured.

To examine whether peptide *F*₁ acts at a presynaptic site to enhance synaptic transmission, parallel measurements were made of peptide effects on nerve-evoked transmitter release (in response to brief trains of stimuli) and on spontaneous transmitter release (MEJPs, measured in the periods between stimuli). As shown in previous experiments, superfusion of peptide *F*₁ over the preparation increases EJP amplitude

(Fig. 5A). Fig. 5B shows the amplitudes and numbers of MEJPs recorded in the same neuromuscular preparation under control conditions (normal saline: open bars) and after 8 min of exposure to peptide *F*₁ (filled bars). In the presence of peptide *F*₁, the number of MEJPs recorded increases by 50 % while the mean MEJP amplitude does not change. An estimate

Fig. 5. Effects of peptide *F*₁ on transmitter release from the excitatory motoneuron. (A) EJP trains recorded intracellularly under control conditions and at the peak of the peptide-induced increase in twitch contractions (after 8 min of exposure to $2 \times 10^{-8} \text{ mol l}^{-1}$ peptide). EJP sizes are increased in the presence of the peptide. Muscle resting potential was -76 mV . (B) A histogram of miniature EJPs (MEJPs) recorded in the same preparation under control conditions (open bars) and at the time of maximal peptide-induced increase in twitch contraction strength (filled bars). Note that MEJP frequency increases approximately 50 % with no change in size distribution. MEJPs were recorded for 120 s under each experimental condition.

Table 1. *Effects of peptide F₁ on mean quantal variables*

		EJP size (mV)	Quantal size (mV)	Quantal content	MEJP frequency (s ⁻¹)
Control (N=21)	First EJP	3.01±1.58	1.80±0.20	1.67±0.88	3.8
	Last EJP	49.30±1.53	—	27.38±0.85	—
Peptide F ₁ (N=21)	First EJP	4.81±1.37*	1.80±0.20	2.67±0.76*	5.8
	Last EJP	59.27±3.22*	—	32.93±1.79*	—

The excitatory motoneuron was simulated at a rate of 35 Hz for 0.3 s at a train rate of 0.1 Hz; EJP size was measured for the first and the last EJP of each train.

The control and experimental values given above were determined for 21 trains under control conditions and after 12 min of exposure of the peptide ($2 \times 10^{-8} \text{ mol l}^{-1}$).

Quantal size was determined by measuring MEJP size during the interval between trains for 120 cumulative seconds under each condition. MEJP frequency was calculated by dividing the number of observed MEJPs by the total recording time.

Quantal content (*m*) was calculated by dividing mean EJP amplitude by quantal size.

Data in this table were obtained from the experiment shown in Fig. 5.

*Experimental values are significantly different from control values in a *t*-test ($P < 0.001$).

Values are mean $\pm$ S.D.

of the mean quantal content of the evoked release based on the MEJP size (Table 1) indicates that treatment with the peptide increases the quantal content of release. These results suggest that the peptide potentiates synaptic transmission by enhancing the probability of release, rather than by increasing the postsynaptic sensitivity of the muscle to the neurotransmitter (see also Skerrett *et al.* 1995).

As shown in Fig. 2, peptide F₁ increases the sizes of the IJPs and of the EJPs. An analysis of the effects of peptide F₁ on IJPs is complicated by the fact that the muscle fiber resting potential is close to the chloride equilibrium potential. In several experiments (not shown), two electrodes were inserted into a single muscle fiber, one to pass depolarizing and hyperpolarizing current and the other to record IJP amplitude as a function of membrane potential. Peptide treatment increases the size of IJPs on both sides of the reversal potential, with no change in the reversal potential itself. This result, and the observations that peptide F₁ causes no consistent changes in muscle fiber resting potential or input resistance, suggests that the peptide potentiates the release of transmitter from inhibitory as well as from excitatory nerve terminals.

To examine whether the peptide is physiologically active in a concentration range consistent with that expected for a blood-borne neurohormal agent, the effects of peptide F₁ on EJP amplitude were measured over a range of concentrations. To eliminate the influence of desensitization of responses with repeated peptide applications (see above), data were collected only during the initial peptide application in each preparation. The effects of the peptides were measured by averaging 20 sequential records (to minimize the influence of quantal fluctuations) and determining the ratio of the response in the presence of peptide to the control response. Fig. 6 shows that both peptide F₁ and FMRFamide increase EJP amplitude, with peptide F₁ eliciting increases in EJP size at concentrations 100-fold lower than does FMRFamide. The threshold for the response to peptide F₁ on this preparation is in the vicinity of $10^{-9} \text{ mol l}^{-1}$. This threshold concentration is similar to that

determined for other neurohormonal agents on this preparation (Battelle and Kravitz, 1978; Schwarz *et al.* 1980).

