Excitatory Actions of FMRFamide-Related Peptides (FaRPs) on the Neurogenic *Limulus* Heart

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Abstract. The actions of FMRFamide-related peptides (FaRPs) on the neurogenic heart of the horseshoe crab, *Limulus polyphemus,* were investigated. Excitatory chronotropic effects were produced by application of TNRNFLRFamide, SDRNFLRFamide, GYNRSFLRFamide, or pQDPFLRFamide to the intact heart preparation. Effects were dose-dependent with a threshold of 10^{-9} *M* or less. TNRNFLRFamide and SDRNFLRFamide increased the burst rate of the isolated *Limulus* cardiac ganglion.

Synthetic FaRPs produced inotropic excitation of the heartbeat as well. GYNRSFLRFamide, TNRNFLRFamide, SDRNFLRFamide, and pQDPFLRFamide increased heart contraction strength at a threshold dose of approximately 10^{-8} *M*. TNRNFLRFamide and SDRNFLRFamide enhanced electrically evoked contractions of the *Limulus* myocardium, elicited contracture in some preparations, and increased the excitability of cardiac muscle fibers.

The presence of cardioactive FaRPs in the *Limulus* central nervous system was suggested by reverse phase HPLC of acidified methanol extracts of *Limulus* nervous tissue. Four peaks of FaRP-like bioactivity were detected with the *Busycon* radula protractor muscle bioassay. These peaks also contained FaRP-like immunoreactivity. Two of these partially purified peaks produced excitatory chronotropic effects on the intact *Limulus* heart preparation similar to those produced by synthetic FaRPs.

Introduction

The FMRFamide-related peptides (FaRPs) are widely distributed in the Arthropoda (reviewed by Price and Greenberg, 1989; Greenberg and Price, 1992). The arthropod FaRPs occur as N-terminally extended variants of the tetrapeptides FMRFamide and FLRFamide. For example, TNRNFLRFamide and SDRNFLRFamide have been isolated from the lobster *Homarus* (Trimmer *et al.,* 1987), DRNFLRFamide and NRNFLRFamide from the crayfish *Procambarus* (Mercier *et al.,* 1991a), and GYNRSFLRFamide from the crab *Callinectes* (Krajniak, 1991). Pharmacological studies with a number of arthropod heart preparations have suggested a cardioregulatory role for N-terminally extended FaRPs (Kravitz *et al.,* 1987; Cuthbert and Evans, 1989; Krajniak, 1991; Mercier *et al.,* 1991b; Groome, 1993; Duve *et al.,* 1993). However, the cellular sites of action targeted by FaRPs on neurogenic arthropod hearts are unknown.

We have begun to examine the cellular and biochemical mechanisms underlying the effects of neuromodulators of the cardiac rhythm in the horseshoe crab, *Limulus polyphemus,* an organism suitable for detailed cellular studies (reviewed by Watson and Groome, 1989). Immunoreactivity to the neuropeptide FMRFamide has been detected in the central nervous system (CNS), cardiac ganglion, peripheral nerves and muscles of the horseshoe crab (Watson *et al.,* 1984; Gaus *et al.,* 1993; Groome, 1993). We decided to determine the actions of synthetic FaRPs on the neurogenic *Limulus* heart, as well as to characterize endogenous cardioactive FaRPs in *Limulus.* Our data indicate that several N-terminally extended FaRPs produce chronotropic and inotropic excitation of the *Limulus* heartbeat. These actions are a result of excitation of cardiac ganglion neurons, and enhancement of cardiac muscle fiber contractility and excitability. Partial purification of *Limulus* prosomal CNS extracts with HPLC reveals several peaks of FaRP-like bioactivity and immunoreactivity; two of these peaks also excite the *Limulus* heartbeat. Pre-

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liminary accounts of portions of this work have appeared in an earlier short report (Groome and Watson, 1991).

Materials and Methods

Preparations

Adult horseshoe crabs (*Limulus polyphemus*) were obtained from the Marine Resources Department at the Marine Biological Laboratory in Woods Hole, Massachusetts. They were maintained at 18 to 20°C in a flow-through seawater tank until used. Hearts were dissected as previously described (Watson *et al.,* 1984). The isolated, intact hearts were pinned in 10-ml resin-lined recording chambers and superfused from a 500-ml reservoir at 5 ml/min with filtered, natural seawater at room temperature (20–23°C) until the cardiac rhythm had stabilized. Contractions were monitored with a force transducer (Grass FT .03 C, Grass Instruments, Quincy, Massachusetts) and polygraph (Grass Model 7D). Test solutions were added at the same flow rate via a 20-ml reservoir, and preparations were washed for 30 min or more prior to the application of the next test solution.

Cardiac ganglia were dissected free from *Limulus* myocardia, pinned by their adhering connective tissue to the bottom of 5-ml recording chambers, and superfused at 5 ml/min. Neuronal activities were recorded extracellularly with a suction electrode; the signals were amplified and filtered with Grass P-15 preamplifiers and displayed with a polygraph. Myocardial rings (3 cm) were stretched over wire electrodes immersed in a 20-ml bath and stimulated with current pulses from a Grass S-88 stimulator. For each evoked contraction, two 50-ms pulses separated by an 80-ms delay were delivered; pulses were equal in intensity (2–5 V) and of opposite polarities. Stimulation parameters were adjusted to obtain contraction strengths of magnitude approximating those recorded from typical intact heart preparations. In other experiments, deganglionated myocardial rings were pinned to the bottom of 10-ml recording chambers. Spontaneous contractions were recorded, and test solutions were delivered as described above.

