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RESEARCH ARTICLE

Distinct or shared actions of peptide family isoforms: I. Peptide-specific actions of pyrokinins in the lobster cardiac neuromuscular system

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ABSTRACT

Although the crustacean heart is modulated by a large number of peptides and amines, few of these molecules have been localized to the cardiac ganglion itself; most appear to reach the cardiac ganglion only by hormonal routes. Immunohistochemistry in the American lobster *Homarus americanus* indicates that pyrokinins are present not only in neuroendocrine organs (pericardial organ and sinus gland), but also in the cardiac ganglion itself, where pyrokinin-positive terminals were found in the pacemaker cell region, as well as surrounding the motor neurons. Surprisingly, the single pyrokinin peptide identified from *H. americanus*, FSPRLamide, which consists solely of the conserved FXPRLamide residues that characterize pyrokinins, did not alter the activity of the cardiac neuromuscular system. However, a pyrokinin from the shrimp *Litopenaeus vannamei* [ADFAFNPRLamide, also known as *Penaeus vannamei* pyrokinin 2 (PevPK2)] increased both the frequency and amplitude of heart contractions when perfused through the isolated whole heart. None of the other crustacean pyrokinins tested (another from *L. vannamei* and two from the crab *Cancer borealis*) had any effect on the lobster heart. Similarly, altering the PevPK2 sequence either by truncation or by the substitution of single amino acids resulted in much lower or no activity in all cases; only the conservative substitution of serine for alanine at position 1 resulted in any activity on the heart. Thus, in contrast to other systems (cockroach and crab) in which all tested pyrokinins elicit similar bioactivities, activation of the pyrokinin receptor in the lobster heart appears to be highly isoform specific.

KEY WORDS: Central pattern generator, Neuromodulation, Neuropeptide

INTRODUCTION

The central pattern generator (CPG)-effector systems of invertebrates have previously and continue to provide valuable contributions to our understanding of peptidergic signaling. For example, the numerically simple systems of decapod crustaceans have been instrumental in refining our understanding of how the actions of individual and unique combinations of neuromodulators, including peptides, are capable of reconfiguring 'hard-wired' neural networks to allow for an almost infinite array of distinct behavioral outputs (e.g. Brezina, 2010; Briggman and Kristan, 2008; Christie et al., 2010a; Dickinson, 2006; Rauscent et al., 2006; Stein, 2009). These CPG-effector systems have also provided important contributions to our understanding of the evolution of neuropeptides and peptidergic signaling systems (e.g. Christie, 2011; Christie et al., 2010a).

The crustacean cardiac neuromuscular system, which consists of the cardiac ganglion (Fig. 1) and the heart musculature, is among the simplest CPG-effector systems known (Cooke, 2002). The decapod heart is neurogenic; its output is driven by the activity of the cardiac ganglion, which, in the American lobster *Homarus americanus*, contains just nine neurons: four small premotor, or pacemaker, neurons and five large motor neurons (Cooke, 2002). The pacemaker neurons are electrically coupled to one another and form chemical synapses onto the motor neurons (Cooke, 2002). The motor neurons, which are likewise electrically coupled to one another, innervate the heart muscle and provide the drive for generating heart contractions; at the same time, they provide feedback to the pacemaker neurons (Cooke, 2002). The heart muscle also provides feedback to the cardiac ganglion via mechanosensitive dendrites (Garcia-Crescioni et al., 2010), as well as through the production and release of the diffusible gas transmitter nitric oxide (Mahadevan et al., 2004).

Despite its apparent simplicity, the output of the crustacean cardiac neuromuscular system is quite diverse; this functional flexibility is imparted largely by the actions of locally released and circulating neuromodulators on the various components that constitute this CPG-effector system (e.g. Christie, 2011; Christie et al., 2010a). Local sources of neuromodulators include the cardiac pacemaker and motor neurons themselves [glutamate in the former (Delgado et al., 2000) and a member of the calcitonin-like diuretic hormone/diuretic hormone 31 peptide family in the latter (Christie et al., 2010b)], and axons of projection neurons whose somata are located elsewhere in the nervous system (Cooke, 2002), which are the local sources of at least GABA (Delgado et al., 2000) and dopamine (Fort et al., 2004). The neuroendocrine pericardial organ, which surrounds the heart and the X-organ–sinus gland (XO–SG) complex, located in the eyestalks, are the primary sources of the hemolymph-borne neuromodulators that influence the cardiac CPG-effector system (Christie, 2011). An extensive array of molecules have been identified in these neuroendocrine organs, including small-molecule transmitters, amines and diffusible gas transmitters, as well as a wide variety of peptides (Christie, 2011).

While a large number of modulators have been shown to be bioactive in the cardiac neuromuscular system, one group of peptides that has yet to be examined on this CPG-effector system is the pyrokinin family. The pyrokinins, a subgroup of the pheromone biosynthesis activating neuropeptide (PBAN)/diapause hormone/pyrokinin/periviscerokinin superfamily, are characterized by the conserved carboxyl (C)-terminal motif FXPRLamide, where X is a variable residue (e.g. Predel and Wegener, 2006; Rafaeli,

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List of abbreviations

CabPK1	*Cancer borealis* pyrokinin 1
CabPK2	*Cancer borealis* pyrokinin 2
CPG	central pattern generator
dn	dorsal nerve
LeucoPK	*Leucophaea maderae* pyrokinin
NDS	normal donkey serum
PBAN	pheromone biosynthesis activating neuropeptide
PevPK1	*Penaeus vannamei* pyrokinin 1
PevPK2	*Penaeus vannamei* pyrokinin 2
sgn	sinus gland nerve
STNS	stomatogastric nervous system
XO-SG	X-organ–sinus gland

2009). While originally isolated from insects (e.g. Holman et al., 1986), members of the PBAN/diapause hormone/pyrokinin/periviscerokinin superfamily have also been identified in a variety of crustaceans (Christie, 2014a,b, 2013; Cooke, 2002; Hui et al., 2012; Ma et al., 2009, 2010, 2008; Saideman et al., 2007; Christie and Chi, 2015). In members of the Decapoda, 20 pyrokinin-like peptides are known, with essentially all being amino (N)-terminally extended variants of the FXPRLamide consensus motif (Christie, 2014a; Hui et al., 2012; Ma et al., 2009, 2010, 2008; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015). The one exception to this rule is the peptide FSPRLamide from the lobster *H. americanus* (Ma et al., 2008), which consists of just the consensus motif FXPRLamide itself and where the X in this case is a serine. FSPRLamide is the only isoform of pyrokinin thus far identified from *H. americanus*, which is also atypical, because essentially all other crustacean species thus far examined possess two or more pyrokinin isoforms (Christie, 2014a,b, 2013; Ma et al., 2009, 2010; Saideman et al., 2007; Torfs et al., 2001; Christie and Chi, 2015). The brachyuran crab *Callinectes sapidus* is currently the only other exception to this rule, although the pyrokinin identified from it is a stereotypical N-terminally extended variant (Hui et al., 2012).

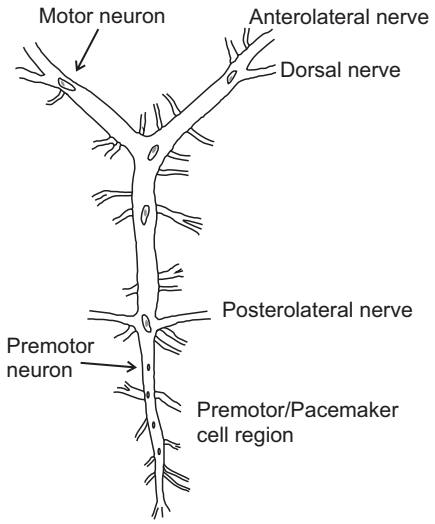

Fig. 1. Schematic representation of the cardiac ganglion of *Homarus americanus*. The five large motor neurons are located in the anterior part of the trunk and in the anterolateral nerves; the four small premotor neurons are located in the posterior part of the main ganglionic trunk. One of each type of neuron is indicated by arrow.

In insects, pyrokinins have been shown to be highly pleiotropic in terms of their physiological actions (e.g. Predel and Wegener, 2006; Rafaeli, 2009), while in crustaceans, information on bioactivity is limited to studies of their effects on the crab (*Cancer borealis*) and lobster (*H. americanus*) stomatogastric nervous system (STNS), a CPG-effector system that controls the rhythmic movement of the foregut, in which all isoforms tested elicit a similar motor output in both species [Saideman et al., 2007; Dickinson et al., 2015 (the companion article to the study presented here)]. Given the lack of information on the cardioactive properties of pyrokinins, we asked here whether members of this peptide family are likely to reach the heart as either local or hormonal modulators, and if so, whether they modulate the output of the lobster cardiac neuromuscular system. We found that pyrokinins are likely to influence the heart via both local and hormonal pathways and they are bioactive in this system. However, to our surprise given the ubiquitous actions different isoforms elicit from the STNS (Saideman et al., 2007; Dickinson et al., 2015), bioactivity in the cardiac neuromuscular system is highly isoform specific.