Comparisons between the actions of peptide F₁ and serotonin

In a general way, the actions of peptide F₁ resemble those of serotonin on this tissue (see Goy and Kravitz, 1989). Both substances enhance the contractility of exoskeletal muscles and

Fig. 6. Peptide F₁ increases the amplitude of EJPs at a lower concentration than does FMRFamide. Each circle is the calculated ratio of the average EJP amplitude in the presence of peptide to the average control EJP amplitude (i.e. EJP size in the presence of peptide/control EJP size). Averages were calculated from 20 sequential EJP records, the standard error for this measure was always less than 15% of the mean (see Fig. 4, for example). The filled circles are ratios calculated for preparations exposed to peptide F₁, while the open circles are ratios calculated for preparations exposed to FMRFamide. Each ratio was calculated from the response of a single preparation to the initial exposure to the peptide.

increase transmitter release from excitatory and inhibitory nerve terminals. This raises the question of whether the two substances share a common signal transduction pathway. To address this, we have compared in detail several of the features of the physiological responses to peptide F_1 and serotonin.

An unusual feature of the peptide F_1 response is its profound desensitization during repeated applications (see Figs 2 and 3). Although this type of desensitization has never been described for serotonin, it seemed important to test the actions of the two

Fig. 7. Response of exoskeletal muscle to sequential repeated applications of peptide F_1 and serotonin. The bars indicate the maximal contractions generated by the muscle in the presence of each agent tested. The open bars indicate the responses to serotonin (5-HT), the black bar indicates the response to peptide F_1 , the shaded bar indicates the response to saline containing an elevated K^+ concentration. The duration of each application of peptide or amine was at least 10 min, and each application was followed by a 1 h period of washing in normal saline. ND indicates that no muscle response was detected.

substances in parallel experiments on single preparations. In Fig. 7, the tension responses of a preparation to alternating applications of amine and peptide are shown. Only the initial application of peptide F_1 elicited muscle contraction in this preparation: no response was seen to later applications, even at higher concentrations. In contrast, contractions can repeatedly be triggered by the application of serotonin and are observable even after the peptide is no longer effective. A similar disparity in the contractile effects of the two agents has been observed in four experiments of this type. It is apparent, therefore, that the peptide and amine responses do not cross-desensitize.

A further comparison of the peptide and amine effects is shown in Fig. 8. Here tension is monitored while alternating trains of stimuli are applied to the excitatory and inhibitory nerves innervating the muscle. The twitch contractions elicited by stimulation of the excitatory nerve are equally potentiated by peptide F_1 and serotonin, but the muscle relaxations elicited by stimulating the inhibitory nerve are much larger in the presence of serotonin. Thus, while both hormones induce an increase in resting muscle tone and twitch tension strength, the response of the muscle to inhibitory motoneuron activity is differentially modulated by the amine and the peptide.

Fig. 8. Effects of peptide F_1 and serotonin on nerve-evoked muscle contractions and relaxations. Muscle tension was recorded in response to alternating trains of stimuli delivered to the excitatory and inhibitory motoneurons; upward deflections show the twitch contractions, downward deflections show the muscle relaxations. Sample tension records are shown in control saline (left side of figure) and after 12 min of exposure to peptide F_1 or serotonin (right side of figure); all traces are from a single preparation. Both hormones potentiate the size of twitch contractions and stimulate a slight increase in muscle tonus. (The dotted line indicates resting tension.) Serotonin also potentiates the strength of nerve-evoked muscle relaxations. The tension traces are aligned with the schematic bars at the bottom of the figure that show the timing of brief trains of stimuli alternately delivered to the excitatory (E) and to the inhibitory (I) motoneurons innervating the muscle. Stimulation rate for excitatory motoneuron, 35 Hz, 0.3 s; for inhibitory motoneuron, 50 Hz, 0.5 s. Stimulus trains were delivered once every 0.1 s.