Peptides were obtained as follows: FMRFamide was obtained from Peninsula Laboratories (Belmont, California); pQDPFLRFamide and TNRNFLRFamide were obtained from Bachem (Torrence, California); SDRNFLRFamide was a gift from Dr. Eve Marder (Brandeis University, Waltham, Massachusetts); GYNRSFLRFamide, RNFLRFamide and FLRFamide were a gift from Dr. Michael J. Greenberg (Whitney Laboratory, St. Augustine, Florida).

Peptide extraction

The extraction procedure for FaRPs in *Limulus* nervous tissue followed the procedure of Trimmer *et al.* (1987). The protocerebra and circumesophageal ganglia (prosomal CNS) from 100 *Limulus* were dissected and placed in ice-cold methanol/acetic acid (99:1) containing 0.05% dithiothreitol; the tissues were then stored in groups of 10 at −20°C. Tissues were homogenized at 0°C, and centrifuged at 25,000 × *g* for 30 min at 4°C. The supernatant was removed, combined with the supernatant resulting from one repetition of this process with the centrifugation pellet, and dried on a rotary evaporator (Savant Instruments, Hicksville, New York). This material was resuspended in 0.1% trifluoroacetic acid (TFA, Pierce Chemical, Rockford, Illinois) and loaded onto Sep-Pak C_{18} cartridges pre-activated with 5 ml methanol and 5 ml 0.1% TFA. The cartridges were washed with 0.1% TFA and peptides were eluted with 60% methanol containing 0.1% TFA. This material was dried, resuspended in 0.1% TFA, and ultracentrifuged (Beckman TL-100, Beckman Instruments, Palo Alto, California) at 36,000 × *g* for 60 min at 4°C. The supernatants were dried and the material stored at −80°C until subjected to reverse phase HPLC.

High performance liquid chromatography (HPLC)

A Perkin-Elmer Series 410 BIO LC pump (Norwalk, Connecticut) and Rheodyne 7125 sample injector with 1-ml loop (Cotati, California) and a 7-μm spherical, 15 × 3.2 mm Polymeric RP guard column (Brownlee Labs, Santa Clara, California) were used in the first three HPLC runs. The extracts were reconstituted in 300 μl 0.1% TFA and filtered (Millex HV4, 0.45 μm, Millipore, Milford, Massachusetts); 250 μl was injected onto a C_8 or C_{18} reverse phase column. The mobile phase consisted of HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, New Jersey), Milli-Q water (Millipore), and TFA. Flow rate was set at 1 ml/min and 1.5-ml fractions were collected. The $O.D._{.204-285}$ of the eluate was monitored with a LKB 2140 Rapid Spectral detector. The fourth HPLC run was accomplished by K. Doble (Whitney Laboratory, University of Florida, St. Augustine, Florida) according to the protocol specified in Price *et al.* (1990). The HPLC columns and protocols employed sequentially were as follows:

Run 1: C_8 column (Aquapore RP-300, 7-μm spherical, 220 × 4.6 mm, Brownlee); 5 min isocratic 2% acetonitrile followed by a linear gradient to 60% acetonitrile over 55 min, with 0.1% TFA throughout.

Run 2: C_8 column (Aquapore RP-300); 5 min isocratic 5% acetonitrile followed by a linear gradient to 30% acetonitrile over 55 min, with 0.1% TFA throughout.

Run 3: C_{18} column (RP-18 Spheri-5, 220 × 4.6 mm, Brownlee) 5 min isocratic 10% acetonitrile and 0.1% TFA followed by a linear gradient to 37% acetonitrile over 55 min, with 0.1% TFA throughout.

Run 4: C_8 column (Aquapore RP-300, 7 μm spherical, 220 × 2.1 mm, Brownlee) with a linear gradient of 16–40% acetonitrile over 30 min, with 0.05% TFA throughout.

Bioassay

Fractions from HPLC runs were dried and resuspended in 1 ml Milli-Q water, and aliquots were analyzed for FaRP-like bioactivity with the *Busycon* radula protractor muscle (RPM, Nagle and Greenberg, 1982). Left-handed whelks (*Busycon contrarium*) were obtained from Gulf Specimens (Panacea, Florida) and maintained at room temperature in aerated natural seawater until used. The paired RPMs were tied at the odontophore and radula, suspended in a 1-ml vertical chamber, and superfused with aerated, filtered seawater. Peptides were added to the chamber with a syringe, and contractures were recorded with a force transducer and polygraph. FaRP-like bioactivities in individual fractions were calculated by comparing the contracture force elicited by samples to that produced by synthetic pQDPFLRFamide at a dose of 10^{-9} *M*, at regular intervals. This preparation was sensitive to picomolar quantities of FMRFamide, FLRFamide, RNFLRFamide, GYNRSFLRFamide, TNRNFLRFamide, SDRNFLRFamide, and pQDPFLRFamide. Active fractions were pooled and dried for further HPLC characterization, for analysis of effects on the *Limulus* heart (after HPLC runs 2 and 3), or for radioimmunoassay (after HPLC run 4).

