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Neuropeptide F peptides act through unique signaling pathways to affect cardiac activity

M. Setzu^a, M. Biolchini^{a,1}, A. Lilliu^{a,1}, M. Manca^{a,1}, P. Muronì^a, S. Poddighe^a, C. Bass^b, A.M. Angioy^a, and R. Nichols^{b,c,*}

^aDipartimento di Biologia Sperimentale, Sezione di Fisiologia Generale, Università di Cagliari-Monserrato, 09042 Monserrato-Cagliari, IT

^bBiological Chemistry Department, University of Michigan Medical School, Ann Arbor, MI 48109-0600, USA

^cVisiting Professor Program, Università di Cagliari-Monserrato, 09042 Monserrato-Cagliari, IT

Abstract

Elucidating how neuropeptides affect physiology may result in delineating peptidergic mechanisms and identifying antagonists for application in basic and translational science. Human neuropeptide Y (NPY) regulates cardiac activity; frequently invertebrates contain orthologs of vertebrate peptides. We report invertebrate NPY-like neuropeptide F (NPF) arrested the signal frequency of the slow phase of the cardiac cycle (EC₅₀ = 1 pM); however, signal frequency of the fast phase was affected only minimally. Neuropeptide F decreased the duration of the slow phase by ~70% (EC₅₀ = 0.6 pM), but increased the duration of the fast phase by ~57% (EC₅₀ = 10 nM). Short NPF-1 (sNPF-1) decreased the signal frequency of the slow phase by ~70% (EC₅₀ = 9 nM); yet, signal frequency of the fast phase was unaffected. Short NPF-1 decreased the duration of the slow phase ~55% (EC₅₀ ~ 50 nM), but increased the duration of the fast phase ~20% without dose dependency. Neuropeptide F and sNPF-1 increased isoelectric period duration. This novel report demonstrated NPY-like peptides are cardioactive but functionally unique. These data contribute to understanding how invertebrate orthologs affect cardiovascular activity. Dipteran fast and slow phases may be generated from separate pacemakers in the abdominal heart and in the anterior thoracocephalic aorta, respectively. Thus, our research suggests NPF and sNPF-1 act through different mechanisms to regulate cardiac activity. Invertebrate NPY-like peptides act in olfaction and feeding yet mechanisms which are associated with their cardioactive effects remain unknown; our work may provide evidence linking their roles in sensory response and cardiac activity.

Keywords

FMRamide; myosuppressin; neuropeptide Y (NPY); short neuropeptide F (sNPF); RFamide-related peptide (RFRP)

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*Corresponding author: tel.: +1 734 764 4467; fax +1 734 763 4581; nicholsr@umich.edu.

¹These authors are listed alphabetically by last name; they made equal contributions. A partial report of this research previously appeared in the thesis each student wrote and presented.

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1. Introduction

Heart failure is the leading cause of death. In order to address the devastating health problem of heart failure, it is imperative to understand how cardiac activity is regulated. The regulation of cardiac contractility is crucial to the proper maintenance of blood flow; improper blood flow can result in heart failure. Peptides are known to regulate cardiac contractility; yet, peptidergic mechanisms involved in cardiac contractility are not fully understood. Elucidating how cardioactive peptides affect activity may identify target molecules for diagnosis and drug design to address heart failure. Research conducted in experimentally versatile invertebrate models may help delineate the mechanisms underlying peptidergic regulation of cardiovascular function.

The vertebrate neuropeptide Y (NPY) is a highly conserved 36-amino acid peptide [32]. Along with peptide YY and pancreatic polypeptide (PP), NPY comprises a family of structurally-related peptides [7]. The three peptides are encoded in different precursors which undergo proteolytic processing to produce the final active products. Neuropeptide Y regulates numerous physiological events by signaling through multiple G protein-coupled receptor subtypes known as Y₁, Y₂, Y₄, Y₅, and Y₆ [6].

Structural and functional orthologs of vertebrate peptides are often present in invertebrates. Peptide purification monitored by PP antisera led to the isolation of a structurally-related peptide from the parasitic flatworm, *Moniezia expansa* [20]. Subsequently, numerous invertebrate NPY-like peptides were identified or predicted from nucleotide sequence data and found to contain –RXRF/Yamide, where F/Y indicates the presence of F or Y [5, 12, 27, 28, 35, 37]. These invertebrate NPY-like peptides were designated NPF due to the C-terminal F present in the majority of molecules identified.

Invertebrate NPF-like materials were discovered based on immunoreactivity and predicted from nucleotide sequences [13, 15, 31, 35, 36]. These peptides differ from NPF by their C terminus, LRLRFamide, and they are encoded in polypeptide precursors. In addition, they contain fewer amino acids; thus, they were designated short neuropeptide F (sNPF) peptides. Short NPF-1 is structurally distinct from NPF except for a C-terminal RFamide.

Vertebrate NPY is abundantly expressed in the cardiovascular system where it modulates cardiac contractility. However, no report describes the influence of the NPY-like invertebrate NPF on cardiac activity. The potato beetle sNPF peptides are cardioactive in isolated heart preparations [15, 18, 19, 30], yet this activity is not extensively analyzed nor are the effects of the peptides described in whole animal or at physiological concentrations. The molecular complexity of the simple blowfly heart makes it an attractive preparation in which to delineate fundamental mechanisms related to peptidergic regulation of cardiac activity [4]. The cardiac pump of the blowfly is a contractile tubular vessel composed of a single layer of myocytes that lies along the dorsal body midline [3, 38]. Fly cardiac activity is composed of a period of low-frequency signal, the slow phase, alternating with a period of high-frequency signal, the fast phase, followed by a period of inactivity, the isoelectric period. The cardiac phases, which are probably generated from two different pacemakers, lead to a pulsatile propagation of electrical activity along the tissue to propel blood, called hemolymph, through the cardiovascular tube.

