[Frontiers in Bioscience 14, 4035-4043, January 1, 2009]

Plasticity in the effects of sulfated and nonsulfated sulfakinin on heart contractions

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TABLE OF CONTENTS

1. ABSTRACT

Neuropeptides regulate the frequency of heart contractions. *Drosophila melanogaster* sulfakinin (drosulfakinin) encodes FDDYGHMRFamide, DSK I, and GGDDQFDDYGHMRFamide, DSK II. Invertebrate sulfakinins are structurally and functionally related to vertebrate cholecystokinins. Naturally-occurring drosulfakinins contain a sulfated or nonsulfated tyrosine and are designated sDSK I, sDSK II, nsDSK I, and nsDSK II. We developed a novel neural-cardiovascular preparation and investigated mechanisms regulating the effect of sulfakinins on *D. melanogaster* heart. We established the preparation in larva, pupa, and adult to examine plasticity in neural regulation of cardiovascular parameters. We discovered sDSK I increased the frequency of larval, pupal, and adult heart contractions; nsDSK I only increased the frequency of larval contractions, not pupal or adult. We also discovered sDSK II and nsDSK II increased the frequency of larval and adult contractions, not pupal. This is the first report of nonsulfated sulfakinin activity on heart, and sulfakinin activity examined in 3 developmental stages within the same animal species. Our data demonstrate a role for plasticity in the effects of sulfakinins on heart contractions, and suggest multiple mechanisms are involved.

2. INTRODUCTION

Failure of the heart to maintain regular and consistent contractions is a significant health problem, it causes morbidity and mortality, yet the mechanisms involved are not well understood. The role of the brain in regulating cardiovascular function is a major focus of scientific research; however, there remains much to be learned. Understanding how a neuropeptide affects heart contractions is significant because it identifies an endogenous signaling agent(s) that could contribute to heart arrhythmias and failure. Knowledge of the molecular mechanisms underlying neuropeptide activity may serve as a basis for the design of agonists and antagonists to regulate cardiovascular function and impact the health and survival of an animal.

Drosophila melanogaster is a model for human cardiovascular research. There is homology between the molecular mechanisms involved in the brains and cardiovascular systems of fruitfly and human. Its small size, short generation time, and the fact it is amenable to molecular genetics and its genome is sequenced, make *D. melanogaster* attractive as a research organism to decipher the neural regulation of the cardiovascular system.

The relevance of *D. melanogaster* to human health research was established many decades ago. However, only relatively recently was the fruitfly cardiovascular system investigated; the primary focus of the publications is heart development [1, 2]. Research is directed toward understanding the molecules involved in regulating heart contractions [3-10], albeit, the number of publications is limited compared to the enormity of the mechanisms involved and its physiological importance.

The *D. melanogaster* cardiovascular system consists of a long tube-like structure which is called the dorsal vessel composed of an anterior aorta and posterior heart [11, 12]. It is a myogenic peristaltic pump which is under neural control [3, 5, 6]. Several methods are described in the literature to investigate the effects of classical transmitters and peptides on *D. melanogaster* heart [3, 5, 6, 10, 13-15]. However, to our knowledge, no *in vivo* preparation is described that includes the central nervous system and the dorsal vessel. In addition, no report analyzes neural mechanisms involved in cardiovascular system at different developmental stages. The role of plasticity in regulating heart contractions is important physiologically and, thus, it is crucial to address scientifically.

Sulfakinin peptides are structurally and functionally related to the vertebrate cholecystokinin (CCK) peptides [16-26]. The *D. melanogaster* sulfakinin gene (Dsk), encodes 2 structurally-related CCK-like peptides, drosulfakinin I (DSK I; FDDYGHMRFNH2) and drosulfakinin II (DSK II; GGDDQFDDYGHMRFNH2) [27]. The drosulfakinin peptides are structurally related; DSK II is a 5 amino acid N-terminal extension of DSK I. Like CCK peptides, naturally-occurring sulfakinins contain a sulfated or nonsulfated tyrosyl residue [16-26]. Drosulfakinins with sulfated or nonsulfated tyrosine residue are designated sDSK I and sDSK II, and nsDSK I and nsDSK II, respectively. Naturally-occurring cholecystokinin peptides contain a sulfated or nonsulfated tyrosine; both forms are biologically active [28-33].