RESULTS

Distribution of pyrokinin-like immunoreactivity in the cardiac ganglion and neuroendocrine organs

The cardiac ganglion

Whole-mount immunohistochemistry was conducted on the isolated *H. americanus* cardiac ganglion to determine if members of the pyrokinin family are present in either the cardiac ganglion pacemaker/motor neurons and/or in input axons projecting to the cardiac ganglion from other portions of the lobster nervous system. The antibody used for these experiments was generated against the sequence FSPRLamide, the only pyrokinin thus far isolated from *H. americanus* (Ma et al., 2008); the production and characterization of this antibody are described in detail in a companion article to this report (Dickinson et al., 2015). In none of the cardiac ganglia examined (N=12) was any evidence for pyrokinin immunoreactivity noted in either the cardiac ganglion pacemaker or motor neurons (Fig. 2). However, in all cardiac ganglia, a single input axon (or fascicle of axons) was labeled on each side of the ganglion by the pyrokinin antibody (Fig. 2A,B). This axon (or fascicle) projects into the ganglion from a secondary root, presumably the dorsal nerve (dn). Within the cardiac ganglion, the dn-derived pyrokinin-immunopositive axon/fascicle gives rise to a number of discrete regions of neuropil, which are typically located near or surrounding each of the motor neuron somata (Fig. 2A–C), as well as in the region of the ganglion where the pacemaker neuron somata are found (Fig. 2D).

Neuroendocrine organs

The pericardial organ

In addition to intrinsically released compounds, the lobster cardiac ganglion is also modulated by a variety of hormones released from the neuroendocrine pericardial organ and/or XO–SG complex (e.g. Christie, 2011). In *H. americanus*, the pericardial organ exists as a system of nerve trunks that ramify, forming diffuse plexuses of release terminals along the lateral walls of the pericardial chamber and on the ligaments of the heart. The somata that give rise to the pericardial organ include both intrinsic and extrinsic somata, with the majority of the latter probably residing within the thoracic nervous system. Processing of pericardial organs (N=14) with the pyrokinin antibody revealed immunopositive axons and release profiles throughout the system (Fig. 3A). In no preparation were any immunopositive somata detected, suggesting that the origin of the

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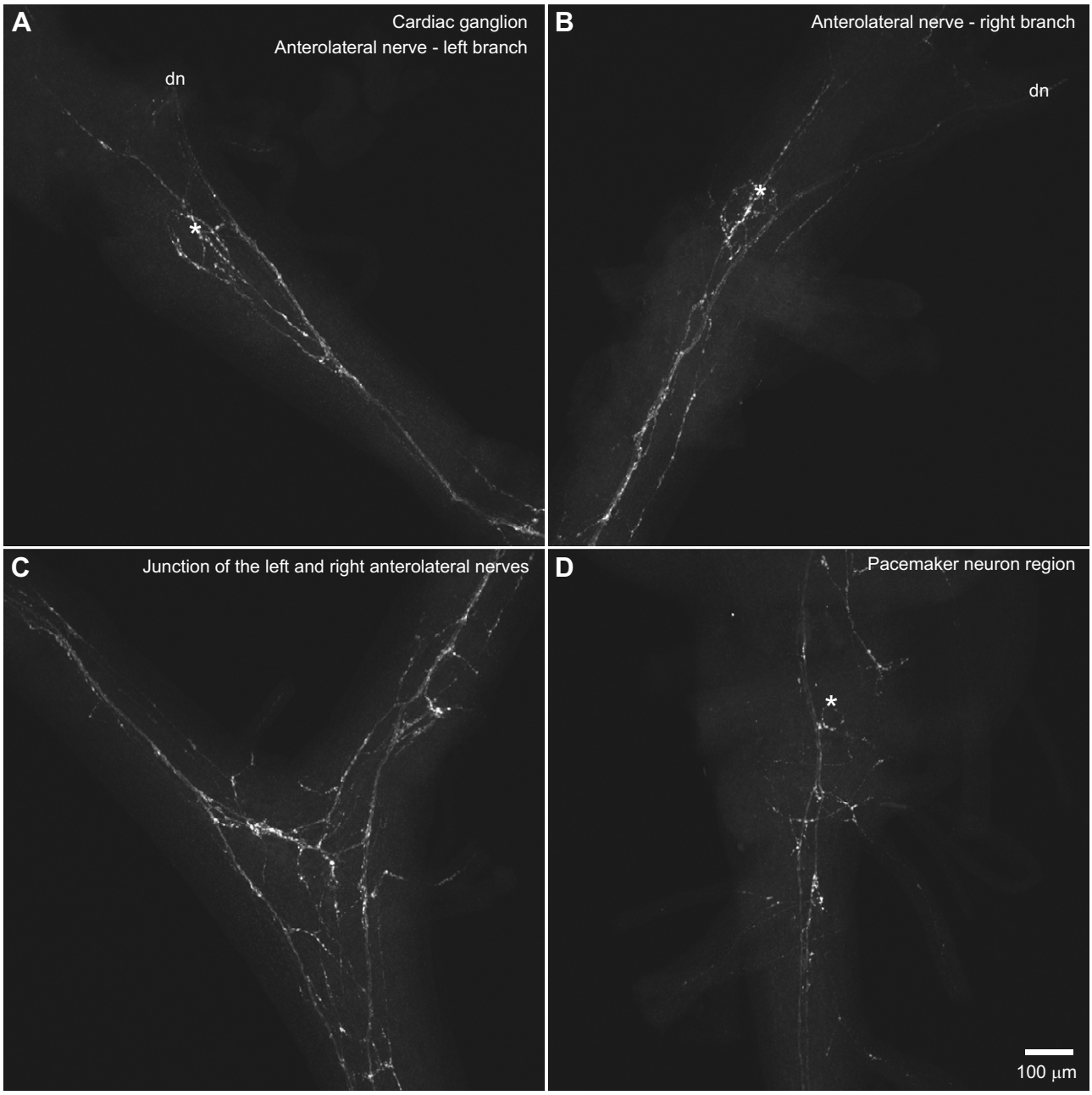

Fig. 2. Distribution of pyrokinin-like labeling in the cardiac ganglion of *Homarus americanus*. (A–D) In the cardiac ganglion, a single pyrokinin-immunoreactive input axon projects into the ganglion from the dorsal nerve (dn). These fibers project down each of the paired anterolateral nerve, arborizing in the nerves and producing extensive regions of neuropile, including processes surrounding each of the motor neuron somata (the location of motor neuron somata identified via bright field optics are indicated by asterisks in A and B). Regions of neuropile were also present in the region of the pacemaker neurons within the ganglion (D), i.e. posterior to the junction of the posterolateral nerves (see Fig. 1); as for the motor neuron somata, pyrokinin-like immunoreactivity appears to surround at least a subset of the pacemaker somata (one denoted by asterisk in D). All images are brightest pixel projections of multiple optical sections (25 in A, 32 in B, 30 in C and 33 in D) collected at approximately 2 µm intervals; all panels are shown at the same scale.

pericardial organ labeling is from somata located outside of this neuroendocrine organ.

The X-organ–sinus gland complex

In *H. americanus*, as in most decapod species, the XO–SG complex is located within the eyestalk (e.g. Christie, 2011). This system is composed of the X-organ, a tightly associated cluster of neurosecretory cells located near the ventral, proximal margin of the medulla terminalis of the eyestalk, and the sinus gland, a well-defined neuroendocrine organ located on the dorsal or dorso-lateral side of the optic ganglia, generally at the level of the medulla interna and/or medulla externa. The axon tract that connects the X-organ and sinus gland is typically referred to as the sinus gland nerve (sgn). In addition to the X-organ, a few somata located in other regions of the central nervous system (e.g. other regions of the eyestalk, the brain and/or the thoracic ganglia) are purported to project to and contribute to the innervation of the sinus gland (e.g. Christie, 2011). Processing of isolated eyestalk ganglia (N=11) with the pyrokinin antibody revealed

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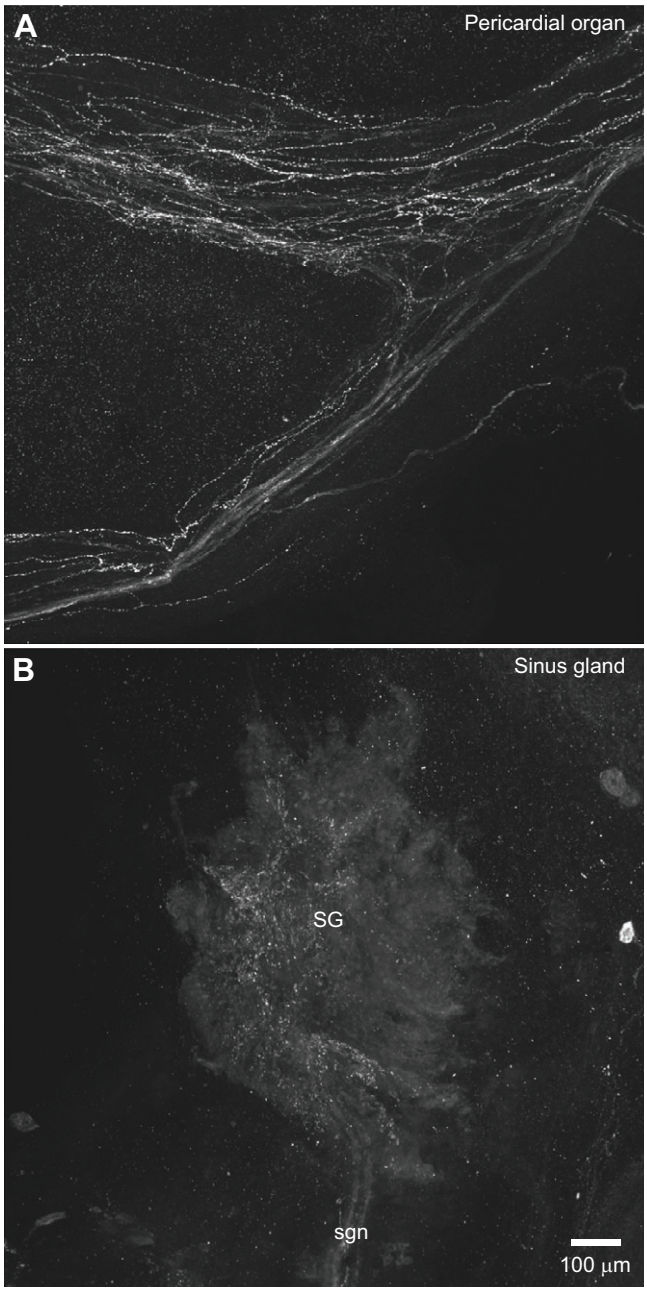