Here we report on the effects of two invertebrate peptides, NPF and sNPF-1, on blowfly cardiac activity *in vivo*. We investigated to what extent these two peptides impacted signal frequency of the slow phase and the fast phase of cardiac activity, duration of each cardiac phase, and duration of the isoelectric period over a range of concentrations. Our research is novel and provides insight into how NPF and NPF-like peptides affect cardiac activity. We propose NPF and sNPF-1 may play a physiological role in regulating invertebrate cardiac

activity. Our results suggest NPF and sNPF-1 act through different mechanisms directly or indirectly at the anterior pacemaker (slow phase) and the posterior pacemaker (fast phase) to uniquely regulate specific parameters of the cardiac cycle and influence contractility. Our data also strengthen the argument invertebrate NPF is a vertebrate NPY ortholog.

2. Materials and methods

2.1 Animals

The experiments were performed on five to six day adult male blowflies, *Protophormia terraenovae* (Diptera, Calliphoridae). The *P. terraenovae* were reared under standard conditions, temperature (22–24°C), humidity (60–70%), and photoperiod (16:8 light: dark cycle). The animals were deprived of food and supplied with water *ad libitum* for 24 hours before experiments.

2.2 Chemicals

The peptides corresponding to NPF (20–36) (FLQDLDTYYGDRARVRFamide) and sNPF-1 (AQRSPSLRLRFamide) were synthesized by standard Fmoc protocol and purified by reversed-phase high-pressure liquid chromatography. The structures of the peptides were confirmed by amino acid analysis and mass spectrometry. Working solutions of the peptides were made fresh by serial dilutions of aliquots of a concentrated, frozen stock solution in a blowfly physiological saline to obtain the concentrations used in the experiments [8].

2.3 Bioassays

In preparation to record cardiac activity the body of a blowfly was fixed dorsal side down on a strip of low-melting-point dental wax and its legs were singly restrained in the wax. These manipulations and recordings were previously described [4]. Cardiac activity was monitored in the intact fly by a surface electrocardiographic (ECG) recording while maintaining climatic conditions as for rearing the colony. Prior to the recording, a small aperture (about 1 mm in diameter) was made in the ventro-lateral surface of the third abdominal sclerite through which the solution being tested was subsequently applied.

Monopolar ECGs were measured using silver/silver-chloride electrodes (150 μ m diameter) which were positioned on the blowfly cuticle. The electrode positions allowed the animals to breathe during the experiment; cardiac activity requires oxygen. Signal data were processed to record, display, and analyze ECG signals.

The ECG was monitored in each animal starting five to ten minutes after positioning the electrodes. If spontaneous contractions of the body musculature affected cardiac activity, additional resting time was allowed until regular slow phase and fast phase were observed [33]. Thereafter, the ECG was recorded continuously starting four cycles before administration of a solution and for the following 10 minutes. Application of 0.5 microliters of solution was made into the abdominal hemocoel. Only one solution was applied per animal; 10 animals ($n = 10$) were tested per solution concentration. The effects of physiological saline solution (control) were tested on a group of animals ($n = 10$).

2.4 Data analysis

The signal frequencies of the slow phase and the fast phase, as well the duration of the two phases and the isoelectric period in four regular cardiac cycles were measured in each blowfly before application of a solution. The mean values collected from each animal before application of the solution were used when determining the percent variation for each phase and for the isoelectric period before and immediately following application ($t = 0$) and at one minute intervals during the following 10 minutes. In this manner each animal served as its

own baseline or control for cardiac activity prior to solution application. Mean values of percent $\pm$ standard errors (S.E.) were determined and their statistical significance was analyzed using the Wilcoxon Test with the p value < 0.05 as a threshold level of significance.

3. Results

To begin to investigate the influence of NPF on cardiac activity 0.5 microliters 10 μ M NPF (20–36) were introduced into the hemolymph of an individual blowfly ($n = 10$) and the cardiac activity monitored by ECG (Fig. 1). The electrical signals recorded before the application of 10 μ M NPF (20–36) exhibited in succession a slow phase followed by a fast phase then a brief isoelectric period (~ 1 sec). A representative recording is shown in Fig. 1. The multiple lines of data are one continuous recording (note arrowheads to the left of the figure). Prior to the application of the peptide (Fig. 1, vertical arrow, first line) the slow phase and the fast phase alternated regularly in a cardiac cycle. The frequency of the slow phase was three-four signals/second for a duration of 17 seconds. The fast phase was composed of signals at a frequency of five-six signals/second with a duration of 11.5 seconds. The duration of the isoelectric period which followed was 1–2 seconds. Cardiac activity prior to peptide application is referred to as the baseline value because it represents basal activity.

Upon application of 0.5 microliters 10 μ M NPF (20–36) a profound and specific effect was observed. Approximately 45 seconds after introduction of the peptide the fast phase had a frequency and duration comparable to baseline values prior to application, however, the slow phase was no longer observed (Fig. 1, second and third lines). In the absence of the slow phase, the duration of the isoelectric period increased to about 21 seconds. This pattern of activity continued (Fig. 1, third and fourth lines) for more than two minutes after which a few signals containing reduced amplitude and frequency were observed during the slow phase of the ECG recording (Fig. 1, fourth line). This activity was present until the end of the recording (Fig. 1, fifth line). After about nine minutes an increase in signal frequency was observed during the slow phase, however, it had not yet recovered to pre-application values of frequency or amplitude (Fig. 1, sixth line). The duration of the isoelectric period reduced with the return of signal during the slow phase. The pre-application levels of cardiac activity did not return by the end of the 10-minute recording period (Fig. 1, end of sixth line).