To emphasize these differences, the experiment shown in Fig. 8 was repeated in preparations in which only inhibitory nerves were stimulated and the measurements of muscle tone were not interrupted by twitch contractions. Under these conditions, when peptide F_1 increased muscle tone it also slightly enhanced nerve-evoked relaxations (Fig. 9A). In the same preparation, serotonin strongly potentiated nerve-evoked relaxations (Fig. 9B) at a dose eliciting a tonic contraction comparable to that induced by peptide F_1 . Next, to test the effect of contraction alone on the inhibitory nerve-evoked relaxations, a different preparation was bathed in saline containing an elevated level of K^+ in order to generate a contraction of a similar size to the ones induced by the test

substances (Fig. 9C). Raising K^+ levels in the bathing solution should lead to muscle fiber depolarizations, which should in turn increase the size of the IJPs. The relaxations resulting from stimulating the inhibitory nerve under these conditions are larger than in the control conditions, but similar in size to those elicited during a peptide F_1 -induced contracture; both are much smaller than the relaxations seen during serotonin-induced contractures.

Thus, despite superficial similarities in the actions of peptide F_1 and serotonin on these preparations, the important differences in their physiological actions shown above suggest that the two neurohormonal substances do not share a common mechanism of action.

Peptide F_1 effects on other contractile tissues

Peptide F_1 increases both the rate (not shown) and strength of the heartbeat (Fig. 10A), with a threshold that is 3–4 orders of magnitude below that of FMRFamide (Fig. 10B). The effects can be large (two- to threefold increases in the amplitude of the heartbeat), and they persist for several minutes after the peptide has been washed from the bath.

Fig. 10. Physiological actions of peptide F_1 on the heart. (A) Tension records from a spontaneously beating heart. The chart speed is slow so that the individual heartbeats have fused; the envelope of each trace gives the amplitude of the heartbeat as a function of time. Peptide F_1 was applied at the indicated concentration for the intervals specified by the horizontal bars. (B) Dose-response curve for peptide F_1 (●) and FMRFamide (○) on heartbeat strength. The data points are the amplitude of the heartbeat at the peak of the response as a percentage of the pre-stimulus amplitude. The data are pooled from experiments on three different preparations. FMRFamide was always less effective than peptide F_1 on the same preparation. The peptides also increase the heart rate; the threshold for the effect on heart rate is about an order of magnitude lower than that for the effect on amplitude.

Table 2. *Cyclic AMP and cyclic GMP levels in lobster tissues after hormone treatment*

Tissue	Treatment	Cyclic AMP level (pmol mg ⁻¹ protein)	Cyclic GMP level (pmol mg ⁻¹ protein)
Dactyl opener muscle	None	29.3±6.3 (N=12)	0.40±0.02 (N=3)
	10 ⁻⁷ mol l ⁻¹ peptide F ₁	30.3±8.5 (N=4)	0.31±0.06 (N=3)
			0.39±0.04 (N=3)*
	10 ⁻⁴ mol l ⁻¹ FMRFamide	25.5±11.3 (N=3)	—
	10 ⁻⁶ mol l ⁻¹ serotonin	80.3±6.7 (N=6)	—
Cardiac ganglion	Peptide G ₁ †	—	4.73±0.44 (N=3)*
	None	39.4±2.0 (N=4)	—
Heart muscle	10 ⁻⁷ mol l ⁻¹ peptide F ₁	31.2±2.8 (N=3)	—
	None	84.2±19 (N=9)	—
	10 ⁻⁷ mol l ⁻¹ peptide F ₁	83.0±13 (N=11)	—

Except where indicated, each tissue was treated for 15 min with the indicated concentration of peptide or amine in the presence of IBMX, as described in Materials and methods.

Values are the mean ± S.E.M. for the number of determinations given in parentheses.

*Measurement made after treatment with peptide for 90 min.

†Saturating dose, as determined by bioassay (Pavloff and Goy, 1990).

Surprisingly, the desensitization described above in this study of the effects of peptide F₁ on exoskeletal muscle is not observed in the responses of the heart. During prolonged exposures to high concentrations of peptide, however, the amplitude of the enhancement often declines (see lower traces Fig. 10A). This may represent a slow adaptation to the peptide or it may represent fatigue, since the amplitude of the heartbeat is sometimes irreversibly depressed after the washout of high doses of the peptide. The oesophagus, a visceral muscle, was also examined as a potential target for peptide F₁. In three experiments, the peptide increased basal tension and either initiated or augmented rhythmic contractile activity (data not shown).