Radioimmunoassay

We used two FaRP antisera, Q2 and S-253, in the radioimmunoassay; the protocol employed has been described in detail (Price *et al.*, 1990). The Q2 antisera was raised against pQDPFLRFamide and boosted with pDDPFLRFamide; it was used in this study at a final dilution of 1:625. The S-253 antiserum was raised against YGGFMRFamide and used at a dilution of 1:25,000.

Results

Chronotropic effects of FaRPs

Synthetic FaRPs were tested for their effects on the rate of contraction of the isolated *Limulus* heart. The peptides TNRNFLRFamide, SDRNFLRFamide, GYNRSFLRFamide, and pQDPFLRFamide consistently increased heart rate in a dose-dependent manner; the threshold dose for each of these peptides was approximately 10^{-9} *M* (Table I, Fig. 1). Chronotropic excitation gradually diminished after the peptide was washed off the preparation. The peptides RNFLRFamide, FLRFamide, and FMRFamide produced only slight increases in heart rate, at doses up to 10^{-5} *M*.

Table I

Effects of FaRPs on the intact heart preparation[a]

Peptide	Threshold[b] (n*M*)	EC_{50}[c] (n*M*)	Maximum[d] increase (%)	Relative[e] efficacy
A. Chronotropic effects				
SDRNFLRFa	0.3	3.4	58.7	1.00
TNRNFLRFa	0.3	2.9	57.0	0.97
GYNRSFLRFa	0.3	3.1	47.3	0.81
pQDPFLRFa	1.0	3.4	53.3	0.91
RNFLRFa	3.0	8.4	23.8	0.41
FMRFa	100	n.d.	5.9	0.10
FLRFa	300	n.d.	10.0	0.17
B. Inotropic effects				
SDRNFLRFa	3.0	40	32.7	1.00
TNRNFLRFa	3.0	30	33.9	1.04
GYNRSFLRFa	10	60	37.0	1.13
pQDPFLRFa	10	110	40.0	1.22
RNFLRFa	1000	n.d.	15.4	0.47
FLRFa	1000	n.d.	6.1	0.19
FMRFa	No response	n.d.	n.d.	n.d.

[a] Values calculated from dose-response data for each peptide (a = amide), over the range of 0.3 n*M* to 1.0 μ*M*. For the tetrapeptides and RNFLRFamide, doses up to 10 μ*M* were tested. A maximum of 6 trials, each with a different dose, were performed with each preparation, for a total *n* of 8 to 12 trials per dose. Where insufficient data were available, calculations were not determined (n.d.).

[b] Threshold defined as the dose at which the mean response exceeded the value of the standard deviation.

[c] EC_{50} calculated as the dose at which the mean response was half that of the maximum.

[d] Maximum increase taken from full or partial dose-response curves, using the mean response.

[e] Relative efficacies compare the maximum increase of each peptide to that for SDRNFLRFamide, for both chronotropic and inotropic effects.

The cellular basis for the chronotropic excitation elicited by application of synthetic FaRPs to the intact heart was investigated with the isolated cardiac ganglion preparation. At 10^{-8} *M*, TNRNFLRFamide and SDRNFLRFamide produced gradual and long-lasting increases in the burst rate of cardiac ganglion neurons (Fig. 2). This effect was associated with decreases in the interburst interval and the duration of individual bursts.

Inotropic effects of FaRPs

FaRPs with potent chronotropic effects increased the contraction amplitude of the *Limulus* heartbeat (Table I, Fig. 1). Dose-dependent inotropic excitation followed the administration of TNRNFLRFamide, SDRNFLRFamide, GYNRSFLRFamide, and pQDPFLRFamide. The peptides RNFLRFamide, FLRFamide, and FMRFamide had little inotropic effect at doses up to 10^{-5} *M*.