Fig. 3. Distribution of pyrokinin-like labeling in the pericardial organ and X-organ–sinus gland complex (XO-SG) of *Homarus americanus*. (A) In the pericardial organ, pyrokinin-like immunoreactivity was restricted to labeled fibers and puncate release terminals. No pyrokinin-like immunoreactivity was seen in any of the intrinsic somata within the pericardial organ. This image is a brightest pixel projection of 63 optical sections taken at approximately 2 μm intervals. (B) Pyrokinin-like labeling in the sinus gland (SG) consisted of a diffuse plexus of small release terminals that appear to be derived from fibers present in the sinus gland nerve (sgn), presumably from somata present in the X-organ, although it was not possible to unambiguously trace the sgn fiber tract for any appreciable distance because of the weak labeling intensity. This image is a brightest pixel projection of 57 optical sections taken at approximately 2 μm intervals. Both A and B are shown at the same scale.

an extensive collection of immunopositive profiles, including numerous cell bodies, neuropilar regions and axon tracts (data not shown), as well as very fine, punctate labeling in the sinus gland (Fig. 3B). The sinus gland immunoreactivity is derived from a tract of small-diameter axons, which are likely to be within the sgn, although the weak intensity of the labeling in these fibers precluded their being traceable to any soma cluster. Consistent with this predicted origin, 8–10 cell bodies in the presumptive region of the X-organ were pyrokinin immunopositive in each of the 10 preparations examined (data not shown).

Table 1. Selected natural and synthetic pyrokinin isoforms

Type	Peptide structure	Acronyms used in text	Reference
Native crustacean isoforms	DFAFSPRLamide	PevPK1	Torfs et al., 2001
	ADFAFNPRLamide	PevPK2	Torfs et al., 2001
	TNFAFSPRLamide	CabPK1	Ma et al., 2010
	SGGFAFSPRLamide	CabPK2	Ma et al., 2010
	FSPRLamide	–	Ma et al., 2008
Synthetic analogs	DFAFNPRLamide	PevPK2[2–9]	This study
	FAFNPRLamide	PevPK2[3–9]	This study
	FNPRLamide	PevPK2[5–9]	This study
	ADFAFSPRLamide	[Ser^{6}]PevPK2	This study
	SDFAFNPRLamide	[Ser^{1}]PevPK2	This study
	LDFAFNPRLamide	[Leu^{1}]PevPK2	This study
	GDFAFNPRLamide	[Gly^{1}]PevPK2	This study

Abbreviations: CabPK1, *Cancer borealis* pyrokinin 1; CabPK2, *Cancer borealis* pyrokinin 2; PevPK1, *Penaeus vannamei** pyrokinin1; PevPK2, *Penaeus vannamei** pyrokinin 2.
*This species is now referred to as *Litopenaeus vannamei*.

PevPK-2 increases heart contraction amplitude and frequency

Six different pyrokinins (Table 1), one identified from the cockroach *Rhyparobia* (formerly *Leucophaea*) *maderae* [LeucoPK, pETSFTPRLamide (Holman et al., 1986)] and five identified from decapod crustaceans, two from the shrimp *Litopenaeus* (formerly *Penaeus*) *vannamei* [PevPK1, DFAFSPRLamide; PevPK2, ADFAFNPRLamide (Torfs et al., 2000)], two from the crab *C. borealis* [CabPK1, TNFAFSPRLamide; CabPK2, SGGFAFSPRLamide (Saideman et al., 2007)] and one from the lobster *H. americanus* [FSPRLamide (Ma et al., 2008)], were perfused through the isolated lobster heart to determine whether they modulated heart contractions. Because pyrokinin immunoreactivity was prevalent in both the pericardial organ and the cardiac ganglion, we tested the effects of both a hormonal concentration (3×10^{-8} mol l^{-1}) and a concentration more likely to be achieved with local release (10^{-6} mol l^{-1}). At each of these concentrations, only PevPK2 resulted in a significant change in contraction amplitude or contraction frequency (Fig. 4). During perfusion with PevPK2, the mean change in frequency at 3×10^{-8} mol l^{-1} was 13.4±2.8%; frequency increased by 27.2±4.7% at 10^{-6} mol l^{-1}. For amplitude, the mean change was 8.7±1.9% at 3×10^{-8} mol l^{-1} and 22.5±3.9% at 10^{-6} mol l^{-1}.

Because PevPK2 was the only pyrokinin that had a significant effect on the output of the cardiac neuromuscular system, we examined the effects of this peptide across a range of concentrations. Concentrations lower than 5×10^{-9} mol l^{-1} had no effect, whereas concentrations higher than 2×10^{-7} mol l^{-1} often caused the heartbeat to become highly irregular, with complex patterns of beating (Fig. 5). Within the relatively narrow range of PevPK2 concentrations from 5×10^{-9} to 2×10^{-7} mol l^{-1}, increasing concentrations of PevPK2 had clear dose-dependent effects on

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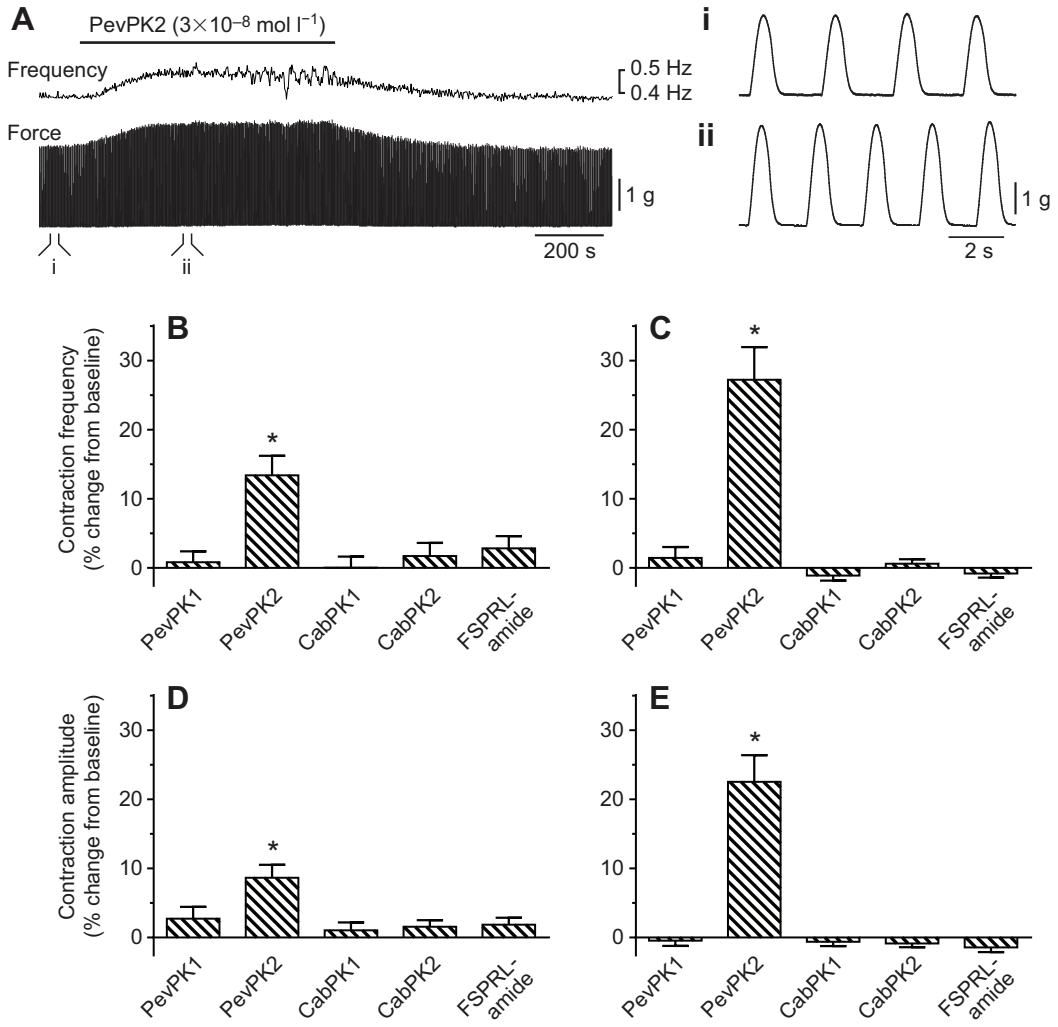