To further study the effect of NPF (20–36) on cardiac activity, the consequences of applying the peptide over a range of concentrations were determined. The results are reported as the percentage of animals displaying arrest of the slow phase; the increased duration of the isoelectric period is also reported over the 10-minute recording period after application (Fig. 2). As seen in a representative recording (Fig. 1), arrest of the slow phase was observed after the application of 0.5 microliters 10 μ M NPF (20–36). The arrest of the slow phase was observed at one minute in 80% of the animals ($n = 10$) in response to 10 μ M NPF (20–36) (Fig. 2, filled columns). The arrest of the slow phase was observed at two and three minutes in more than 37% of the animals. At four, five, six, and seven minutes, arrest of the slow phase was observed in 20% to 22% of the animals. The percentage of animals with arrest of the slow phase was reduced to 11% at eight minutes. At nine and ten minutes no animals demonstrated arrest of the slow phase; however, an increased duration of the isoelectric period was observed in a large percentage of animals, 90%, even at 10 minutes after application of the peptide. At one minute 100% of the animals showed an increased duration of the isoelectric period (Fig. 2, open columns). From two to ten minutes 80% to 90% of the animals demonstrated an increased duration of the isoelectric period.

After the application of 0.5 microliters 1 nM, 1 pM, 100 fM, or 1 fM NPF (20–36), no animal demonstrated arrest of the slow phase in the 10-minute recording period (Fig. 2). About 50% or more of the animals showed an increased duration of the isoelectric period after application of 1 nM, 1 pM, or 100 fM NPF (20–36) (Fig. 2, open columns). However, in response to 1 fM NPF (20–36) only 8% to 25% of the animals showed an increased duration of the isoelectric period (Fig. 2, open columns). No animals in which only the carrier, physiological saline, was applied (Fig. 2, control) showed arrest of the slow phase or increased duration of the isoelectric period.

The effect of NPF (20–36) on the mean value of the duration of the isoelectric period was determined over a range of concentrations (Fig. 3). The application of 0.5 microliters 10 μ M NPF (20–36) significantly increased the duration of the isoelectric period at each time point compared to baseline (Fig. 3). Significant increases in the duration of the isoelectric period also occurred in response to 1 nM NPF (20–36) and 1 pM NPF (20–36), although the increases were reduced compared to 10 μ M NPF (20–36) (Fig. 3). The duration of the isoelectric period increased in response to 100 fM NPF (20–36); however, it was only significant compared to baseline at eight, nine, and ten minutes. At 1 fM NPF (20–36) there was no significant effect on the duration of the isoelectric period. Also, the application of saline only (control) did not significantly affect isoelectric period duration (Fig. 3).

The effect of NPF (20–36) on the signal frequency of the slow phase and the fast phase was determined over a range of concentrations (Fig. 4). Application of 0.5 microliters 10 μ M NPF (20–36) significantly and dramatically decreased the signal frequency of the slow phase by 99% compared to baseline until it leveled off to a 68% decrease at the end of the 10-minute recording period. In contrast, the peptide increased the signal frequency of the fast phase moderately by 12–16% at each time point (Fig. 4). The effect of 0.5 microliters 1 nM NPF (20–36) on the signal frequency of the slow phase initially was to decrease it by 61%; the frequency then steadily increased until it leveled off to a 20% decrease compared to baseline by the end of the 10-minute recording period. The signal frequency of the fast phase decreased significantly albeit only about 3–10% and not at all time points in response to 1 nM NPF (20–36) (Fig. 4). Application of 0.5 microliters 1 pM NPF (20–36) caused the signal frequency of the slow phase to decrease by 26–42% which was sustained but fluctuated over the 10-minute recording period. Little or no change in signal frequency of the fast phase was observed in response to 1 pM NPF (20–36). At 100 fM NPF (20–36) or 1 fM NPF (20–36) there were no significant effects on signal frequency of the slow phase nor fast phase compared to baseline. Also, the application of saline (control) did not significantly affect signal frequency (Fig. 4).

The effect of NPF (20–36) on the duration of the slow phase and the fast phase was determined over a range of concentrations (Fig. 5). The application of 0.5 microliters 10 μ M NPF (20–36) significantly decreased the duration of the slow phase by 95% at one minute then by 60% at two minutes. The decrease in duration of the slow phase continued to grow smaller until four minutes when it returned to baseline and then decreased steadily again until it was down 48% compared to baseline at nine minutes, returning finally to near baseline at 10 minutes (Fig. 5). The effect on the duration of the fast phase was to significantly increase it by 34–57% from two to six minutes, after which it leveled off to a 21–25% increase for the remainder of the 10-minute recording period. At 1 nM NPF (20–36) an initial decrease in the duration of the slow phase of 43% compared to baseline occurred at one minute; the duration of the slow phase then steadily returned to near baseline by the end of the 10-minute recording period. Additionally, at 1 nM NPF (20–36) the duration of the fast phase increased significantly by 20% compared to baseline at one, four, and five minutes, 13% at eight minutes, and 20% at nine minutes (Fig. 5). At 1 pM NPF (20–36) the duration of the slow phase decreased significantly by about 50% for the first

three minutes and then showed a steady return to baseline. Additionally, 1 pM NPF (20–36) significantly increased the duration of the fast phase by 31% at one minute, 28% at six minutes, and 23% at ten minutes with similar but not significant effects at other times. At 100 fM NPF (20–36) and at 1 fM NPF (20–36) there were fewer significant decreases in the duration of the slow phase or significant increases in the duration of the fast phase compared to other concentrations tested. Also, the application of saline only (control) did not significantly affect duration of the slow phase or the duration of the fast phase (Fig. 5).

The effective concentrations for the half-maximal (EC₅₀) induced effects of NPF (20–36) on the signal frequency and the duration of the slow phase were 1 pM or 1×10^{-12} M and 0.6 pM or 0.6×10^{-12} M (Fig. 6), respectively. The EC₅₀ for the NPF (20–36)-induced effects on the duration of the fast phase was 10 nM or 1×10^{-8} M (Fig. 6); the effects of the peptide on the signal frequency of the fast phase were minimal and not dose dependent.