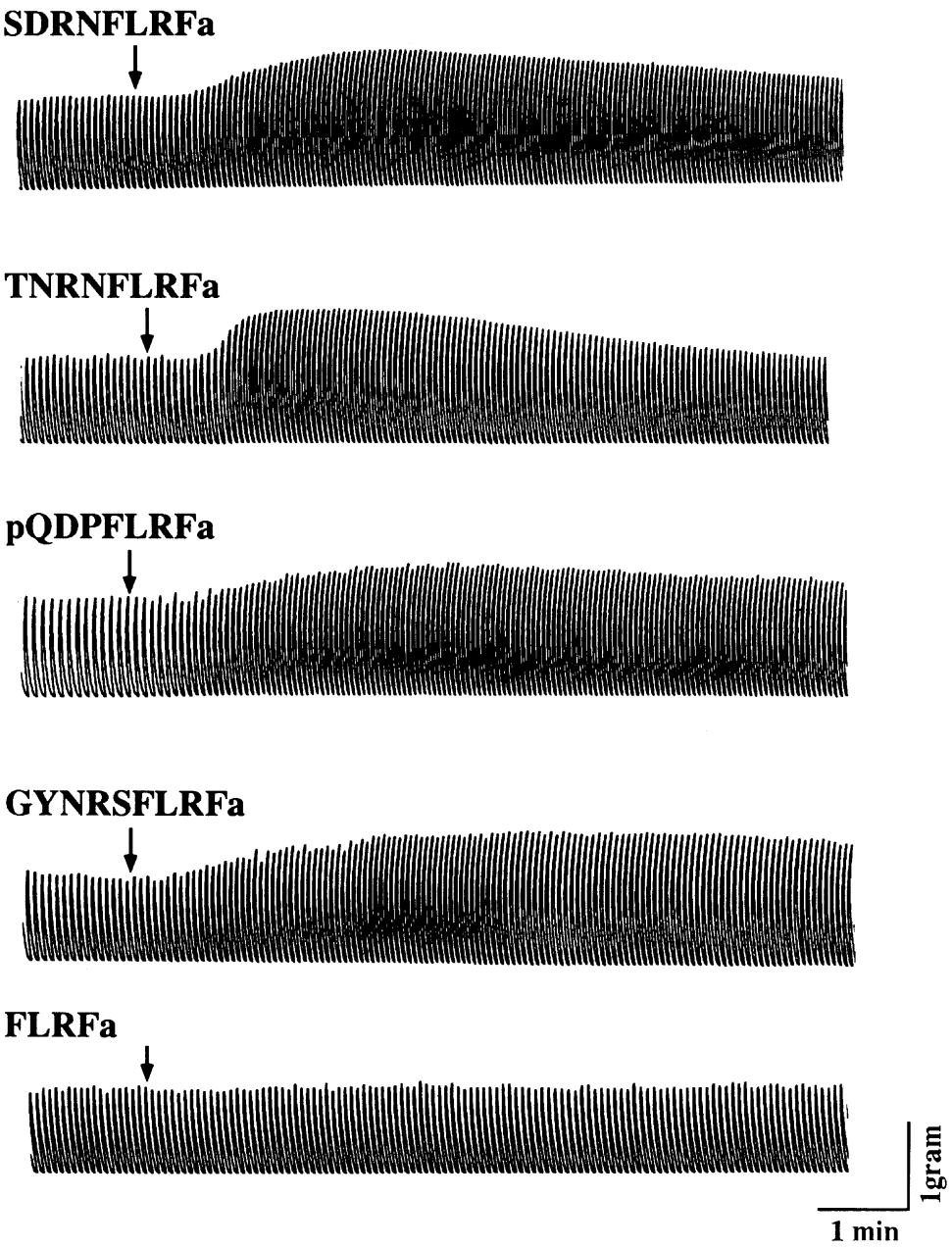

Figure 1. Effect of FaRPs (a = amide) on the intact *Limulus* heart preparation. At 10^{-7} *M*, N-terminally extended FaRPs produced chronotropic and inotropic excitation of the heartbeat, whereas FLRFamide, at 10^{-6} *M*, had little effect on this preparation.

To determine the effect of synthetic FaRPs on cardiac muscle contractility, deganglionated muscle rings were stimulated with current pulses at fixed intensities and rates. At 3×10^{-7} *M*, both TNRNFLRFamide and SDRNFLRFamide produced a gradual and long-lasting increase in the amplitude of evoked contractions (Fig. 3). At this dose, the inotropic effect was significantly greater than that observed with the same dose of TNRNFLRFamide, or SDRNFLRFamide, applied to the intact heart preparation. This difference may be attributable to negative inotropic effects associated with increased burst rate in the cardiac ganglia of intact heart preparations (Watson and Groome, 1989).

In a few experiments, these peptides increased the baseline tension as well as the amplitude of evoked contractions (Fig. 3A). Small oscillations in muscle tension between stimulating pulses also occurred occasionally during peptide administration. These observations sug-