First identified as myostimulatory gut peptides (16, 17), sulfakinins also elicit a decrease in food intake (34-37); the gut and neural activities of sulfakinins are similar to the effects of cholecystokinins (28-33). Sulfakinins structurally related to sulfated DSK I and sulfated DSK II increase the frequency of contractions of adult cockroach heart (23). Sulfakinins structurally related to sulfated DSK I and sulfated DSK II increase the frequency of contractions in adult lobster heart (36). However, to our knowledge, the effects of sulfakinins on *D. melanogaster* heart and the role of plasticity, change in gene expression, and its impact on the cardiovascular system are not reported. Nor is there a report which investigates the effects of nonsulfated sulfakinins on heart contractions. In order to elucidate the role (s) of the sulfakinins in biology it is crucial to investigate all of the naturally-occurring peptides, otherwise, the data collected and the interpretations are limited, ambiguous, or incorrect.

Here, we describe a novel preparation to investigate neural molecular mechanisms involved in *D. melanogaster* cardiovascular physiology. We also present the first analysis of the effects of sulfated and nonsulfated sulfakinins on the frequency of heart contractions at 3 different developmental stages; larva, pupa, and adult. We discovered all forms of the sulfated and nonsulfated drosulfakinin peptides affected the frequency of *D. melanogaster* heart contractions. However, the structurally-related but distinct sulfated and nonsulfated DSK I and DSK II peptides differed in their effects on *D. melanogaster* heart contractions which is consistent with developmental plasticity of mechanisms involved in the cardiovascular activities of drosulfakinins.

3. METHODS

3.1. Animals

The *D. melanogaster* Oregon R wild type strain was used. The animals were maintained on cornmeal molasses media at 24°C under a 12h light/dark cycle. *D. melanogaster* assayed were feeding third instar wandering larvae, white prepupae, and 1 day adults. Both females and males were analyzed and no difference in response was observed.

3.2. Chemicals

The sulfakinin peptides were synthesized by standard Fmoc protocol with a Protein Technologies Symphony synthesizer and purified by high pressure liquid chromatography. Fmoc sulfated tyrosine sodium salt was used to generate the sulfated forms of DSK I and DSK II. The structure of each peptide was confirmed by amino acid analysis and mass spectrometry. Reagent grade chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA.

3.3. Assays

An individual cold-anesthetized *D. melanogaster* larva or pupa was pinned dorsal side down onto Sylgard 184 (Dow Corning, Midland, MI, USA) and covered with physiological saline (5mM HEPES, 128 mM NaCl, 36 mM sucrose, 4 mM $MgCl_2$, 2 mM KCl, and 1.8 mM $CaCl_2$, pH 7.1). The cuticle of the larva or pupa was cut along the midline and its abdominal contents removed; the dorsal fat bodies were not removed. The gut and salivary glands were also removed. The brain, trachea, ventral nerve cord, dorsal vessel, and dorsal fat bodies remained intact. The dorsal vessel was visible along the entire length of the animal. The anterior dorsal vessel (aorta) and posterior dorsal vessel (heart) beat at the same rate, 60 - 150 contractions/minute.

An individual cold-anesthetized *D. melanogaster* adult was pinned, dorsal side down, to Sylguard 184 and covered with physiological saline. The cuticle posterior to the thorax was opened, perpendicular to a slit made along the abdominal midline. The digestive system was removed posteriorly at the proventriculus and anteriorly at the rectum. Reproductive organs and Malpighian tubules were removed to visualize the dorsal vessel along the entire