Cyclic nucleotides and peptide F₁

In many invertebrate and vertebrate preparations, hormones that elevate cyclic AMP levels enhance neurosecretion and/or alter muscle contractility (see Goy and Kravitz, 1989, for a review). Moreover, in molluscan heart muscle, FMRFamide elevates cyclic AMP levels in parallel with its physiological actions (Higgins, 1977; Higgins *et al.* 1978; Painter, 1982b). We therefore tested peptide F₁ and FMRFamide for their effects on cyclic AMP levels in various lobster tissues (Table 2). Both peptides failed to increase cyclic AMP levels, while serotonin generated a readily detectable increase. Another second-messenger system that may play a role in muscle contractility in vertebrates (George *et al.* 1970; Lee *et al.* 1972; Rapoport and Murad, 1983) and invertebrates (Beam *et al.* 1977; Painter, 1982a; Matsuura, 1984) is cyclic GMP. We therefore examined whether peptide F₁ might stimulate cyclic GMP levels in the dactyl opener muscle, the tissue which is the main focus of these studies. When tested at 10⁻⁷ mol l⁻¹, a high concentration compared with the physiological threshold, peptide F₁ has no effect on cyclic GMP levels measured in isolated neuromuscular preparations (Table 2). In contrast, parallel incubations with peptide G₁

(Pavloff and Goy, 1989) produced substantial increases in cyclic GMP levels. These results suggest that neither cyclic AMP nor cyclic GMP is a good candidate for mediating the effects of peptide F₁. We cannot rule out the possibility, however, that the peptide raises cyclic nucleotide levels in some restricted tissue compartments where the nucleotide content is small relative to the total background levels of nucleotides.

Discussion

In this study, the lobster peptide F₁ has been found to modulate several aspects of nerve-muscle function. In a skeletal muscle preparation, peptide F₁ increases the strength of neurally evoked twitch contractions and acts directly on skeletal muscle fibers to induce sustained increases in tone in the absence of evoked nerve activity. The tonic muscle contractions induced by peptide F₁ are independent of the activity of the excitatory motoneuron, since they are observed under conditions where stimulation frequency of the excitatory motoneuron is sufficiently high to evoke twitch conditions (Fig. 1A) and when there is no stimulation of the excitatory motoneuron (Figs 3, 7 and 9). In addition to potentiating muscle contractility, peptide F₁ presynaptically enhances transmitter release from the motoneurons innervating the muscle, resulting in an increase in the sizes of EJPs and IJPs recorded postsynaptically. Peptide F₁ also augments contractility in lobster cardiac and visceral muscle preparations, although the mechanism of these actions has not been studied extensively. Taken together, these results suggest that this peptide may play a general role in lobsters as a systemic neurohormone.

A similar potentiation of neurally evoked contractions by members of the FaRP family has been seen in neuromuscular preparations from locusts, crayfish and moths (Cuthbert and Evans, 1989; Schiebe and Walther, 1989; King *et al.* 1990;

Mercier *et al.* 1990). In addition, a presynaptic enhancement of transmitter release has been shown previously for the lobster peptides F₁ and F₂ and their crayfish analogs N₁ and D₂ in a crayfish phasic neuromuscular system (Mercier *et al.* 1990; Skerrett *et al.* 1995). In each case, FaRPs increase the probability of transmitter release, with no effect on the postsynaptic responsiveness of the muscle to the released transmitter compound. In contrast, in studies with snail muscles and neurons, FMRFamide has been shown to reduce the responsiveness of muscles to the excitatory transmitter acetylcholine (Zoran *et al.* 1989) and to inhibit neurotransmission between neurons *via* a presynaptic mechanism (Haydon *et al.* 1991). Thus, members of the FaRP family may potentiate or suppress synaptic effectiveness and muscle contractility in different neuromuscular systems in different organisms.

While the actions of peptide F₁ on lobster synaptic transmission and twitch contractions are similar to those reported in other muscle systems, a direct stimulation by FaRPs of tonic skeletal muscle contractions has not been reported previously. The mechanism by which the peptide generates contractures of this type is not known. One possibility is that they are a consequence of the peptide-F₁-mediated increase in frequency of spontaneous transmitter release from the excitatory motoneuron innervating the muscle (see Fig. 5). However, no changes in muscle membrane potential or input resistance are seen accompanying the contractures, both of which would result from a neurotransmitter-mediated mechanism. This suggests that the contractures result from direct actions on the muscle fibers through mechanisms that are independent of membrane voltage. These observations also rule out a mechanism of the type observed in leech heart muscle, where FaRPs directly induce an inward Na⁺ current. This current depolarizes muscle fibers into a voltage range in which excitation-contraction coupling is activated and modulation of voltage-dependent K⁺ and Ca²⁺ conductances becomes apparent (Thompson and Calabrese, 1992).