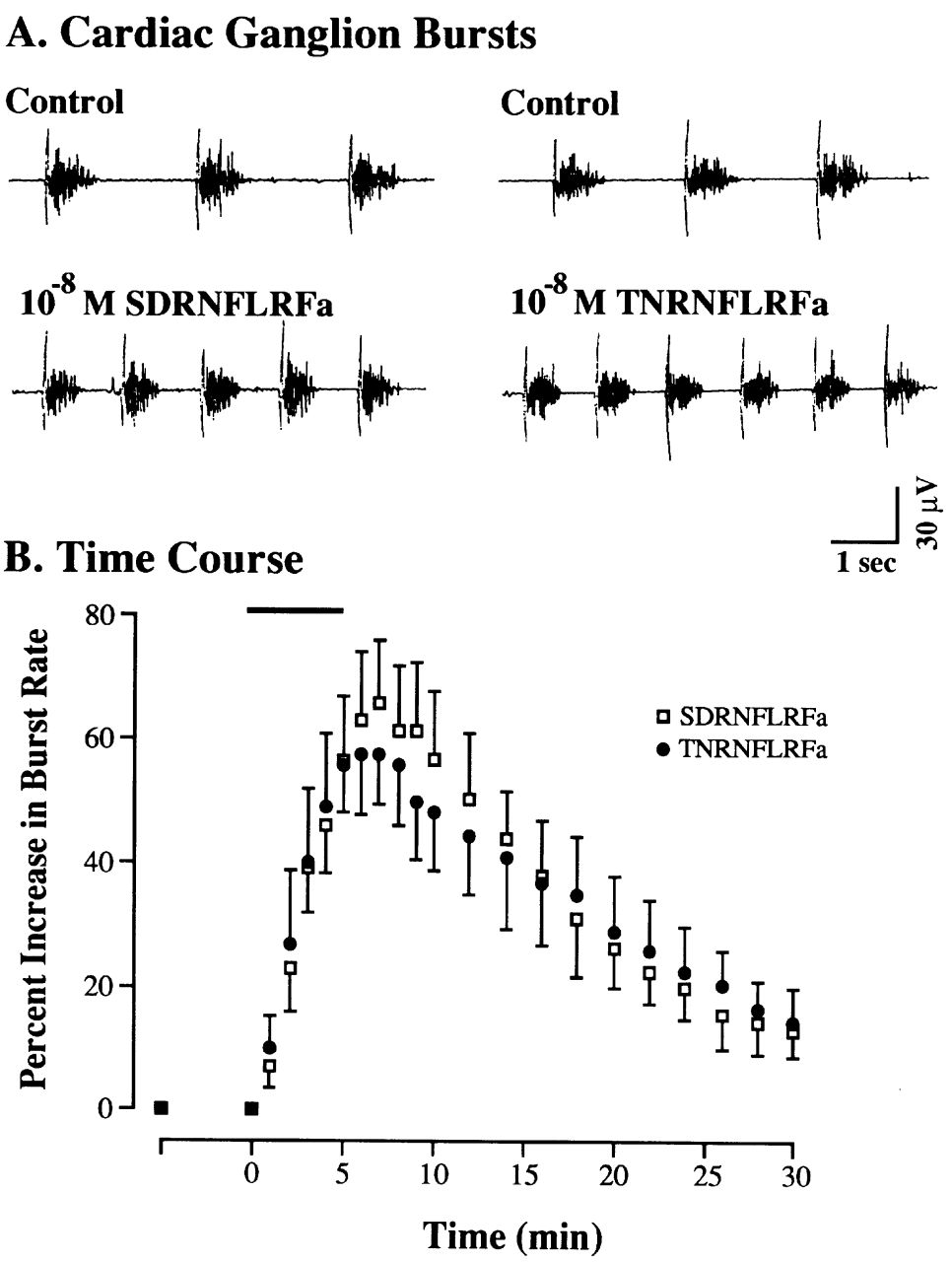

Figure 2. The chronotropic action of TNRNFLRFamide and SDRNFLRFamide (a = amide) on the *Limulus* heart is due to excitation of cardiac ganglion neurons. A. At 10^{-8} *M*, these peptides increased the rate of bursting in isolated ganglia, while decreasing the interburst interval and the duration of individual bursts. B. Time course of the effect of FaRPs on the isolated ganglion. The bar depicts the duration of peptide application.

gested that FaRPs alter the excitability of cardiac muscle fibers. To test this hypothesis, we applied FaRPs to deganglionated muscle rings. Both TNRNFLRFamide and SDRNFLRFamide, at 3×10^{-7} *M*, caused these preparations to beat irregularly in the absence of neural or electrical stimulation (Fig. 4). Peptide-induced contractions were abolished with the addition of 20 m*M* magnesium to the superfusion (data not shown).

Partial characterization of Limulus *FaRPs*

Acidified methanol extracts of the *Limulus* prosomal CNS were partially purified by reverse phase HPLC in three consecutive protocols; the purification was monitored with the *Busycon* RPM bioassay. With a linear gradient of 2 to 60% acetonitrile with the C_8 column, we detected two peaks of FaRP-like bioactivity. The sepa-