Fig. 4. Heart contraction frequency and amplitude in *Homarus americanus* increases with application of PevPK2. At concentrations reflecting both hormonal (3×10^{-8} mol l^{-1}) and local (10^{-6} mol l^{-1}) release, perfusion through the heart of one of the pyrokinins tested, PevPK2, elicited significant increases in both contraction frequency and amplitude in the isolated lobster heart; the other pyrokinins had no significant effects. (A) Recording of heart contractions (force) and frequency during perfusion with 3×10^{-8} mol l^{-1} PevPK2 illustrates the visible increases in contraction frequency and amplitude; the effect washed out upon removal of the peptide. (i) and (ii) are enlarged portions of regions highlighted in A. (B–E) Perfusion with PevPK2 results in significant changes in frequency (B,C) and amplitude (D,E), whereas perfusion with PevPK1, CabPK1, CabPK2 and FSPRLamide does not significantly change either contraction frequency (B,C) or amplitude (D,E). The effects of PevPK2 on both frequency (*t*-test, $P<0.05$) and amplitude (*t*-test, $P<0.01$) were significantly larger at the higher concentration (10^{-6} mol l^{-1}; C,E) than at the lower concentration (3×10^{-8} mol l^{-1}; B,D). 3×10^{-8} mol l^{-1}, $N=13$; 10^{-6} mol l^{-1}, $N=5$; * indicates a mean value that was significantly different from zero. Error bars show the s.e.

both amplitude and frequency (Fig. 6A,B). Significant increases in frequency ranged from means of 3.7±1.6% at 5×10^{-9} mol l^{-1} to 19.6±4.3% at 2×10^{-7} mol l^{-1}. Similarly, the changes in amplitude ranged from means of 5.0±1.6% at 2×10^{-8} mol l^{-1} to 13.4±3.1% at 2×10^{-7} mol l^{-1}.

In addition to its effects on contraction amplitude and duration, perfusion of PevPK2 resulted in small, but significant, decreases in contraction duration (Fig. 6C). These decreases ranged from −1.0±0.4% at 5×10^{-9} mol l^{-1} to −4.9±1.1% at 2×10^{-7} mol l^{-1}. This decrease in duration appeared to be due to an increase in the rate of muscle relaxation, because the time taken to return to baseline (relaxation time) decreased, while the time required for contraction (rise time) did not change (Fig. 6D). The decreases in relaxation time were similar to those recorded for contraction duration, ranging from −1.2±0.5% at 5×10^{-9} mol l^{-1} to −7.2±1.3% at 2×10^{-7} mol l^{-1} (Fig. 6E). Because the increases in frequency were greater than the decreases in contraction duration, the duty cycle of the contractions tended to increase as the concentration of PevPK2 was raised (5.8±1.7% at 2×10^{-8} mol l^{-1} to 12.5±2.8% at 2×10^{-7} mol l^{-1}; Fig. 6F).

Increases in duty cycle and burst duration in the cardiac ganglion probably contribute to the effects of PevPK2 on heart contraction amplitude

Among the factors that control the amplitude of heart contraction in the lobster are both the frequency of bursts in the cardiac motor neurons and the duty cycle of those bursts (Williams et al., 2013). Because contraction frequency is determined directly by burst cycle frequency, the changes in burst frequency are the same as those seen for contraction frequency (Fig. 4B). To quantify the effects of PevPK2 on the other parameters that might contribute to the observed increases in contraction amplitude, we recorded the activity of the cardiac motor neurons, using extracellular electrodes on the anterolateral nerves, in the semi-intact lobster heart.

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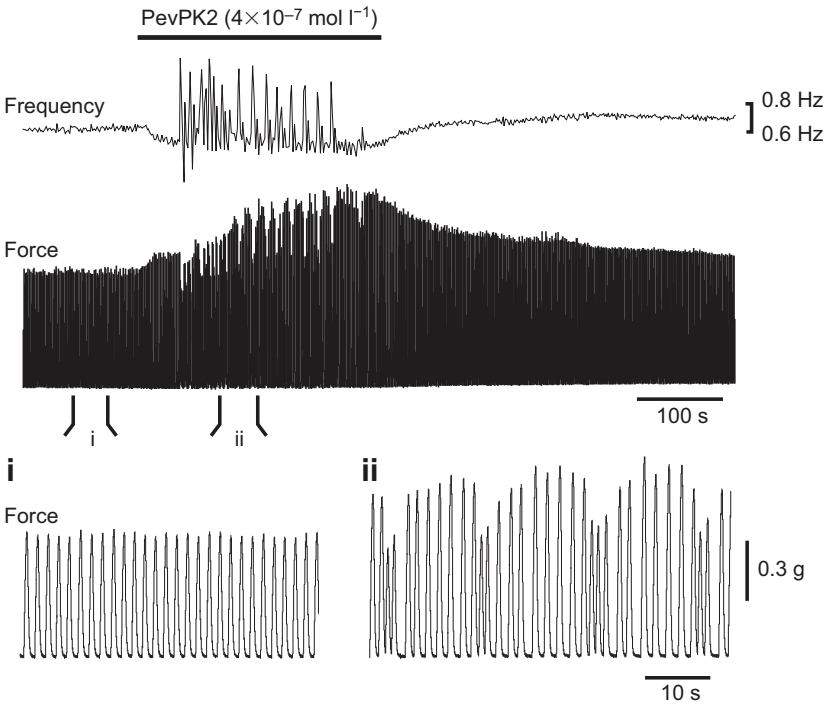

Fig. 5. Higher concentrations of PevPK2 often result in complex patterns of beating. Results obtained with a PevPK2 concentration of 4×10^{-7} mol l^{-1} are shown. Although average heart rate increased, it was highly irregular in the presence of PevPK2. (i,ii) Enlarged portions of the recording above show that high PevPK2 concentrations induced complex contraction patterns, in which the heart exhibited a series of rapid contractions without full relaxation, followed by a full relaxation and longer period between successive beats.

Although there was considerable variation among preparations, as illustrated for two hearts in Fig. 7A,B, both burst duration and burst duty cycle tended to increase during perfusion with PevPK2. Burst duration increased in the higher concentrations of PevPK2, with significant increases at 4×10^{-8} mol l^{-1} and 6×10^{-8} mol l^{-1} (Fig. 7C). These changes in burst duration were relatively small, but there was a trend towards an increase with increasing peptide concentration (ANOVA, P=0.051). However, because both cycle frequency and burst duration tended to increase with increasing concentrations of PevPK2, burst duty cycle increased significantly with increasing PevPK2 concentrations, with increases ranging from 18.5±7.6% at 2×10^{-8} mol l^{-1} to 32.2±7.6% at 2×10^{-7} mol l^{-1} (Fig. 7D). In addition to changes in cycle frequency and duty cycle, increases in spike frequency or in the number of spikes per burst would be expected to alter contraction amplitude. However, neither of these parameters changed during PevPK2 perfusion (Fig. 7E,F).

Although increases in burst duration in the motor neurons could theoretically account for an increase in contraction amplitude, we were somewhat surprised to see such an increase, albeit modest, in burst duration, since we recorded a decrease in contraction duration during PevPK2 perfusion (see Fig. 6C). Because these changes were modest and varied somewhat between individual lobsters, we compared the changes in duration of contractions and motor neuron bursts in individual lobsters during perfusion of 4×10^{-8} mol l^{-1} PevPK2. In nearly all preparations, there was a clear increase in burst duration (Fig. 8), but a small decrease in contraction duration, suggesting the possibility that the peptide alters the rate of relaxation at the level of the cardiac muscle itself, or that changes in the internal patterning of action potentials within the burst are responsible for the changes in contraction duration.

Synthetic pyrokinin analogs

Because FSPRLamide, the only known native *H. americanus* pyrokinin (Ma et al., 2008), had no measurable effect on the cardiac neuromuscular system in the lobster, and because only one of the other pyrokinins we tested, PevPK2 (ADFAFNPRLamide), altered cardiac contractions, we wanted to understand how the structure of the pyrokinin amino acid sequence affects its modulation of the cardiac system. Strikingly, PevPK1 (DFAFSPRLamide), which had no effect, and PevPK2, which increased both frequency and amplitude of cardiac contractions, differed at only two residues, one being the variable amino acid in the conserved sequence –FXPRLamide, the other being the addition of an alanine to the N-terminus in PevPK2. Thus, we compared the effects of PevPK2 with the effects of a number of synthetic sequences, each of which had specific amino acid changes relative to PevPK2 (Table 1). These synthetic analogs allowed us to examine the roles that might be played by three facets of the amino acid sequence: (1) the overall length of the peptide, (2) the variable amino acid (X) within the conserved sequence –FXPRLamide, and (3) the identity of the N-terminal amino acid.

To determine whether the length of the peptide was critical, we tested three pyrokinins that were identical to PevPK2, except that they were N-terminally truncated by one, two or four amino acids, i.e. DFAFNPRLamide (PevPK2[2–9]), FAFNPRLamide (PevPK2 [3–9]) and FNPRLamide (PevPK2[5–9]). None of these peptides had any effect on the lobster heart (Fig. 9). Even the elimination of a single amino acid from the N-terminus eliminated all activity by the peptide, suggesting that the length of the peptide was critical.

As stated earlier, a second difference between the PevPK1 (DFAFSPRLamide) and PevPK2 (ADFAFNPRLamide) sequences lies in the variable amino acid within the conserved sequence –FXPRLamide. The variable residue in the non-bioactive PevPK1 is a serine, whereas the bioactive PevPK2 possesses an asparagine at this position. We thus tested a PevPK2 sequence in which the asparagine was replaced with a serine, i.e. [Ser^6]PevPK2 (ADFAFSPRLamide), and found that, like PevPK1, it had no physiological effects (Fig. 9), suggesting that the variable amino acid, like the overall length of the peptide, is essential for bioactivity.