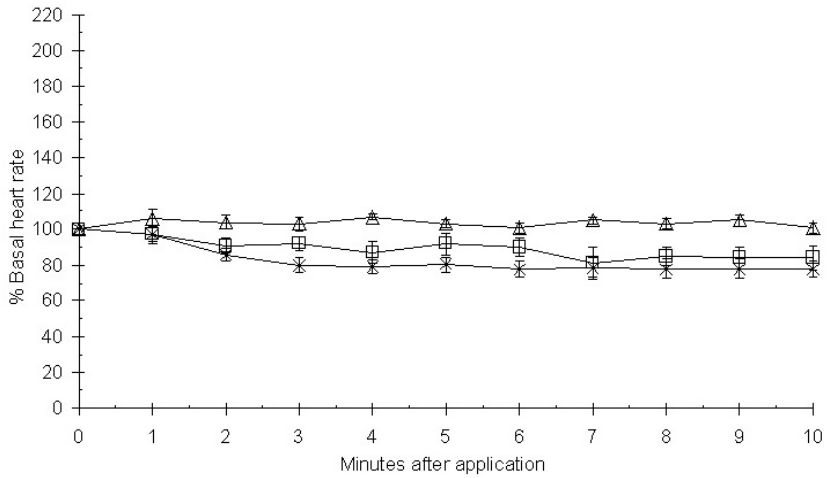

Figure 1. The effects of saline on the frequency of larval (unfilled rectangles), pupal (unfilled circles), and adult (unfilled triangles) heart contractions reported as a percent of basal rate over a 10 minute recording period. The values at 1 minute after application of saline to larval (97 ± 3%), pupal (97 ± 4%), and adult (106 ± 5%) heart were not statistically different from basal rate.

length of the abdomen. The aorta and heart beat at the same rate, 115 - 150 contractions/minute.

All dissections and experiments were conducted at room temperature to promote physiological conditions; solutions, kept on wet ice, were warmed to ambient temperature immediately before use. After the dissection, the saline was removed and replaced with fresh saline. After recording the frequency of basal heart contractions for 2 minutes, saline was removed and replaced with the control or experimental solution, rinsing with 2 changes of the same solution and counting contractions per minute. The frequency of heart contractions was recorded optically and continuously for 10 minutes immediately after the application of a fresh aliquot of saline or a peptide. The number of contractions per minute was reported as percent basal heart rate. ANOVA followed by Student Newman Keuls test were used for analysis; $p < 0.5$ was considered significant.

4. RESULTS AND DISCUSSION

4.1. Saline effects on *D. melanogaster* heart contractions

Here we report the effect of a reagent (control, saline; experimental, 1μM peptide) on the frequency of heart contractions at 3 different developmental stages – third instar wandering larva, white prepupa, and 1 day adult – as percent basal rate measured on each individual animal prior to the application of the reagent tested. Typically, 5 to 8 animals were tested for each reagent at each developmental stage; each animal was used in only 1 experiment. The average effects and percent error are reported at 1 minute after application of the reagent, which was, typically, the time of maximal response.

The application of saline to the neural-cardiovascular preparations did not statistically affect the rate of heart contractions in any of the 3 developmental stages (Figure 1). Within error, the frequencies of heart contractions observed after application of saline to the preparations were not statistically different from basal rates (100%) measured prior to application of the saline; larva (97 ± 3%), pupa (97 ± 4%), and adult (106 ± 5%). The lack of effects of the control on the frequency of heart contractions supports the validity of using the neural-cardiovascular preparations to measure the effects of an experimental reagent.

4.2. sDSK I and nsDSK I effects on *D. melanogaster* heart contractions

Sulfated sulfakinins are reported to be biologically active in numerous animal species in several physiological processes including the frequency of heart contractions (16-26). However, no study reports the effects of both sulfated and nonsulfated sulfakinin peptides on heart, or the role development plays in the effects of any sulfakinins.

In order to investigate the effect of the 4 forms of sulfakinins on *D. melanogaster* cardiovascular function we began our work with the analysis of sulfated DSK I (sDSK I) on heart contractions. The application of sDSK I to the neural-cardiovascular preparations did statistically affect the rate of heart contractions in all 3 developmental stages tested (Figure 2). The frequencies of heart contractions observed 1 minute after application of sDSK I to the preparations were statistically different from basal heart rates (100%) measured prior to application of the sulfated form of DSK I; larva (134 ± 7%), pupa (127 ± 2%), and adult (176 ± 6%). These data suggest sDSK I may play a role as a cardio-acceleratory peptide in regulating heart contractions in each of the 3 developmental stages analyzed.