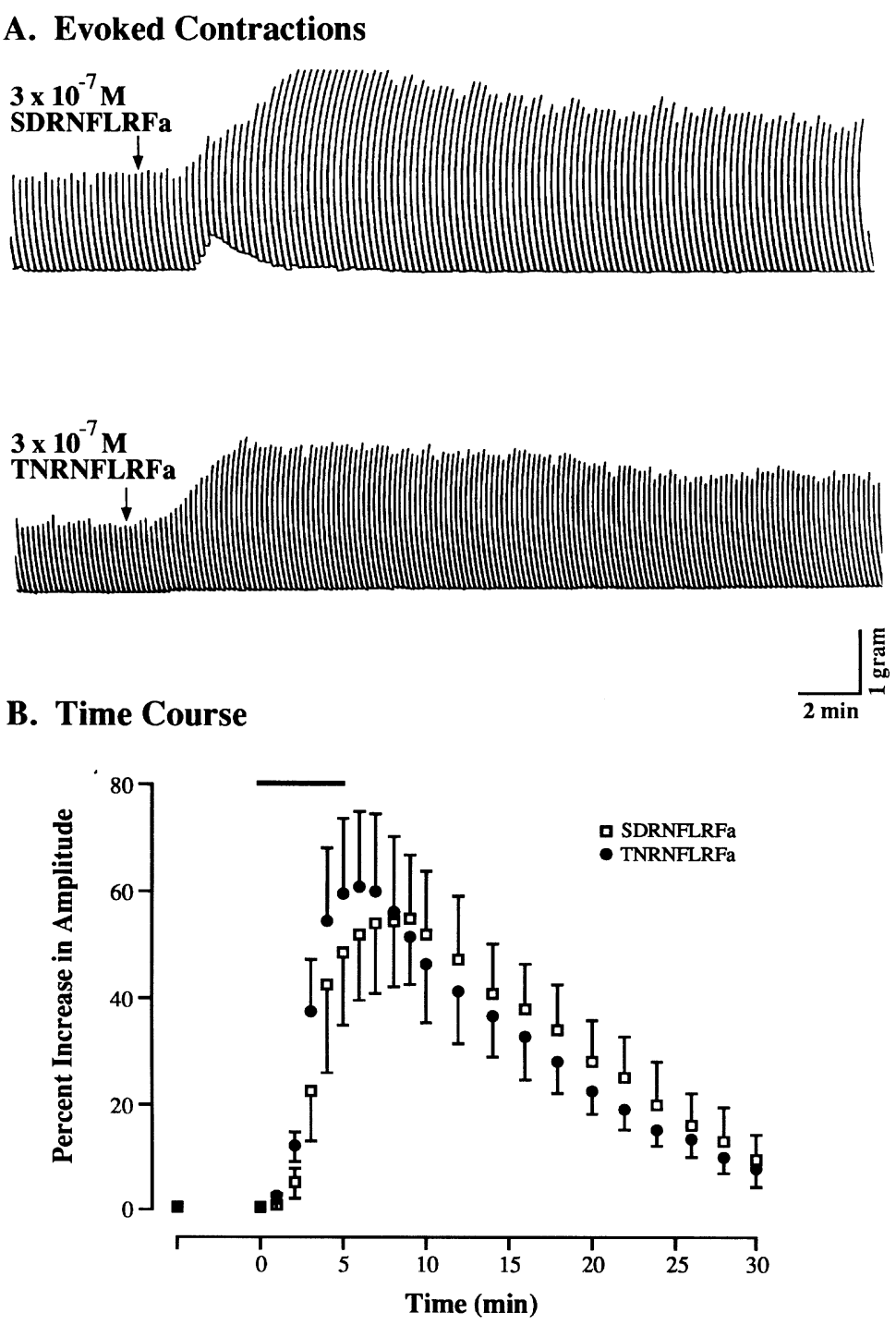

Figure 3. The peptides TNRNFLRFamide and SDRNFLRFamide (a = amide) increased the amplitude of electrically evoked contractions of the deganglionated *Limulus* myocardium. A. At 3×10^{-7} *M*, these peptides enhanced contraction amplitude, and occasionally increased baseline tension. B. Time course of the inotropic effect of FaRPs. The bar depicts the duration of peptide application.

rately pooled fractions from each peak were subjected to a linear gradient of 5 to 30% acetonitrile with the C_8 column, from which we detected three principal peaks of FaRP-like bioactivity. Aliquots from each of these bioactive peaks were tested on the *Limulus* heart, and marked cardioexcitation was elicited with application of the first and second peaks (data not shown). The separately pooled fractions from each of these three peaks were subjected

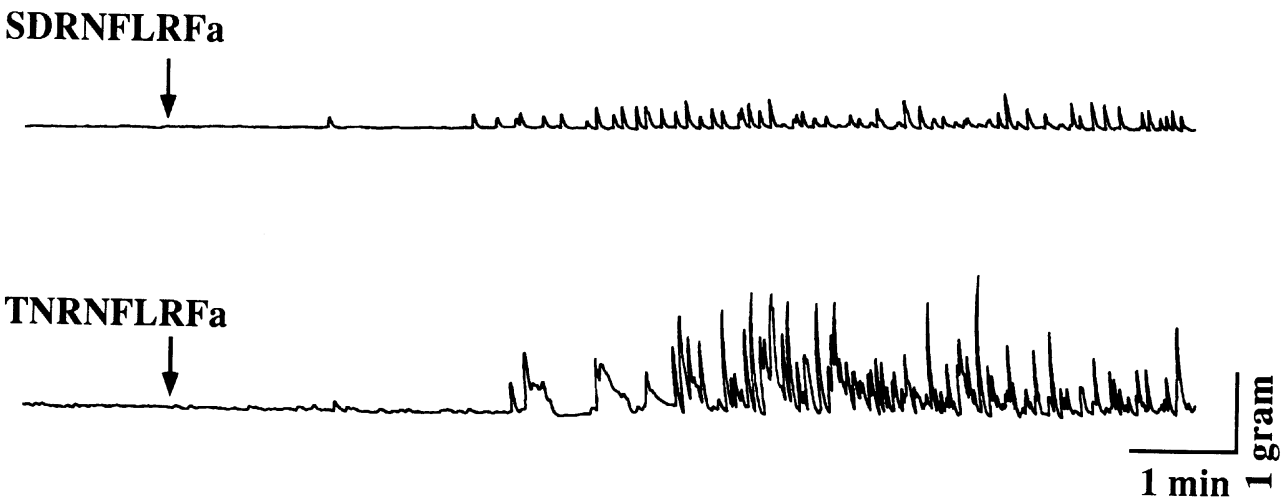

Figure 4. The peptides TNRNFLRFamide and SDRNFLRFamide (a = amide), each at 3×10^{-7} *M*, produced rhythmic contractions in unstimulated, deganglionated *Limulus* myocardia. This effect appeared after several minutes of superfusion of peptide and persisted for several minutes after the peptide was removed from the bath.

to 10 to 37% acetonitrile gradient with a C_{18} column, from which four peaks of FaRP-like bioactivity were detected (Fig. 5). The first three peaks had elution times near those for FMRFamide, TNRNFLRFamide, and pQDFLRFamide, while the fourth had an elution time later than that for any FaRP standard tested.