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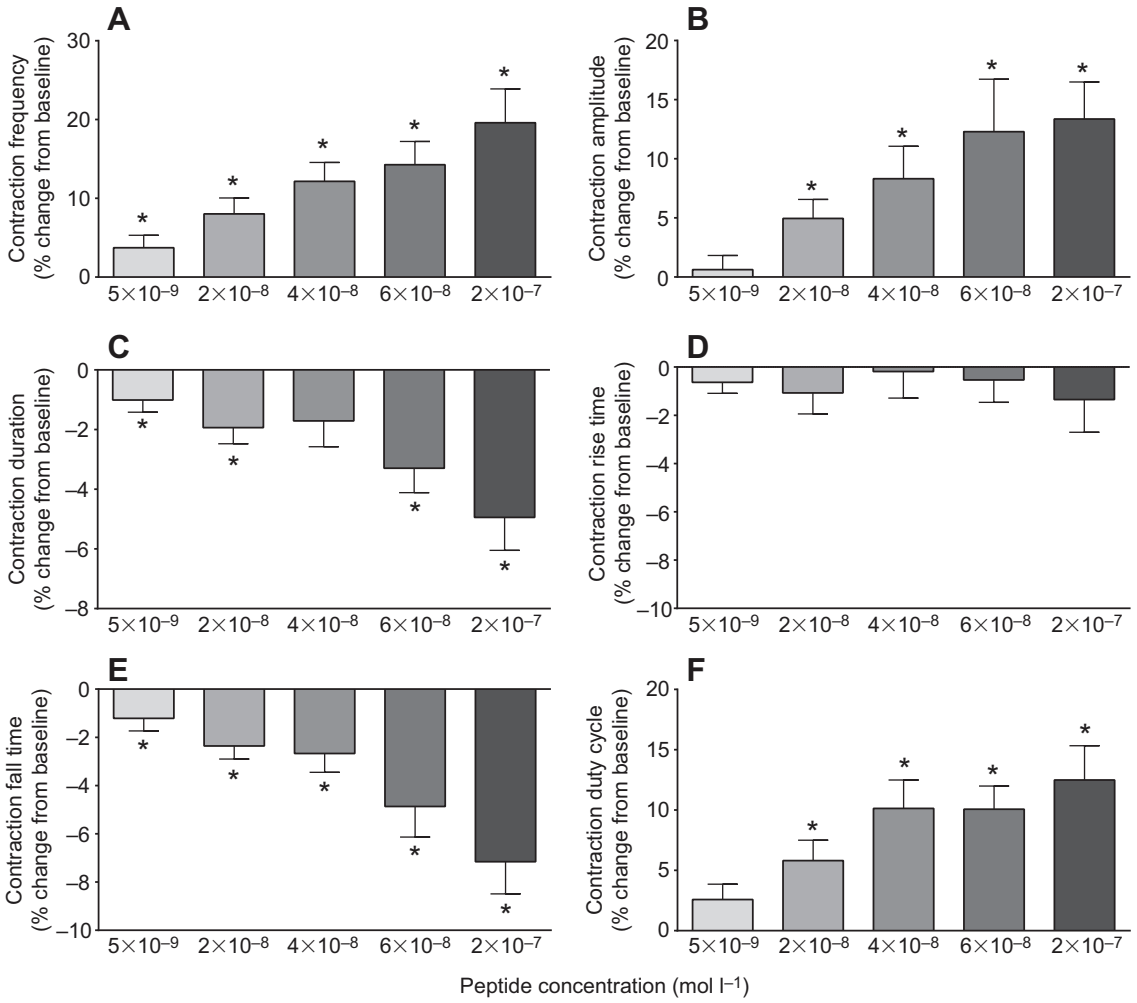

Fig. 6. Perfusion of PevPK2 through the heart of *Homarus americanus* alters most contraction parameters in a dose-dependent manner. Both contraction frequency (A) and amplitude (B) increased at concentrations of 5×10^{-9} mol l^{-1} (frequency) or 2×10^{-8} mol l^{-1} (amplitude) and above (single sample *t*-tests, denoted by *). These increases were larger at higher concentrations of the peptide (ANOVA, $P<0.01$ for both parameters). (C) Contraction duration decreased slightly relative to baseline, with significant changes (*, in single sample *t*-tests) at all concentrations except 4×10^{-8} mol l^{-1}. Like amplitude and frequency, the changes tended to be larger for higher peptide concentrations (ANOVA, $P<0.01$). Although the rise time (D) was not affected by the peptide, the change in duration was mirrored by dose-dependent changes in relaxation (E) (ANOVA, $P<0.0001$). (F) The duty cycle also showed a consistent increase with increasing concentrations of the peptide ($P<0.01$). $N=22$ for 5×10^{-9} mol l^{-1}, 2×10^{-8} mol l^{-1} and 4×10^{-8} mol l^{-1}; $N=15$ for 6×10^{-8} mol l^{-1} and 2×10^{-7} mol l^{-1}. * indicates a mean value that was significantly different from zero (single sample *t*-test). Error bars show the s.e.

To determine whether the identity of the N-terminal amino acid is crucial, we designed three peptides with serine, leucine or glycine substitutions for the N-terminal alanine of PevPK2, i.e. [Ser^1]PevPK2 (SDFAFNPRLamide), [Leu^1]PevPK2 (LDFAFNPRLamide) and [Gly^1]PevPK2 (GDFAFNPRLamide). Of these peptides, only [Ser^1]PevPK2 elicited any significant changes on the heart's frequency and amplitude (Fig. 9). This peptide caused a small increase in both frequency and contraction amplitude; effects of the peptide on other contraction parameters mirrored the effects of PevPK2 on all contraction parameters (i.e. decreased duration and relaxation time); however, the threshold for effects was considerably higher than that recorded for the original PevPK2 sequence (Fig. 10). [Ser^1]PevPK2 had no effects at concentrations below 2×10^{-7} mol l^{-1}; moreover, it did not cause disruption of beating even at concentrations as high as 2×10^{-6} mol l^{-1}. Additionally, the magnitude of the effects of [Ser^1]PevPK2 was much smaller than that of PevPK2 (Fig. 11).

DISCUSSION

Pyrokinin is likely to reach the cardiac neuromuscular system from both local and hormonal sources

Immunohistochemical mapping showed that pyrokinin is located within the cardiac ganglion itself, as well as in the pericardial organ and XO-SG systems, two well-known neuroendocrine organs known to release cardiomodulatory hormones (e.g. Christie, 2011). Specifically, there was extensive staining in neuropil surrounding the pacemaker and motor neurons of the cardiac ganglion, as well as in endocrine release terminals in both the pericardial organ and sinus gland. These results suggest that pyrokinin is likely to act on the cardiac ganglion both as a locally released neuromodulator and as a circulating neurohormone. In support of this hypothesis, we found that the concentration range over which PevPK2 enhanced activity of the cardiac neuromuscular system spanned the ranges that would be expected for both hormonal and local release. While concentrations of pyrokinins in the lobster hemolymph have not been measured, measurements of

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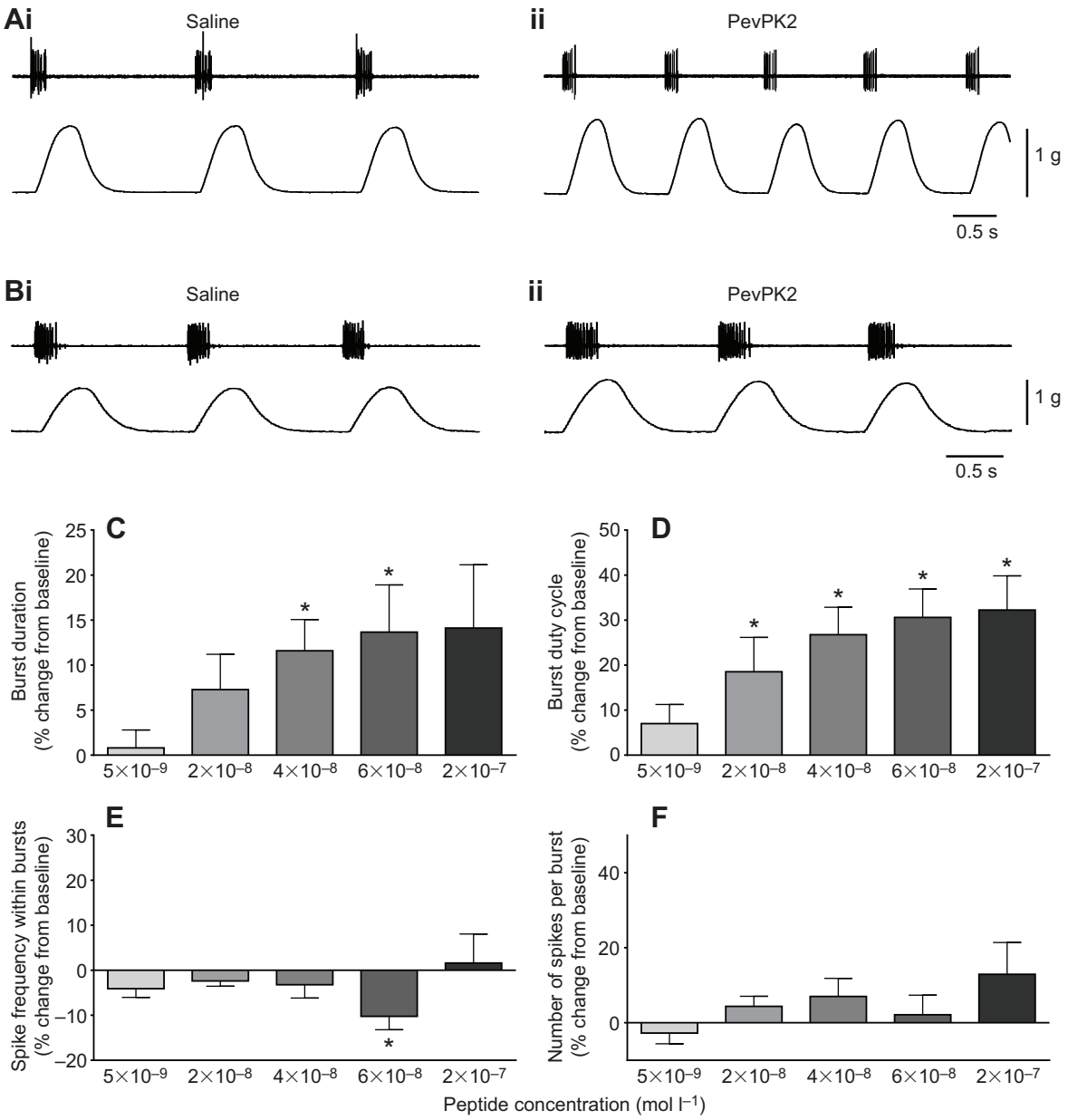