Next, the influence of sNPF-1 on cardiac activity was investigated. The arrest of the slow phase was observed in about 22% of all animals ($n = 10$) at one, two, and three minutes in response to 10 μ M sNPF-1 (Fig. 7, filled columns). In response to application of 0.5 microliters 1 μ M sNPF-1 arrest of the slow phase was observed in 11% – 22% of all animals at one, two, three, and four minutes. In response to application of 0.5 microliters 100 nM sNPF-1 arrest of the slow phase was observed in 8% of the animals at one minute only. The arrest of the slow phase was not observed in any animal in response to application of 0.5 microliters 1 nM sNPF-1. The duration of the isoelectric period was also determined in response to sNPF-1 over a range of concentrations. The duration of the isoelectric period increased in response to the application of 0.5 microliters 10 μ M sNPF-1 in 68%–100% of all animals throughout the 10-minute recording period (Fig. 7, open columns). The duration of the isoelectric period also increased in response to 1 μ M sNPF-1, 100 nM sNPF-1, and 1 nM sNPF-1 in 58%–90%, 40%–60%, and 10%–50% of the animals, respectively.

The effect of sNPF-1 on the mean value of the duration of the isoelectric period was determined over a range of concentrations. Application of 0.5 microliters 10 μ M sNPF-1 significantly increased the duration of the isoelectric period at each minute during the 10-minute recording period (Fig. 8). The mean value of the duration of the isoelectric period also increased in response to 1 μ M sNPF-1 and 100 nM sNPF-1 over the recording period; however, it was significantly different only at one, two, and ten minutes and five, eight, and nine minutes, respectively. No significant increase in duration of the isoelectric period was observed in response to 1 nM sNPF-1.

The effects of sNPF-1 on the signal frequency of the slow phase and the fast phase were determined over a range of concentrations. Application of 0.5 microliters 10 μ M sNPF-1 significantly decreased the signal frequency of the slow phase throughout the 10-minute recording period (Fig. 9) with an initial decrease of 76% that steadily grew smaller and leveled off to a 31% decrease. At 10 μ M sNPF-1 the signal frequency of the fast phase decreased by 8% initially, then rose to an increase of 7% by 10 minutes; the effects were only significant at one and two minutes. Significant decreases in the signal frequency of the slow phase also occurred in response to 1 μ M sNPF-1, which showed an initial decrease of 56% that then rose steadily and leveled off to a 6% decrease by 10 minutes. A decrease in the mean value of the signal frequency of the fast phase of 7–14% occurred in response to 1 μ M sNPF-1; the effect was significant at only six, seven, and eight minutes. At 100 nM sNPF-1 there was an initial significant decrease in the signal frequency of the slow phase of 36%, the frequency then leveled off and fluctuated around a 12% decrease that was significant only at seven and eight minutes. The signal frequency of the fast phase significantly decreased in response to 100 nM sNPF-1 by 5–9% at three, four, five, six, and seven minutes after which the frequency returned to baseline at eight minutes. The return to

baseline was significant at nine minutes only. At 1 nM sNPF-1 the signal frequency of the slow phase was only slightly decreased, which was not significant. At 1 nM sNPF-1 the signal frequency of the fast phase decreased by about 4–14% which was significant from seven to ten minutes, only.

The effect of sNPF-1 on the duration of the slow phase and the fast phase was also determined over a range of concentrations. In response to the application of 0.5 microliters 10 μ M sNPF-1 there was a decrease in the duration of the slow phase of 46%, after which it rose and leveled off to baseline at seven minutes; the decrease was only significant at one, two, and three minutes (Fig. 10). After seven minutes, the duration of the slow phase increased by 13–19%, but the response was not significant. In response to the application of 0.5 microliters 10 μ M sNPF-1, the duration of the fast phase increased by 15–16% from two to four minutes and then returned to baseline, the only significant change was at five minutes. Significant decreases in the duration of the slow phase also occurred in response to 1 μ M sNPF-1, showing an initial decrease of 64% that steadily rose and leveled off to an increase in duration of about 25% by nine minutes. Changes were significant at one, two, three, four, and five minutes only. At 1 μ M sNPF-1 an increase occurred in the duration of the fast phase which reached 25% and was significant at five minutes, then returned to baseline at nine and ten minutes. At 100 nM sNPF-1, the duration of the slow phase was significantly decreased by 43% at one minute, returning to near baseline at three minutes. Changes were only significant at one and two minutes; the duration of the slow phase fluctuated around the baseline from three minutes to the end of the recording. At 100 nM sNPF-1 there was a significant effect on the duration of the fast phase only at seven minutes, which showed a 14% increase. At 1 nM sNPF-1 there was an initial significant decrease in the duration of the slow phase of 25% at one minute that leveled off to about an 8% increase and was significant at nine and ten minutes. There was no significant effect on the duration of the fast phase in response to 1 nM sNPF-1.

The EC₅₀ for the sNPF-1-induced effects on the signal frequency and the duration of the slow phase were 90 nM or 9×10^{-8} M and 50 nM or 5×10^{-8} M (Fig. 11), respectively. The effects on the signal frequency and the phase duration of the fast phase were minimal and not dose dependent (Fig. 11).

4. Discussion

Our results are the first to describe the effects of invertebrate NPY-like peptides on the cardiac cycle in the whole animal. We measured the effects of the peptides on multiple cardiovascular parameters over a range of concentrations. We discovered that the application of NPF (20–36) arrested or decreased the signal frequency of the slow phase (EC₅₀ = 1 pM), but increased the signal frequency of the fast phase only slightly. Neuropeptide F decreased the duration of the slow phase by ~70% (EC₅₀ = 0.6 pM), but increased the duration of the fast phase by ~57% (EC₅₀ = 10 nM). We also discovered NPF (20–36) increased the duration of the isoelectric period, a time of electrical activity absence. The duration of the isoelectric period increased when the duration of the slow phase decreased, suggesting it was physiologically important to compensate for the total length of time of the cardiac cycle, and NPF (20–36) participated in this crucial response.