Aliquots from each of the peaks generated by the third HPLC run were subjected to a final characterization on another HPLC system, with analysis by radioimmunoassay with the S253 and Q2 antisera (Fig. 6). Immunoreactivity to one or both antisera was detected in each peak. Peak three from the third HPLC run displayed strong immunoreactivity to both antisera in fractions that had an elution time slightly longer than that of pQDPFLRFamide.

The four peaks of FaRP-like bioactivity from the third HPLC run were also tested on the *Limulus* heart preparation (Fig. 7). In each test, aliquots comprising 10 prosomal CNS equivalents, with respect to the original extraction, were used. Excitatory chronotropic effects were more consistently elicited than were increases in heart contraction amplitude, especially with peaks one and two. Peak one increased with heart rate by 42.2 ± 4.6% SEM (n = 8), and peak two produced even greater chronotropic excitation (61.4 ± 8.7% SEM increase, n = 9). Peak three increased heart rate by 15.8 ± 3.9% SEM (n = 9), and the effect of peak four was negligible (7.5 ± 2.7% SEM increase, n = 9). Inotropic effects were slight, ranging from an increase of 9.3 ± 3.3% SEM elicited by peak two, to the 13.0 ± 3.9% SEM increase elicited by peak one. Peaks three and four elicited increases in amplitude of 9.9 ± 3.0% SEM and 12.1 ± 4.5% SEM, respectively.

Discussion

This study shows that several FaRPs have pronounced excitatory actions on the neurogenic heart of the chelicerate arthropod *Limulus*. The crustacean peptides TNRNFLRFamide, SDRNFLRFamide, and GYNRSFLRFamide all increase the rate and strength of heart contractions at low doses, as does the molluscan peptide pQDPFLRFamide. Endogenous factors purified from the *Limulus* nervous system produce FaRP-like cardioexcitatory effects. These factors may play a cardioregulatory role in the horseshoe crab, and they may, in fact, be FaRPs.

Cellular actions of FaRPs on the neurogenic Limulus *heart*

Several N-terminally extended FaRPs increase both the rate and strength of *Limulus* heart contractions. In crustaceans, FaRPs have been detected in the pericardial organs and have excitatory effects on the heartbeat. These peptides are predominantly chronotropic in the blue crab *Callinectes* (Krajniak, 1991), whereas the peptide TNRNFLRFamide produces both chronotropic and inotropic excitation of the heartbeat in the lobster *Homarus* (Kravitz *et al.*, 1987). Similar cardioexcitatory effects of TNRNFLRFamide, SDRNFLRFamide, and native FaRPs are observed in the crayfish *Procambarus* (Mercier *et al.*, 1991a, b).

The cardioexcitatory effects of FaRPs on the *Limulus* heartbeat are mimicked by several biogenic amines (Augustine *et al.*, 1982). These amines elicit chronotropic excitation by increasing the firing frequency of pacemaker neurons and thus the burst rate of the *Limulus* cardiac ganglion (Augustine and Fetterer, 1985). The peptides