One way in which peptide F₁ might regulate muscle tone is suggested by a report that tetrapeptides of the FaRP family inhibit a Na⁺/Ca²⁺ exchanger in vertebrate cardiac muscle sarcolemmal vesicles (Khananashvili *et al.* 1993). In cardiac muscle, the sarcolemmal Na⁺/Ca²⁺ exchanger plays an important role in the electrogenic extrusion of intracellular Ca²⁺, thereby influencing muscle tone and relaxation. If a similar exchanger protein is present in the sarcolemma of lobster skeletal muscle fibers and if it is inhibited by peptide F₁, the resulting increase in intracellular Ca²⁺ concentration could activate a number of Ca²⁺-dependent processes that might regulate muscle tone and contractility. For example, increases in cytoplasmic Ca²⁺ concentration could (1) stimulate enzymatic pathways or second-messenger cascades; (2) regulate muscle contractile proteins; or (3) potentiate the release of Ca²⁺ from internal stores. Further studies will be required to explore these and other such possibilities.

The long latency to onset and persistent effects of the peptide in increasing muscle tone suggest the involvement of

a second-messenger system, but the appropriate second messenger has not yet been identified. The cyclic nucleotide systems appear to be unlikely, as neither cyclic AMP nor cyclic GMP levels are elevated by the peptide in any lobster tissues tested. Peptide F₁, therefore, is one of several neuromodulators of the dactyl opener system (including the amines serotonin and octopamine and the peptide proctolin) for which the cellular mechanism of contractile activation is unknown (Kravitz *et al.* 1985; Goy and Kravitz, 1989). The skeletal muscle responses to peptide F₁ differ from those produced by the other lobster neurohormones, however, in that the muscle and nerve terminal responses desensitize with repetitive bath applications of the peptide. Little is known of the mechanism of desensitization in either nerve or muscle. In muscles, however, it apparently is not at the level of activation of the contractile proteins, since muscles desensitized to peptide F₁ continue to respond to neural stimulation and to bath application of other hormones (see Fig. 7). Furthermore, although the contractile effects of the peptide on exoskeletal muscle desensitize strongly, those on the heart can be elicited repeatedly with little decrease in potency. The desensitization phenomenon is therefore tissue-specific. Similar observations have been made in crayfish studies with analogs of peptide F₁. Here too, the peptide effects on neuromuscular transmission decline with repetitive application but no desensitization is observed in studies of heartbeat amplitude or rate (Skerrett *et al.* 1995). These observations raise the possibility that multiple receptor types or second-messenger systems may mediate the actions of these substances. Such a possibility is consistent with the findings that the pharmacological profiles of locust heart and skeletal muscle show differences in response to particular FaRP sequences (Cuthbert and Evans, 1989).

The pericardial organs, segmentally arranged pairs of neurosecretory glands found within the pericardial sinus at the openings of the branchiocardiac veins, are an important site of FaRP accumulation in lobsters (Trimmer *et al.* 1987). When applied to the lobster heart in nanomolar concentrations, peptide F₁ increases both the amplitude and the frequency of the heartbeat. This raises the possibility that the release of peptide F₁ from pericardial organs is one important way of regulating cardiac activity *in vivo*. Cardioexcitatory effects of peptide F₁ have also been observed on locust heart (Cuthbert and Evans, 1989), crab heart (Krajniak, 1991) and crayfish heart (Mercier *et al.* 1990). In each of these systems, peptide F₁ was more potent than any other FaRP tested and at least an order of magnitude more potent than the tetrapeptide FMRFamide. It is not known whether the peptide is acting on neurons of the cardiac ganglion that provide pacemaker activity to the heart or on heart muscle directly. An increase in heartbeat amplitude could reflect actions on either tissue, but an increased heart rate probably reflects a change in ganglionic neuronal activity. Effects of FaRPs on the neuronal networks that function as pattern generators or bursting pacemakers have been described in the leech heart (Kuhlman *et al.* 1985), *Aplysia* buccal and abdominal ganglia (Sossin *et al.* 1987; Ruben *et al.* 1984) and the lobster and crab pyloric and gastric

rhythm central pattern generators (Hooper and Marder, 1984; Weimann *et al.* 1993).

In summary, peptide F₁ acts at low concentrations to potentiate synaptic transmission and to regulate the contractility of skeletal, cardiac and visceral muscles in lobsters. These observations, and the demonstration that neurosecretory regions are rich sources of such peptides, suggest that peptides of this family act as blood-borne hormones capable of influencing multiple peripheral targets in lobsters. These results are consistent with an emerging picture of FaRPs as important regulators of nerve and muscle activity in a wide variety of invertebrate species. In *Homarus*, peptide F₁ is only one of several neurohormones that influence muscle contractility and nerve–muscle communication. Understanding the behavioral contexts in which peptide F₁ and related peptides are released, and elucidating the cellular mechanisms by which they act, remain significant challenges for the future.

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