The first reports of sulfakinin activities indicated that only the sulfated forms of the peptides were active;

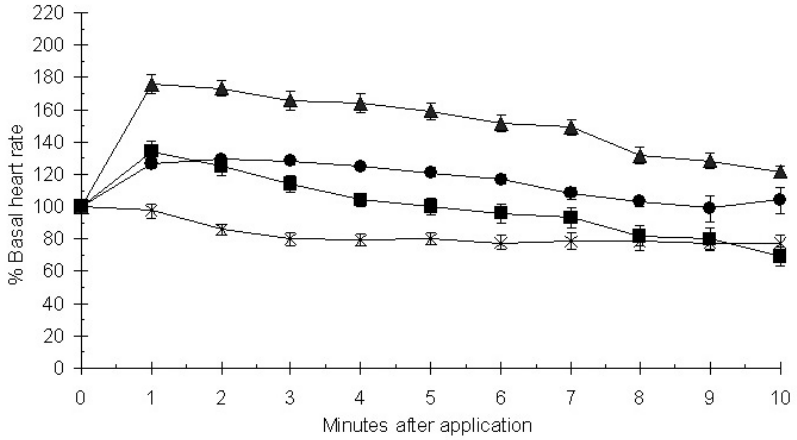

Figure 2. The effects of 1μM sDSK I on the frequency of larval (filled rectangles), pupal (filled circles), and adult (filled triangles) heart contractions reported as a percent of basal rate over a 10 minute recording period. The values at 1 minute after application of sDSK I to larval (134 ± 7%), pupal (127 ± 2%), and adult (176 ± 6%) heart were statistically different from basal rate. The effect of saline on larval heart (x) is shown for reference; see Figure 1 and Table 1 for a developmental profile of the effects of saline on heart contractions.

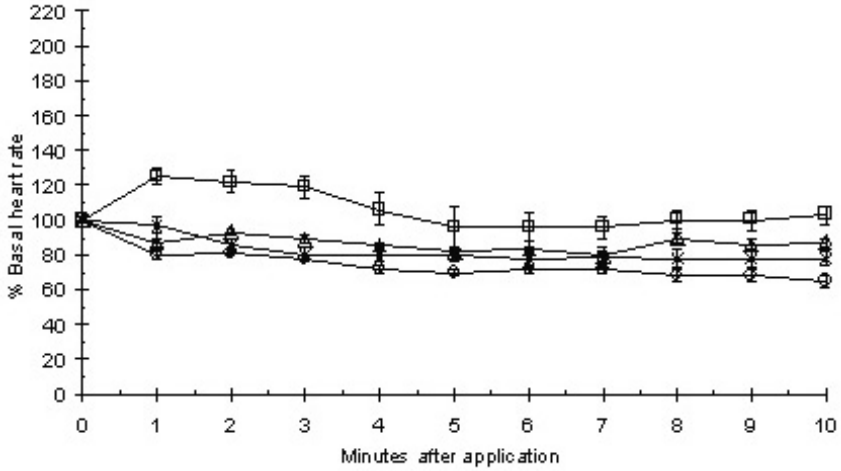

Figure 3. The effects of 1μM nsDSK I on the frequency of larval (unfilled rectangles), pupal (unfilled circles), and adult (unfilled triangles) heart contractions reported as a percent of basal rate over a 10 minute recording period. The value at 1 minute after application of nsDSK I to larval (125 ± 5%) heart was statistically different from basal rate. The values at 1 minute after application of nsDSK I to pupal (80 ± 8%) and to adult (87 ± 2%) heart were not statistically different from basal rate. The effect of saline on larval heart (x) is shown for reference; see Figure 1 and Table 1 for a developmental profile of the effects of saline on heart contractions.

however, recently we reported the activity of nonsulfated DSK I on gut (39). In addition, other investigators report the isolation of naturally-occurring nonsulfated sulfakinin and sulfakinin-like peptides (23, 44). Thus, in addition to analyzing the effect of the sulfated form of DSK I on heart rate, we tested the nonsulfated form of DSK I (nsDSK I) on the frequency of heart contractions. The application of nsDSK I to the neural-cardiovascular preparations did statistically affect the frequency of heart contractions but in only 1 of the 3 developmental stages analyzed; in larva, but not in pupa and not in adult (Figure 3). The frequency of heart contractions observed 1 minute after application of nsDSK I to the larval preparations was statistically different from basal heart rates (100%) measured prior to application of the nonsulfated form of DSK I; larva (125 ± 5%). However, nsDSK I showed a marked difference in its effect on the frequency of heart contractions in other developmental stages; it was not statistically different from basal rate in pupa (80 ± 8%) and adult (87 ± 2%). These data suggest nsDSK I may only play a role as a cardio-acceleratory peptide in regulating heart rate in the larval developmental stage.