We also tested another invertebrate peptide known as a NPY-like peptide, sNPF-1. Short NPF-1 decreased the signal frequency and duration of the slow phase ~70% (EC₅₀ = 9 nM) and ~55% (EC₅₀ ~50 nM), respectively. It increased the duration of fast phase ~20% with no dose dependency and had no effect on the signal frequency of the fast phase. Short NPF-1 increased the duration of the isoelectric period in the absence of the slow phase, again suggesting it was physiologically important to compensate for the total length of time

of the cardiac cycle, and sNPF-1 participated in this crucial response. In comparison, saline did not alter the signal frequency nor duration of the slow phase or the fast phase, nor duration of the isoelectric period.

Based on these data, we believe that both NPF (20–36) and sNPF-1 act to regulate the slow phase of cardiac activity. Effects of both peptides on the signal frequency and duration of the slow phase are pronounced, long-lasting, and show clear dose-dependency. The slight effect of the peptides on the fast phase is likely a consequence of the significantly altered slow phase and the stress this puts on the heart rather than a direct peptide effect. Previous studies have shown that stress to the heart can indirectly cause alterations in the slow and fast phases of cardiac activity [3,38,39]. This conclusion is supported by the short-lived nature of the peptide effects on the fast phase, lack of dose dependency, and higher EC₅₀ values observed compared to the slow phase. In addition, for true peptide effects on a cardiac phase, variations in phase duration are most often directly related to a variation in signal frequency, as was observed for the peptide effects on the slow phase [25, 34]. However, for the fast phase, a significant variation was observed only for the phase duration and just a slight variation was seen in the signal frequency. Because there is no correlation of effects on the fast phase, the effects observed on the fast phase are consistent with an indirect result of peptidergic regulation of the slow phase.

Research on molecular mechanisms underlying cardiac contractility is driven by the devastating economic and emotional costs of heart failure, the leading cause of death. Delineating peptidergic regulation of cardiac contractility may identify progenitor molecules for the development of diagnostic tests and therapeutic strategies to help address cardiovascular dysfunction. Neuropeptides affect cardiovascular function; however, much remains to be learned about the mechanisms through which they regulate cardiac activity. We chose to explore the role of invertebrate peptides referred to as neuropeptide Y-like in cardiac activity because the vertebrate NPY peptide family regulates mammalian cardiovascular function. The invertebrate peptides had not been analyzed for their effects on the cardiac cycle prior to our report. We chose to study invertebrate peptides because conservation of mammalian cardioactive peptides in lower animals provides the opportunity to elucidate peptidergic mechanisms involved in cardiovascular function in simple systems with complex molecular physiology and translate the findings across the animal kingdom. Neuropeptide Y is an abundant mammalian neuropeptide which acts in olfaction [26], feeding behavior [17], and cardiovascular function [29]. Invertebrate NPY-like peptides act in olfaction and feeding behavior [reviewed in 22], whether they influenced cardiac activity was unreported. Olfactory and gustatory chemosensory stimulation are known to influence invertebrate blood flow [1, 2, 34].

The peptides we investigated were NPF (20–36) and sNPF-1. The structure of blowfly NPF is not yet identified. Although antisera to structurally-related vertebrate peptides identified cross-reactive material, blowfly NPF was not isolated [see 22 for review]. However, NPF is a peptide of 28 to 36 amino acids in length in other invertebrates. We predicted NPF (20–36) might contain the active core and possibly exist as a naturally-occurring peptide because it is highly conserved in diptera (Table 1) and bounded by proteolytic cleavage sites in its precursor molecule. We demonstrated NPF (20–36) is active at physiological concentrations, which is consistent with our assumptions regarding the potential for this molecular species to be biologically active.

The structure of blowfly sNPF-1 is not known. Although antisera to structurally-related vertebrate peptides identified cross-reactive material, blowfly sNPF-1 was not isolated [see 22 for review]. However, sNPF-1 is highly conserved in diptera represented by the consensus structure X¹X²RSPSLRLRFamide (Table 2) where 88% are represented by X¹ =

A/S and 81% by $X^2 = Q/V$. Our decision to test sNPF-1 was further weighed by the likelihood the strictly conserved C-terminal amidated nonapeptide contained the active core. Previously, 1 μ M potato beetle sNPF-1 was applied to cardiac tissue and visually observed to decrease heartbeat frequency and amplitude of contractions [30]. Our research advanced this initial study. We extensively tested the effects of sNPF-1 over a range of physiological concentrations in a whole animal preparation monitoring activity by continuously recording ECGs to collect data on multiple, individual parameters of the cardiac cycle. We demonstrated sNPF-1 is active at physiological concentrations, which is consistent with our assumptions regarding the potential for its biological activity.

The name for invertebrate NPF originates from the similarity of its C-terminal structure to vertebrate NPY, RxRF/Yamide versus RxRYamide, respectively. The similarity extends beyond the C-terminal structures; the peptides are each encoded in a precursor which undergoes proteolytic processing to produce a single, highly conserved, gene product. Additionally, the lengths of NPF and NPY are similar and both peptides play a role in olfaction and feeding. We propose the results of our current study further support the conclusion invertebrate NPF and vertebrate NPY peptides are orthologous.