Fig. 7. Recordings of motor neuronal activity on the anterolateral nerve suggest that changes in burst duration and duty cycle contribute to the increased contraction amplitude in PevPK2. (A,B) Recordings from the anterolateral motor nerve in two different preparations illustrate the changes that were seen in burst parameters, with simultaneous recordings of heart contractions (force), when two different hearts were perfused with 6×10^{-8} mol l^{-1} PevPK2. In the preparation shown in Ai and ii, burst duration increased only slightly, but cycle frequency increased substantially, with the result that duty cycle increased. In the heart shown in Bi and ii, there was little change in cycle frequency, but a large increase in burst duration, again leading to a substantial increase in duty cycle. (C–F) Data pooled from multiple preparations. (C) Burst duration increased at higher concentrations, with a trend (ANOVA, P=0.051) towards larger increases with higher concentrations. (D) Burst duty cycle increased in the presence of PevPK2, with larger increases at higher peptide concentrations (ANOVA, P<0.01). In contrast, neither spike frequency within bursts (E) nor the number of spikes per burst (F) changed during peptide perfusion. N=10 for all concentrations except 2×10^{-7} mol l^{-1}, where N=9. * indicates a mean value that was significantly different from zero. Error bars show the s.e.

other peptides in arthropods range from ~10^{-10} mol l^{-1} (Fastner et al., 2007; Kang et al., 2014; Žitňan et al., 1999) to as high as 3–4×10^{-8} mol l^{-1} [e.g. ecdysis triggering hormone and vitellogenin inhibiting hormone (Fastner et al., 2007; Kang et al., 2014; Žitňan et al., 1999)]. The threshold concentrations of PevPK2 in the lobster heart were around 3×10^{-8} mol l^{-1}, which corresponds to the highest levels measured in arthropod hemolymph. Nonetheless, because the pericardial organ is located within the pericardial sinus in which the heart lies, hormones released from this neuroendocrine organ are likely to reach the heart initially at concentrations that are even higher than those found in the circulating hemolymph. Thus, it is likely that lobster pyrokinins are able to modulate the motor patterns produced by the cardiac ganglion when released hormonally.

Interestingly, the heartbeat became irregular when pyrokinin was applied at concentrations greater than approximately 2×10^{-7} mol l^{-1}. These patterns resemble the higher-order modulation superimposed on the baseline that was observed for the effects of high concentrations of a FLRFamide-related peptide on the crab heart (Fort et al., 2007) and may reflect a similar process. At concentrations of 10^{-6} mol l^{-1}, nearly all hearts showed these more

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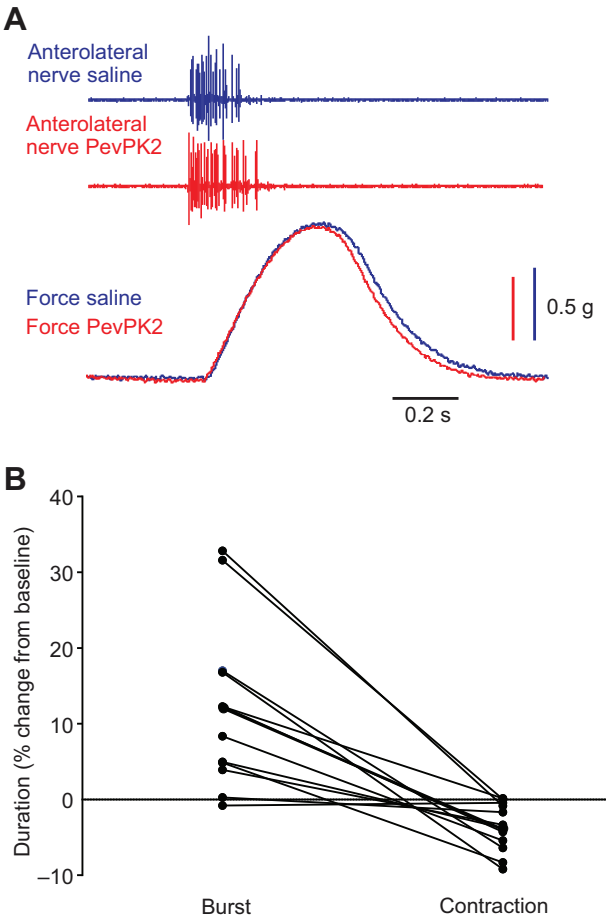

Fig. 8. In individual hearts, burst duration nearly always increases, whereas contraction duration decreases slightly. (A) Simultaneous recordings of the changes in one lobster heart, showing a typical motor neuron burst and resultant contraction force before (blue) and during (red) perfusion with PevPK2 (4×10^{-8} mol l^{-1}). The motor neuron burst is longer in PevPK2, whereas the contraction is slightly shorter in duration. The recordings of contraction force have been scaled to the same amplitude (see scale bars), so that the decrease in duration is seen more accurately. (B) Comparison of percentage changes in burst duration and contraction duration in individual lobsters during the application of PevPK2 at 4×10^{-8} mol l^{-1}. Lines show the changes that took place in each of 10 lobster hearts.

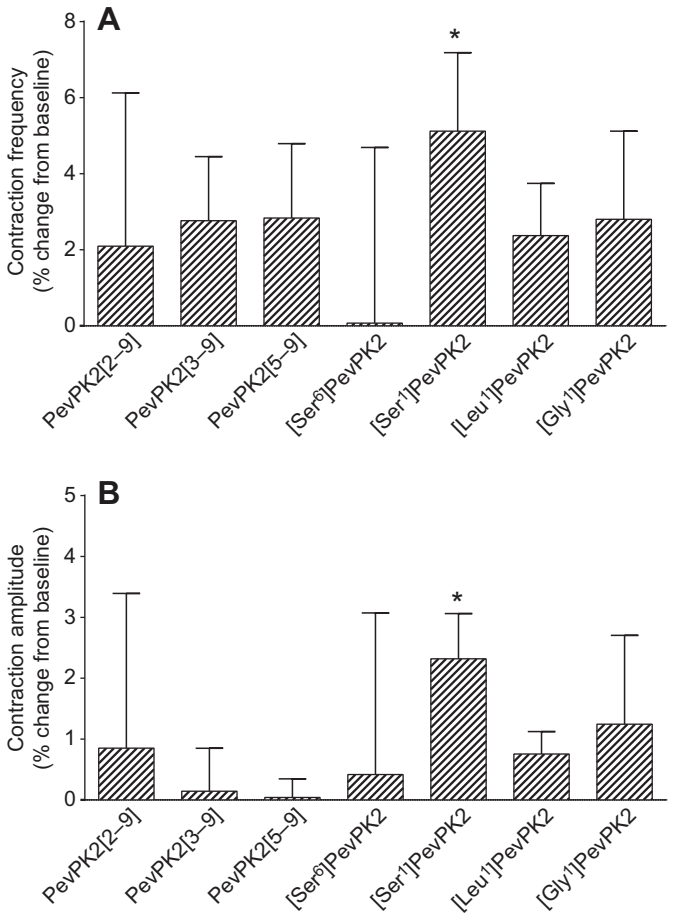

Fig. 9. Most changes to the sequence of PevPK2 eliminate its modulatory effects on the heart. Of the peptides tested, only [Ser1]PevPK2 resulted in changes in either frequency (A) or amplitude (B) when perfused through the heart. *N*=5 for all peptides, except [Ser^1]PevPK2, in which *N*=7. * indicates a mean value that was significantly different from zero ($P<0.05$). Error bars show s.e.m.

complex patterns of heartbeat, suggesting that such concentrations might generate an irregular cardiac output. Nonetheless, the presence of labeled release terminals surrounding the pacemaker and motor neuron somata suggest that local release, which is usually predicted to result in higher concentrations, is likely to occur. However, although pyrokinin immunoreactivity is found widely distributed in the cardiac ganglion, including the region containing the pacemaker neurons and surrounding the motor neurons, it is possible that perfusion with pyrokinins results in higher concentrations in some regions than would be found during local neuronal release, and that this contributes to the generation of the complex patterns. It is also possible that the pyrokinin is released in the cardiac ganglion with a co-transmitter, which helps to stabilize the pattern and thus prevent the irregular bursting that we saw with perfusion of the peptide.