4.3. sDSK II and nsDSK II effects on *D. melanogaster* heart contractions

In order to continue our analysis of the effect of sulfakinin peptides on the frequency of heart contractions we tested the effect of sulfated DSK II (sDSK II) on heart rate; DSK II is a 5-amino acid N-terminal extension of DSK I. The application of sDSK II to the neural-cardiovascular preparations did statistically affect the rate of heart contractions but only in 2 of the 3 developmental

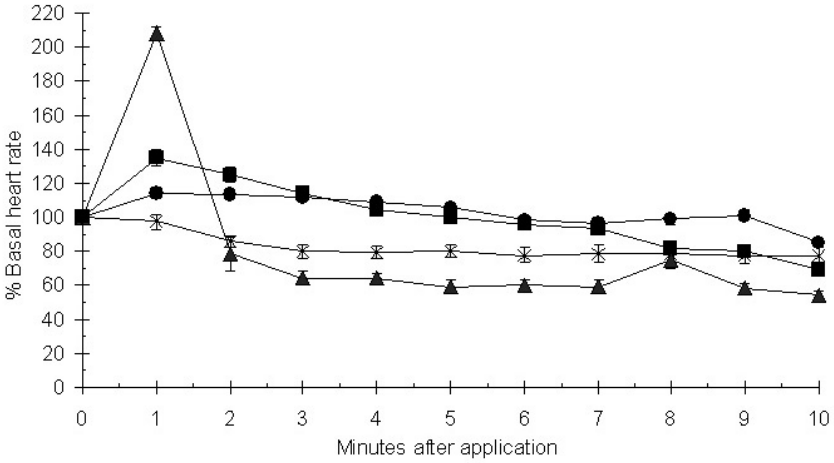

Figure 4. The effects of 1μM sDSK II on the frequency of larval (filled rectangles), pupal (filled circles), and adult (filled triangles) heart contractions reported as a percent of basal rate over a 10 minute recording period. The values at 1 minute after application of sDSK II to larval (135 ± 9%) and to adult (208 ± 4%) heart were statistically different from basal rate. The value at 1 minute after application of sDSK II to pupal (114 ± 2%) heart was not statistically different from basal rate. The effect of saline on larval heart (x) is shown for reference; see Figure 1 and Table 1 for a developmental profile of the effects of saline on heart contractions.

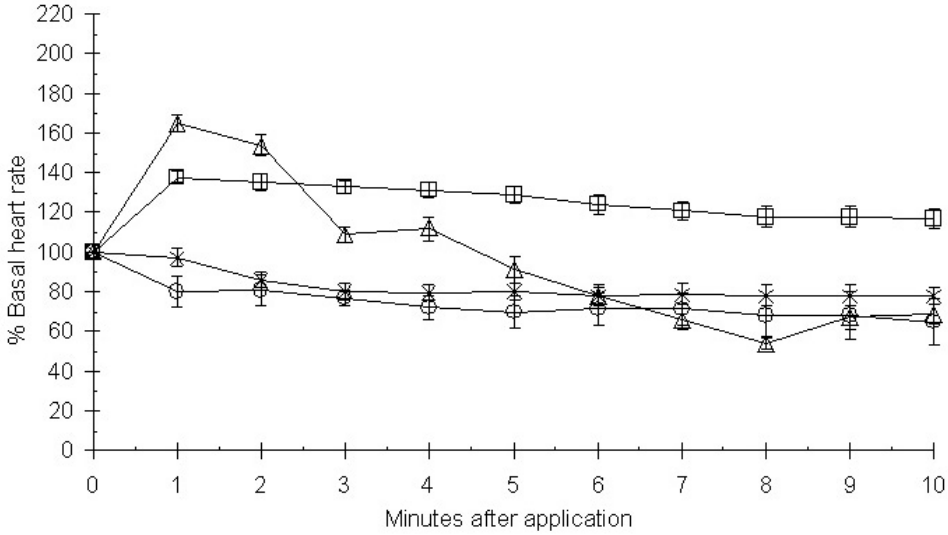

Figure 5. The effects of 1μM nsDSK II on the frequency of larval (unfilled rectangles), pupal (unfilled circles), and adult (unfilled triangles) heart contractions reported as a percent of basal rate over a 10 minute recording period. The values at 1 minute after application of nsDSK II to larval (138 ± 3%) and to adult (165 ± 4%) heart were statistically different from basal rate. The value at 1 minute after application of nsDSK II to pupal (80 ± 8%) heart was not statistically different from basal rate. The effect of saline on larval heart (x) is shown for reference; see Figure 1 and Table 1 for a developmental profile of the effects of saline on heart contractions.