Another invertebrate peptide, short NPF-1, was named due to its immunoreactivity to NPF antisera, however, the C terminus of sNPF-1 is structurally different from NPF and NPY, RLRFamide versus RxRF/Yamide and RxRYamide, respectively. Short NPF-1 is encoded in a polypeptide precursor which undergoes proteolytic processing to produce multiple, highly conserved, structurally-related gene products. We propose sNPF-1 may be more like vertebrate RFamide-related peptide-1 (RFRP-1) which contains a LRFamide C-terminal structure. The sNPF-1 C terminus is also identical with the invertebrate myosuppressin peptide, DMS [23]. Short NPF-1 is biologically similar to myosuppressin and RFRP-1 which both dramatically decrease cardiac function [4, 24]. Inference of an evolutionary, functional, and/or structural relationship between peptides based on their immunoreactivity to polyclonal antisera alone is problematic. Thus, naming based on the initial means of identification is often handy for reference, however in this case it is most likely a misnomer.

The vertebrate NPY family is comprised of multiple, structurally-related peptides encoded by separate genes. The conservation of vertebrate bioactive peptides across the animal kingdom and presence of NPF suggests the invertebrate NPY-like peptide family is comprised of multiple, structurally-related peptides encoded in separate precursors. Although sNPF-1 may not be NPY-like, other invertebrate candidates exist including dRYa-1 and dRYa-2 which contain RYamide, bind a receptor activated by NPY, and affect feeding in blowfly [16].

Based on our data we conclude NPF and sNPF-1 may act differentially, directly or indirectly, at cardiac pacemakers to regulate the slow phase of cardiac activity. Our results are consistent with NPF and sNPF-1 working through different mechanisms to affect cardiac activity which is in agreement with published binding data demonstrating the peptide ligands bind to different receptors [9, 10–14, 21]. We predict the cardiac distribution of the NPF receptor and sNPF-1 receptor is different, and/or their cardiac signaling pathways are distinct. However, no data are available for the presence of NPF receptor or sNPF-1 receptor in cardiac tissue. Although the structurally-related DMS peptide also acts at the pacemakers there are no data to indicate NPF or sNPF-1 binds to myosuppressin receptors [4]. Our studies report bioactivities of NPF, sNPF-1, and DMS which reflect the complexity of peptidergic regulation of the cardiac cycle to control the frequency and direction of blood flow. Based on their high degree of conserved structure and influence on cardiovascular, olfactory, and gustatory processes, NPF, sNPF-1, and DMS may play crucial roles in

regulating blood flow to deliver blood-borne messengers and/or nutrients to maintain homeostasis and/or respond to sensory cues and physiological conditions.

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References

1. Angioy AM. Reflex cardiac response to a feeding stimulus in the blowfly, *Calliphora vomitoria* L. *J Insect Physiol.* 1988; 34:21–7.
2. Angioy AM, Tomassini Barbarossa I, Crnjar R, Liscia A, Pietra P. Reflex cardiac response to various olfactory stimuli in the blowfly, *Protophormia terraenovae*. *Neurosci Lett.* 1987; 81:263–6. [PubMed: 3431742]
3. Angioy AM, Boassa D, Dulcis D. Functional morphology of the dorsal vessel in the adult fly *Protophormia terraenovae* (diptera, calliphoridae). *J Morphol.* 1999; 240:15–31.
4. Angioy AM, Muroi P, Barbarossa IT, McCormick J, Nichols R. Evidence dromyosuppressin acts at posterior and anterior pacemakers to decrease the fast and the slow cardiac activity in the blowfly *Protophormia terraenovae*. *Peptides.* 2007; 28:585–93. [PubMed: 17141921]
5. Brown MR, Crim JW, Arata RC, Cai HN, Chun C, Shen P. Identification of a *Drosophila* brain-gut peptide related to the neuropeptide Y family. *Peptides.* 1999; 20:1035–42. [PubMed: 10499420]
6. Cabrele C, Beck-Sickinger AG. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J Pept Sci.* 2000; 6:97–122. [PubMed: 10759209]
7. Cerdá-Reverter JM, Larhammar D. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. *Biochem Cell Biol.* 2000; 78:371–92. [PubMed: 10949087]
8. Chen AC, Friedman S. An isotonic saline for the adult blowfly, *Phormia regina*, and its application to perfusion experiments. *J Insect Physiol.* 1975; 21:529–36. [PubMed: 1117154]
9. Chen ME, Pietrantoni PV. The short neuropeptide F-like receptor from the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Arch Insect Biochem Physiol.* 2006; 61:195–208. [PubMed: 16552771]
10. Feng G, Reale V, Chatwin H, Kennedy K, Venard R, Ericsson C, Yu K, Evans PD, Hall LM. Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster*. *Eur J Neurosci.* 2003; 18:227–38. [PubMed: 12887405]
11. Garczynski SF, Brown MR, Shen P, Murray TF, Crim JW. Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster*. *Peptides.* 2002; 23:773–80. [PubMed: 11897397]
12. Garczynski SF, Crim JW, Brown MR. Characterization of neuropeptide F and its receptor from the African malaria mosquito, *Anopheles gambiae*. *Peptides.* 2005; 26:99–107. [PubMed: 15626509]
13. Garczynski SF, Brown MR, Crim JW. Structural studies of *Drosophila* short neuropeptide F: Occurrence and receptor binding activity. *Peptides.* 2006; 27:575–82. [PubMed: 16330127]
14. Garczynski SF, Crim JW, Brown MR. Characterization and expression of the short neuropeptide F receptor in the African malaria mosquito, *Anopheles gambiae*. *Peptides.* 2007; 28:109–18. [PubMed: 17140700]
15. Gaus G, Doble KE, Price DA, Greenberg MJ, Lee TD, Battelle BA. The sequences of five neuropeptides isolated from *Limulus* using antisera to FMRFamide. *Biol Bull.* 1993; 184:322–9.
16. Ida T, Takahashi T, Tominaga H, et al. Identification of the novel bioactive peptides dRYamide-1 and dRYamide-2, ligands for a neuropeptide Y-like receptor in *Drosophila*. *Biochem Biophys Res Commun.* 2011; 410:872–7. [PubMed: 21704020]
17. Levine AS, Morley JE. Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides.* 1984; 5:1025–9. [PubMed: 6549409]