Pyrokinin receptors in the cardiac ganglion show a high level of sequence specificity

In the one previous study of the effects of pyrokinins on the decapod nervous system, Saideman et al. (2007) found that both of the crab pyrokinins they identified had similar modulatory effects on the pattern generators of the *Cancer borealis* stomatogastric nervous system: both activated the gastric mill pattern similarly, while having no effect on the output of the pyloric motor pattern generator. Additionally, they found that the non-native sequence pETSFTPRLamide (LeukoPK), which had been previously identified in cockroach (Holman et al., 1986), had virtually identical effects on the crab stomatogastric system. Similarly, Nachman et al. (1986) found that the sequence of the cockroach pyrokinin could be altered at the N-terminus with relatively minimal effects on activity. Moreover, the peptide was still able to activate movements of the cockroach hindgut when the three N-terminal amino acids were removed, leaving a five-residue peptide, FTPRLamide, which differs by only one amino acid from the lobster FSPRLamide. This contrasts sharply with the effects we recorded on the lobster cardiac neuromuscular system. In the lobster heart, most of the pyrokinins tested had no effect. Indeed, of the five crustacean pyrokinins we tested [FSPRLamide, CabPK1, CabPK2, PevPK1 and PevPK2 (Ma et al., 2008; Saideman et al., 2007; Torfs et al., 2000)], only one had any effects, suggesting that the receptor in the lobster heart is much more constrained than the receptor that mediates the effects of pyrokinins in the crab stomatogastric system. Such differences in receptor specificity have been seen, for example, in *Drosophila*, in which one receptor for tachykinin-related peptides

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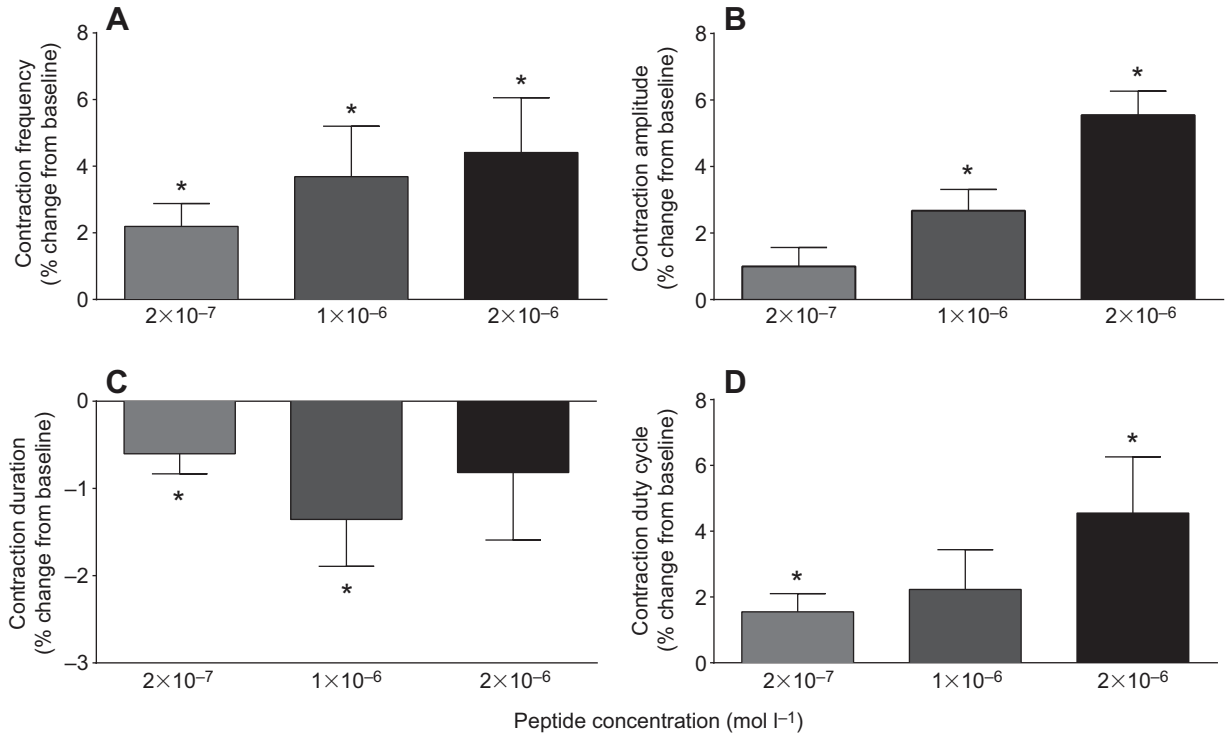

Fig. 10. The qualitative effects of [Ser1]PevPK2 on the lobster heart mimic those of PevPK2. Increases are observed in contraction frequency (A) and amplitude (B). Similarly, contraction duration (C) decreased during perfusion of [Ser1]PevPK2. Because the decreases in contraction duration were very small relative to increases in cycle frequency, contraction duty cycle (D) increased at two of the peptide concentrations tested. However, the thresholds for the effects of [Ser^1]PevPK2 were higher than for PevPK2 (2×10^{-7} mol l^{-1} for frequency, duration and duty cycle; 10^{-6} mol l^{-1} for amplitude). Only contraction amplitude (B) showed a significant increase with higher peptide concentrations (ANOVA, $P<0.01$); other variables did not change significantly as a function of peptide concentration. $N=11$ for all concentrations. * indicates a mean value that was significantly different from zero. Error bars show the s.e.

is highly specific, responding only to a single isoform, whereas a second receptor is less specific (Poels et al., 2009). Whether this is a difference in the two species or in the receptors in the two parts of the nervous system is not clear from this study alone [but see our companion article (Dickinson et al., 2015)].

Predicted characteristics of the native lobster pyrokinins

The lobster *H. americanus* is unusual among crustaceans in having just one pyrokinin, which consists of only five amino acids, identified in its nervous system. All but one of the other crustaceans in which pyrokinins have been identified have more than one isoform (Christie, 2014a,b, 2013; Hui et al., 2012; Ma et al., 2009, 2010; Saideman et al., 2007; Torfs et al., 2000; Christie and Chi, 2015) and the vast majority of these are N-terminally extended peptides, with 2–16 additional amino acids on their N-termini. Surprisingly, the peptide identified as the native *H. americanus* isoform (FSPRLamide; Ma et al., 2008) had no effect on the cardiac neuromuscular system of this species, while one of the extended pyrokinins was able to activate the system at concentrations as low as 2×10^{-9} mol l^{-1}. Thus, we hypothesize that the sequence previously identified as the native lobster isoform is in fact a truncated peptide, which is either a product of proteolytic

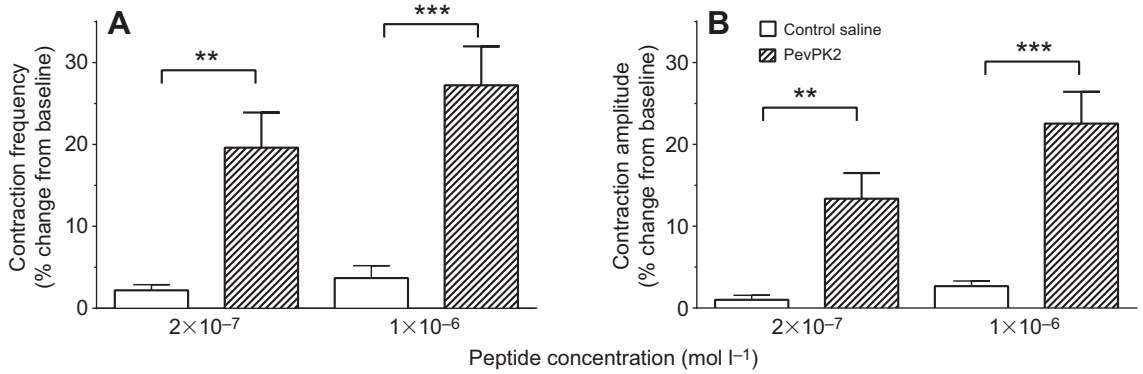

Fig. 11. PevPK2 has a stronger effect than [Ser^1]PevPK2 on frequency and amplitude of contractions when applied to lobster heart at supra-threshold concentrations. Two supra-threshold concentrations (2×10^{-7} mol l^{-1} and 10^{-6} mol l^{-1}) of PevPK2 caused a significant increase in both frequency (A) and amplitude (B) compared with [Ser^1]PevPK2. Brackets indicate values that differ significantly from one another: **$P<0.01$; ***$P<0.001$. $N=11$ for both [Ser^1]PevPK2 concentrations; $N=13$ for PevPK2 at 2×10^{-7} mol l^{-1}; $N=5$ for PevPK2 at 10^{-6} mol l^{-1}.