stages analyzed; in larva and adult, but not in pupa (Figure 4). The frequency of heart contractions observed at 1 minute after application of sDSK II to the larval and adult preparations was statistically different from basal heart rates (100%) measured prior to application of the sulfated form of DSK II; larva (135 ± 9%) and adult (208 ± 4%). However, sDSK II shows a marked difference in its effect on heart rate in another developmental stage; it was not statistically different from basal rate in pupa (114 ± 2%). These data suggest sDSK II may only play a role as a cardio-acceleratory peptide in regulating heart rate in the larval and adult developmental stages.

We continued our analysis by testing the nonsulfated form of DSK II (nsDSK II) on the frequency of heart contractions. The application of nsDSK II to the preparations did statistically affect the frequency of heart contractions but in only 2 of the 3 developmental stages analyzed; larva and adult, but not in pupa (Figure 5). The frequency of heart contractions observed 1 minute after

Table 1. The effects of saline on the frequency of heart contractions

	Larva	Pupa	Adult
Saline	97 ± 3%	97 ± 4%	106 ± 5%

Table 2. The effects of sDSK I and nsDSK I peptides on the frequency of heart contractions

	Larva	Pupa	Adult
sDSK I	134 ± 7%	127 ± 2%	176 ± 6%
nsDSK I	125 ± 5%	80 ± 8%	87 ± 2%

application of nsDSK II to the larval and adult preparations were statistically different from basal heart rates (100%) measured prior to application of the nonsulfated form of DSK II; larva (138 ± 3%) and adult (165 ± 4%). However, nsDSK II shows a marked difference in its effect on heart rate in another developmental stage; it was not statistically different from basal rate in pupa (80 ± 8%). These data suggest nsDSK II may only play a role as a cardio-acceleratory peptide in regulating heart rate in the larval and adult developmental stages.

4.4. DSK effects on heart contractions: proposed mechanisms

Frequently neuropeptides precursors encode multiple gene products. The importance of peptides in regulating and signaling crucial physiological functions and potential to be target molecules for drug development demands the elucidation of the mechanisms underlying their processing and signaling. This knowledge is critical to basic research and the applied health sciences. The Dsk gene is an example of how posttranslational processing generates related but structurally unique members of a peptide family from a single precursor molecule. Additional mechanisms including the presence of multiple signal transduction mechanisms and/or sequence-specific proteolysis may exist to broaden the diversity of physiological roles in which the structurally-related peptides act as messengers or regulators.

An approach to gain insight into the role (s) of peptides in physiology is to test the effects of the naturally-occurring molecules in biological preparations. Our study provides the most extensive analysis of sulfakinin peptide activities to date. We tested the bioactivity of all 4 forms of drosulfakinin – sulfated and nonsulfated DSK I and sulfated and nonsulfated DSK II – in 3 different developmental stages of the same animal species, *D. melanogaster*, a model organism. Our results demonstrate that the mechanisms involved in sulfakinin biology are complex. Our data are consistent with the conclusion that multiple and/or different mechanisms are involved in the activities of the DSK peptides in cardiovascular biology. The mechanisms involved may be, but are not limited to, processing of the drosulfakinin precursor and regulation of synthesis to produce DSK I and DSK II in the sulfated and nonsulfated forms. Additionally, the signaling pathway (s) involved in transducing the effects of the sulfakinins on heart may be different in larva, pupa, and adult. Proteolysis or degradation of a specific form (s) of sulfakinin may also affect whether a DSK peptide acts on heart contractions.