18. Marciniak P, Grodecki S, Konopińska D, Rosiński G. Structure-activity relationships for the cardiotropic action of the Led-NPF-I peptide in the beetles *Tenebrio molitor* and *Zophobas atratus*. *J Pept Sci*. 2008; 14:329–34. [PubMed: 17929328]
19. Marciniak P, Slocinska M, Bednarski P, Grodecki S, Konopińska D, Rosiński G. Effects of the arginine on the cardioinhibitory activity of the Led-NPF-I neuropeptide. *Pestycydy/Pesticides*. 2009; 1–4:71–8.
20. Maule AG, Shaw C, Halton DW, Thim L, Johnston CF, Fairweather I, Buchanan KD. Neuropeptide F; a novel parasitic flatworm regulatory peptide from *Moniezia expansa* (Cestoda: Cyclophyllidae). *Parasitology*. 1991; 102:309–16.
21. Mertens I, Meeusen T, Huybrechts R, De Loof A, Schoofs L. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. *Biochem Biophys Res Commun*. 2002; 297:1140–8. [PubMed: 12372405]
22. Nässel DR, Wegener C. A comparative review of short and long neuropeptide F signaling in invertebrates: Any similarities to vertebrate neuropeptide Y signaling? *Peptides*. 2011; 32:1335–55. [PubMed: 21440021]
23. Nichols R. Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J Mol Neurosci*. 1992; 3:213–8. [PubMed: 1390001]
24. Nichols R, Demers LA, Larsen BM, Robinson D, Converso K, Russell MW, Westfall MV. Human RFamide-related peptide-1 diminishes cellular and integrated cardiac contractile performance. *Peptides*. 2010; 31:2067–74. [PubMed: 20797420]
25. Normann TC. Heart activity and its control in the adult blowfly, *Calliphora erythrocephala*. *J Insect Physiol*. 1972; 18:1793–1810.
26. Ohm TG, Braak E, Probst A, Weindl A. Neuropeptide Y-like immunoreactive neurons in the human olfactory bulb. *Brain Res*. 1988; 451:295–300. [PubMed: 3251589]
27. Reale V, Chatwin HM, Evans PD. The activation of G-protein gated inwardly rectifying K⁺ channels by a cloned *Drosophila melanogaster* neuropeptide F-like receptor. *Eur J Neurosci*. 2004; 19:570–6. [PubMed: 14984407]
28. Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science*. 2002; 298:172–5. [PubMed: 12364794]
29. Rioux F, Bachelard H, Martel JC, St-Pierre S. The vasoconstrictor effect of neuropeptide Y and related peptides in the guinea pig isolated heart. *Peptides*. 1986; 7:27–31. [PubMed: 3754963]
30. Skonieczna M, Grodecki S, Konopinska D, Rosinski G. New biological activity of NPF-related peptides in insects. *Pestycydy*. 2005; 4:91–8.
31. Spittaels K, Verhaert P, Shaw C, Johnston RN, Devreese B, Van Beeumen J, De Loof A. Insect neuropeptide F (NPF)-related peptides: isolation from Colorado potato beetle (*Leptinotarsa decemlineata*) brain. *Insect Biochem Mol Biol*. 1996; 26:375–82. [PubMed: 8814784]
32. Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*. 1982; 296:659–60. [PubMed: 6896083]
33. Thon B. Habituation of cardiac and motor responses to a moving visual stimulus in the blowfly *Calliphora vomitoria*. *J Comp Physiol Psychol*. 1980; 94:886–93. [PubMed: 7430471]
34. Tomassini Barbarossa I, Muroli P, Liscia A, Crnjar R, Angioy AM. Modulation of the reflex cardiac response in *Protophormia terraenovae* to gustatory stimulation. *Boll Zool*. 1992; 59:41–4.
35. Vanden Broeck J. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. *Peptides*. 2001; 22:241–54. [PubMed: 11179818]
36. Veenstra JA, Lambrou G. Isolation of a novel RFamide peptide from the midgut of the american cockroach, *Periplaneta americana*. *Biochem Biophys Res Commun*. 1995; 213:519–24. [PubMed: 7646507]
37. Walker RJ, Papaioannou S, Holden-Dye L. A review of FMRFamide- and RFamide-like peptides in metazoa. *Invert Neurosci*. 2009; 9:111–53. [PubMed: 20191373]
38. Wasserthal LT. Functional morphology of the heart and of a new cephalic pulsatile organ in the blowfly *Calliphora vicina* (Diptera:Calliphoridae) and their roles in hemolymph transport and tracheal ventilation. *Int J Insect Morphol Embryol*. 1999; 28:111–29.

39. Wasserthal LT. *Drosophila* flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and ‘venous’ channels. J Exp Biol. 2007; 210:3707–19. [PubMed: 17951411]

Highlights

- NPF arrested slow phase signal frequency; fast phase frequency was affected lesser.
- NPF decreased the slow phase duration, but increased the fast phase duration.
- sNPF-1 decreased signal frequency of the slow phase; the fast phase was unaffected.
- sNPF-1 decreased the slow phase duration, but increased the fast phase duration.
- NPF and sNPF-1 increased isoelectric period duration.

Fig. 1.

Neuropeptide F (20–36)-induced effects on cardiac activity. Prior to the application of peptide solution (arrow, first line), baseline cardiac activity is recorded which exhibits a slow phase and a fast phase followed by an isoelectric period. In response to the application of 0.5 microliters 10 μ M NPF (20–36), the slow phase was arrested, but the fast phase remains (second and third lines). Two minutes after application limited signal was observed during the slow phase (fourth line), but did not return to pre-application values (fifth and sixth lines) during the 10-minute recording period.

Fig. 2. Neuropeptide F (20–36)-induced effects on cardiac activity over a range of concentrations. Animals (y-axis, percent) displaying slow-phase arrest (filled columns) or increased duration of the isoelectric period (open columns) are reported compared to baseline value for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control).