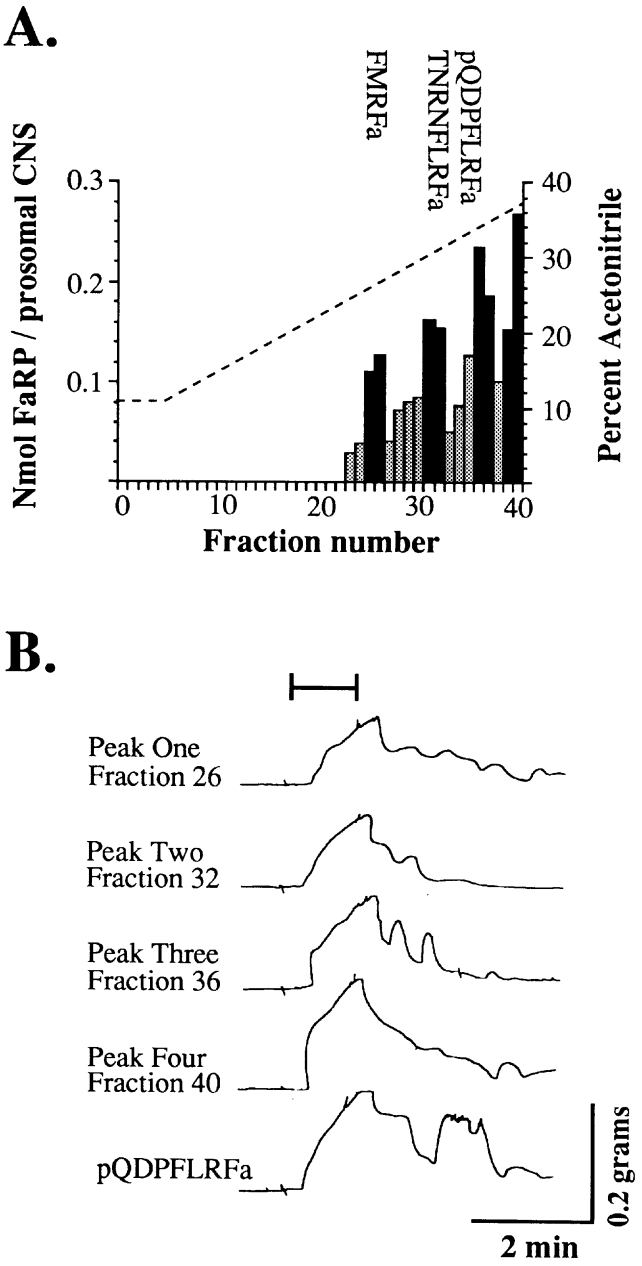

Figure 5. Reverse phase HPLC of acidified methanol extracts of the *Limulus* prosomal CNS as assayed for FaRP-like bioactivity with the RPM of *Busycon*. A. composite HPLC profile of the third HPLC run. The HPLC gradient used and the elution times of standard FaRPs (a = amide) are shown. Four bioactive peaks were detected (black), and these fractions were assayed further. B. Contractures of the *Busycon* RPM produced by peak fractions and by synthetic pQDPFLRFamide.

TNRNFLRFamide and SDRNFLRFamide also increase cardiac ganglion burst rate, probably by a similar cellular mechanism. The positive inotropic actions of FaRPs on the *Limulus* heart are, at least in part, due to an enhancement of cardiac muscle contractility. However, the effect of these FaRPs on transmitter release at the cardiac neuromuscular junction is not known. We have not failed to notice that the actions of TNRNFLRFamide and SDRNFLRFamide on the *Limulus* myocardium are similar to those produced by dopamine or proctolin (Watson and Groome, 1989). FaRPs might act *via* similar mechanisms, or might induce the release of these modulators from cardiac ganglion neurons.

Structure-activity relationship of synthetic FaRPs

The *Limulus* heart is responsive to several N-terminally extended FaRPs, whereas RNFLRFamide is less effective, and the tetrapeptides FLRFamide and FMRFamide are essentially inactive on this preparation. The relative po-

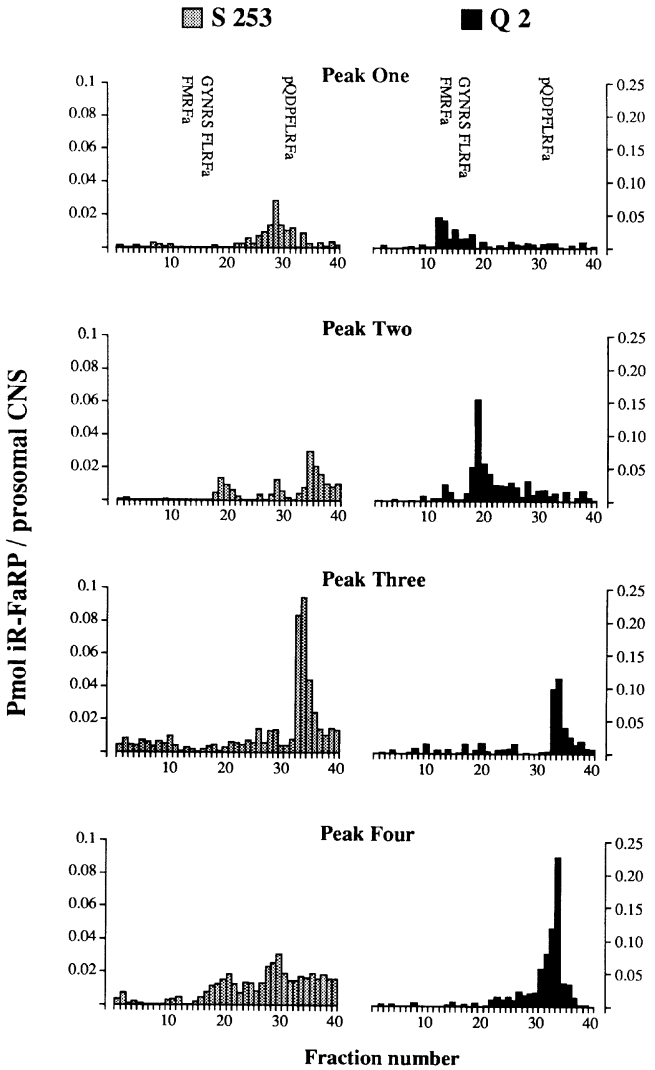

Figure 6. FaRP-like immunoreactivity in partially purified *Limulus* extracts. Each of the four bioactive peaks from the third HPLC run was subjected to further purification with a linear gradient of 16 to 40% acetonitrile. The profiles of immunoreactivity to the antisera S-253 and Q2 are shown, as are the elution times of synthetic FaRPs (a = amide).