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processing of a longer isoform (or potentially isoforms) or is an artifact of the tissue/extract processing done for the study in which it was identified. We predict that the native *H. americanus* isoform (or isoforms) is an extended peptide, since removal of even one amino acid from PevPK2, the only isoform that activated the lobster cardiac system in this study, completely eliminated all activity. Moreover, replacing the variable amino acid in the conserved portion of the pyrokinin sequence, which is an asparagine in PevPK2 with a serine, as is present in the identified *H. americanus* fragment, likewise eliminated its ability to modulate the cardiac neuromuscular system, suggesting that at least one native sequence in lobster contains an asparagine rather than a serine as the variable amino acid. In fact, only one of the other pyrokinin peptides tested, the synthetic peptide SDFAFNPRLamide ([Ser^1]PevPK2), had any significant effect on the cardiac system, suggesting that the native *H. americanus* pyrokinin is very similar to PevPK2.

However, the fragment FSPRLamide is clearly present in the *H. americanus* nervous system, suggesting that an extended peptide with this sequence may also be present. The fact that peptides with this sequence were not physiologically active suggests the possibility that there are two pyrokinins (one with asparagine and one with serine as the variable amino acid) in the *H. americanus* nervous system, or that the extended sequence differs sufficiently from any of those we tested that the differences in the sequence are compensated for, enabling the native *H. americanus* peptide and PevPK2 to bind to the same receptor despite differences in sequence. In insects, the existence of multiple isoforms of pyrokinins generally reflects the presence of two different genes that encode pyrokinin peptides: the *capa* gene, which normally encodes a single pyrokinin (often with a well-conserved FGPRLamide C-terminus) as well as non-pyrokinin peptides (Loi and Tublitz, 2004; Predel and Wegener, 2006), and the *hugin* (*Drosophila*, Lepidoptera) or *pk/PBAN* (other insects) gene, which most often encodes more than one FXPRLamide peptide (Choi et al., 2011; Hellmich et al., 2014; Stepanyan et al., 2006; Towle and Smith, 2006). Although a relatively extensive collection of lobster expressed sequence tags (ESTs) exists (Stepanyan et al., 2006; Towle and Smith, 2006), it does not contain any pyrokinin sequences (Christie and Chi, 2015); it will thus be of interest to examine any genomic or transcriptomic information that becomes available for the gene(s) encoding *H. americanus* pyrokinin to determine its identity, and whether there are genes homologous to one or both of the insect pyrokinin genes.

MATERIALS AND METHODS

Animals and tissue collection

Animals

American lobsters, *Homarus americanus* Milne-Edwards 1837, were purchased from local (Brunswick, ME, USA) seafood retailers. All animals were housed in recirculating natural seawater aquaria at 10–12°C and were fed approximately weekly on a diet of chopped squid.

Dissections

For the isolation of the heart and dissection of the cardiac ganglion contained within it, animals were cold-anesthetized by packing in ice for approximately 20–30 min. The heart was then removed and the cardiac ganglion was dissected from the surrounding musculature in chilled (approximately 4°C) physiological saline (composition in mmol l^{-1}: 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20.00 $MgSO_4$, 3.91 Na_2SO_4, 11.45 Trizma base and 4.82 maleic acid, pH=7.45). The eyestalk ganglia, including the sinus gland, were similarly dissected from each eyestalk, while the paired pericardial organs were isolated from the musculature of the lateral pericardial chamber.

Whole-mount immunohistochemistry

Immunoprocessing

The cardiac ganglion and neuroendocrine tissues were immunolabeled as whole-mount preparations. In brief, dissected tissues were pinned out in Sylgard-lined Petri dishes and fixed for 12–24 h in a solution of 4% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 mol l^{-1} sodium phosphate buffer (PB), pH 7.4. Following fixation, tissues were rinsed five times at 1 h intervals in phosphate buffer containing 0.3% Triton X-100 (PBT), after which they were incubated for approximately 72 h in pyrokinin antibody (code R3-30; see Dickinson et al., 2015 for a complete description and vetting of the specificity of this antibody in *H. americanus*) diluted to a final concentration of 1:10,000–1:15,000 in PBT containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; cat. no. 017-000-121). After incubation in primary antibody, tissues were rinsed five times at 1 h intervals in PBT and then incubated for 12–24 h in DyLight 488-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; cat. no. 711-485-152) diluted to 1:300 in PBT containing 10% NDS. Following incubation in secondary antibody, tissues were rinsed five times at 1 h intervals in PB and then mounted between a glass microscope slide and coverslip in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA; cat. no. H-1000). Fixation and incubations in both primary and secondary antibody were conducted at 4°C, while tissue dissection and all rinses were conducted at room temperature (approximately 22°C). Incubation in secondary antibody and subsequent processing was conducted in the dark and slides were stored at 4°C in the dark until examined for labeling.

Epifluorescence and confocal microscopy

Data were collected and digital images were generated using an Olympus BX-51 upright compound microscope (Olympus America, Center Valley, PA, USA) outfitted with epifluorescence and an OPTRONICS MacroFire digital camera (OPTRONICS, Goleta, CA, USA) or an Olympus Fluoview 1000 confocal system that utilizes an Olympus IX-81 inverted microscope and blue diode, multi-argon, green HeNe and red HeNe lasers. For the production of figures, digital images were exported from the Olympus confocal system as tiff files and then arranged using Photoshop software (v7.0; Adobe Systems Inc., San Jose, CA, USA). It should be noted that the contrast and brightness of the final figures were adjusted as needed to optimize the clarity of the printed images.

Electrophysiology

Intact heart recordings

The methods used for the recording of whole heart physiology were identical to those described in Stevens et al. (2009). Briefly, lobsters were packed in ice for 20–30 min before the heart, still attached to the dorsal carapace to maintain appropriate levels of stretch, was removed. The carapace was pinned in a small, Sylgard 184 (KR Anderson, Santa Clara, CA, USA)-lined dish filled with cold (4°C) physiological saline. One of the two posterior arteries was cannulated and connected to a perfusion system (RP-1 Peristaltic Pump; Rainin Instrument Co., Emeryville, CA, USA), which was used to pump cooled physiological saline (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA) through the heart at a rate of 2.5 ml min^{-1}. The cannula was inserted until it passed through the heart valve to ensure that the saline entered the lumen of the heart. An additional cold saline stream was directed over the top of the heart, enabling us to maintain temperature between 9°C and 12°C.

Suture silk (6-0) was tied around the three anterior arteries and attached to a Grass FT03 force-displacement transducer (Astro-Med, West Warwick, RI, USA) at an angle of approximately 30 deg from horizontal. The tied arteries and the cannulated artery were both pulled away from the heart proper to create tension of 2 g of force, which is sufficient to ensure continued heart contractions (Stevens et al., 2009). The heart was allowed to stabilize for at least 50 min prior to the initiation of physiological experimentation. The output from the force transducer was amplified with an ETH-250 Bridge/Bio Amplifier (Filter: 50 Hz; 16 iWorx Amplifiers, Dover, NH, USA) and a Brownlee Precision Instrumentation amplifier (Low Pass Filter: 2 kHz), Model 440, and was recorded using a CED Micro 1401 digitizer and Spike2V7.03 (Cambridge Electronic Design, Cambridge, UK).

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Recordings of activity in semi-intact hearts

Semi-intact hearts were initially set up similar to those used for whole heart recordings. After the force transducer was attached, the ventral side of the heart was slowly cut open near the posterior end. If necessary, some fibers were cut away to reveal the cardiac ganglion, which lies on the dorsal side of the heart (Cooke, 2002). A suction electrode was inserted, and attached to one of the anterolateral motor nerves of the cardiac ganglion, enabling the simultaneous recording of motor output and heart contractions. The signal from the suction electrode was amplified with a model 1700 A-M Systems Differential AC Amplifier (Low Pass Filter: 300 Hz, High Pass Filter: 5 kHz; Sequim, WA, USA) and a Brownlee Precision amplifier. Data were recorded using the CED system, as described above.

Peptides

The peptides used here included both previously identified native arthropod pyrokinins and synthetic pyrokinin analogs (Table 1). Initial samples of LeucoPK, PevPK1, PevPK2, CabPK1 and CabPK2 were gifts from Dr Michael Nusbaum (University of Pennsylvania School of Medicine [Philadelphia, PA, USA]). Additional PevPK2, as well as FSPRLamide and the synthetic pyrokinin analogs PevPK2[2–9], PevPK2[3–9], PevPK2 [5–9], [Ser^{1}]PevPK2, [Leu^{1}]PevPK2, [Gly^{1}]PevPK2 and [Ser^{6}]PevPK2, were custom synthesized by GenScript Corporation (Piscataway, NJ, USA). All peptides were dissolved in deionized water to a concentration of 10^{-3} mol l^{-1}, aliquoted and stored at −20°C, and then diluted with physiological saline just before use. Peptide solutions were perfused through the cannulated artery for 10 min. After each peptide application, the heart was washed with saline for at least 50 min.

Data analysis

Data from recordings of heart contractions were analyzed using custom-written scripts and the built-in Spike2 functions. Further analysis was done using Excel templates. The recordings from each heart were converted into percentage change from baseline, using an average of contractions during the 200 s just before peptide perfusion, and the 200 s recorded after 5–7 min of peptide perfusion. Significance from a theoretical mean of zero was tested using one-sample two-tailed *t*-tests ($P<0.05$) in Prism v.5.0 (GraphPad Software, San Diego, CA, USA). To compare the means of two treatments, paired *t*-tests were used; to determine whether means of three or more treatments differed significantly, we used ANOVA followed by Tukey's *post hoc t*-tests. Data are presented as means±s.e.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

P.S.D. and A.E.C. conceived and designed this study. Experiments were executed and data were analyzed by P.S.D. A.S., M.A.K. and A.E.C. The manuscript was written by P.S.D., A.S. and A.E.C.

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