Fig. 3.

Neuropeptide F (20–36)-induced effects on the duration of the isoelectric period of cardiac activity over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the duration of the isoelectric period are reported (y-axis, seconds) and for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). An asterisk indicates significance relative to baseline (B) calculated with the Wilcoxon test using $p < 0.05$.

Fig. 4.

Neuropeptide F (20–36)-induced effects on the signal frequency of the slow phase and the fast phase over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the variation in the signal frequency (y-axis, percent) of the slow phase (triangles) and the fast phase (squares) are reported for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). Filled symbols indicate significance relative to baseline calculated with the Wilcoxon test using $p < 0.05$.

Fig. 5.

Neuropeptide F (20–36)-induced effects on the duration of the slow phase and the fast phase over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the variation in the duration (y-axis, percent) of the slow phase (triangles) and fast phase (squares) are reported for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). Filled symbols indicate significance relative to baseline calculated with the Wilcoxon test using $p < 0.05$.

Fig. 6.

Dose-response curves for the NPF (20–36)-induced effects on the signal frequencies and duration of the slow phase and the fast phase. Both periods of cardiac activity are analyzed independently (slow phase, triangles, and fast phase, squares). The y-axis represents the percent variation from baseline of the variable being measured, and the x-axis represents the concentration of NPF (20–36) in molarity. Mean values of the maximum change in response observed during the 10-minute recording period $\pm$ S.E. (vertical bars) are reported.

Fig. 7. Short NPF-1-induced effects on cardiac activity over a range of concentrations. Animals (y-axis, percent) displaying slow-phase arrest (filled columns) or increased duration of the isoelectric period (open columns) are reported compared to baseline value for a 10-minute recording time (x-axis, minutes) in response to peptide or control.

Fig. 8. Short NPF-1-induced effects on the duration of the isoelectric period over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the duration of the isoelectric period are reported (y-axis, seconds) for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). An asterisk indicates significance relative to baseline (B) calculated with the Wilcoxon test using $p < 0.05$.

Fig. 9.

Short NPF-1-induced effects on the signal frequency of the slow phase and the fast phase over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the variation in the signal frequency (y-axis, percent) of the slow phase (triangles) and the fast phase (squares) are reported for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). Filled symbols indicate significance relative to baseline calculated with the Wilcoxon test using $p < 0.05$.

Fig. 10.

Short NPF-1-induced effects on the duration of the slow phase and the fast phase over a range of concentrations. Mean values $\pm$ S.E. (vertical bars) of the variation (y-axis, percent) of the slow phase (triangles) and the fast phase (squares) are reported for a 10-minute recording time (x-axis, minutes) in response to peptide or saline (control). Filled symbols indicate significance relative to baseline calculated with the Wilcoxon test using $p < 0.05$.

Fig. 11.

Dose-response curves for the sNPF-1-induced effects on the signal frequencies and duration of the slow phase and the fast phase. Both periods of cardiac activity are analyzed independently (slow phase, triangles, and fast phase, squares). The y-axis represents the percent variation from baseline of the variable being measured, and the x-axis represents the concentration of sNPF-1 (molarity). Mean values of the maximum change in response observed during the 10-minute recording period $\pm$ S.E. (vertical bars) are reported.

Table 1

<i>D. melanogaster</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. simulans</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. ananassae</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. sechellia</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. persimilis</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. pseudoobscura</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. willistoni</i>	F	L	Q	D	L	D	L	D	T	Y	G	D	R	A	R	V	R	F	amide
<i>D. yakuba</i>	F	L	Q	D	L	D	L	D	S	Y	G	D	R	A	R	V	R	F	amide
<i>D. erecta</i>	F	L	Q	D	L	D	L	D	S	Y	G	D	R	A	R	V	R	F	amide
<i>D. mojavensis</i>	Y	L	Q	D	L	D	L	D	V	Y	G	D	R	A	R	V	R	F	amide
<i>D. virilis</i>	Y	L	Q	D	L	D	L	D	V	Y	G	D	R	A	R	V	R	F	amide
<i>D. grimshawi</i>	Y	L	Q	D	L	D	L	D	V	Y	G	D	R	A	R	V	R	F	amide

Table 2

<i>D. melanogaster</i>	A	Q	R	S	P	S	L	R	L	R	F	amide
<i>D. mojavensis</i>	A	Q	R	S	P	S	L	R	L	R	F	amide
<i>D. virilis</i>	A	Q	R	S	P	S	L	R	L	R	F	amide
<i>D. grimshawi</i>	A	Q	R	S	P	S	L	R	L	R	F	amide
<i>Anopheles gambiae</i>	A	V	R	S	P	S	L	R	L	R	F	amide
<i>Aedes aegypti</i>	A	V	R	S	P	S	L	R	L	R	F	amide
<i>Culex quinquefasciatus</i>	A	V	R	S	P	S	L	R	L	R	F	amide
<i>Bombus terrestris</i>	S	Q	R	S	P	S	L	R	L	R	F	amide
<i>Camponotus floridanus</i>	S	Q	R	S	P	S	L	R	L	R	F	amide
<i>Acromyrmex echinator</i>	S	Q	R	S	P	S	L	R	L	R	F	amide
<i>Apis mellifera</i>	S	Q	R	S	P	S	L	R	L	R	F	amide
<i>Harpegnathos saltator</i>	S	Q	R	S	P	S	L	R	L	R	F	amide
<i>Acrythosiphon pisum</i>	N	Q	R	S	P	S	L	R	L	R	F	amide
<i>Daphnia pulex</i>	S	D	R	S	P	S	L	R	L	R	F	amide
<i>Tribolium castaneum</i>	S	G	R	S	P	S	L	R	L	R	F	amide
<i>Ixodes scapularis</i>	G	G	R	S	P	S	L	R	L	R	F	amide