Little information is available on sulfakinin precursor processing and regulation of synthesis (21, 22), and sulfakinin proteolysis. The most information available regarding mechanisms involved in sulfakinin biology identifies 2 candidate G-protein coupled receptors (GPCRs), DSK-R1 and DSK-R2 identified from the *D. melanogaster* genome project (40, 41). The binding requirements of 1 sulfakinin receptor candidate, DSK-R1, were examined using sulfated and nonsulfated forms of a DSK I analog, Leu^7-DSK I (FDDYGHLRFNH2) (42). Binding the analogs to *in vitro* expressed protein led the authors to conclude only a sulfated DSK I activated DSK-R1; they report the nonsulfated DSK I analog binds the expressed receptor protein about 3000-fold less. Unfortunately, no data are provided to demonstrate the Leu^7-substituted DSK I analogs accurately reflect the binding of the naturally-occurring sulfakinin peptides. Nor do binding data reflect biological activities. Additionally, the report does not investigate DSK-R2 binding requirements, nor does it examine sulfated or nonsulfated DSK II ligand-receptor binding. There are no data for the mechanisms responsible for the observed sulfated and nonsulfated DSK peptides on the frequency of heart contractions.

Based on the potential for 2 DSK receptors and mechanisms involved in the physiology of other neuropeptides, in particular CCK, models which explain the data can be formulated and tested. Sulfakinins are structurally and functionally similar to vertebrate CCK peptides which act through 2 GPCRs designated CCK1R and CCK2R (43). CCK1R shows high specificity for sulfated CCK; CCK2R has the same affinity for both sulfated and nonsulfated CCK. The selective binding and/or developmental expression of DSK receptors may help explain the effects of the DSK peptides on the frequency of heart contractions; precursor processing and/or proteolysis may also be involved.

First, both forms of DSK I peptides, sulfated and nonsulfated, increase the frequency of larval heart contractions. However, only sDSK I increases the frequency of pupal and adult heart contractions; nsDSK I does not affect the frequency of pupal and adult heart contractions (Figures 2 and 3, and Table 2). The reason for this difference is not known. An explanation is nsDSK I only plays a role in larval cardiovascular physiology and, thus, the mechanism (s) involved in the effect of nonsulfated drosulfakinin I is only present in larva. Sulfated drosulfakinin I may be the true biological ligand and nsDSK I may activate the sDSK I signaling pathway in larval; however, a molecular change (s) may prevent nsDSK I from activating the sDSK I signaling pathway in pupal and adult. Alternatively, proteolysis may degrade nsDSK I in pupa and adult, but not in larva in which case a difference in stability, not signaling, prevents the nonsulfated from activating the mechanisms required to affect heart contractions.

Compared to the drosulfakinin I peptides, the drosulfakinin II peptides show similar activities over the developmental stages tested; both sDSK II and nsDSK II

Table 3. The effects of sDSK II and nsDSK II peptides on the frequency of heart contractions

	Larva	Pupa	Adult
sDSK II	135 ± 9%	114 ± 2%	208 ± 4%
nsDSK II	138 ± 3%	80 ± 8%	165 ± 4%

increase the frequency of contractions for larval and adult heart, but not pupal heart. An explanation of the data (Figures 4 and 5, and Table 3) is the receptor to which the DSK II peptides bind is only expressed in larval and adult. Alternatively, only 1 receptor may be present throughout the 3 developmental stages tested and other mechanisms such as processing or proteolysis explain the activity differences.

The time courses of the activities observed argue for multiple processes associated with the effects of the peptides. The mechanisms involved in the different activities may include but are not limited to differences in the susceptibility of the peptides to proteolysis. Additionally or alternatively, the mechanisms may involve how a peptide interacts with a receptor (s) and signal transduction processes. The mechanisms involved may be neural or cardiovascular in nature, or both. No evidence to date provides insight into the site (s) of activities; the preparation we describe and our data provide a basis to decipher the mechanisms involved.

The isolation of the first sulfakinin and subsequent discovery of the peptide family posed several interesting questions about their role (s) in physiology, and the evolutionary relationship of the invertebrate sulfakinins to vertebrate cholecystokinins and to other RFamide-containing peptides (16, 17, 44). Our report identifying biological activities for both nonsulfated and sulfated sulfakinins strengthens the similarity between the invertebrate and vertebrate peptide families. Our data further argue nonsulfated sulfakinins are biologically active and are consistent with the conclusion developmental plasticity is involved in sulfakinin biology. Our results widen the breath of scientific questions necessary to explore and answer in elucidating the physiological functions of this conserved peptide family.

5. SUMMARY AND PERSPECTIVE

The discovery of sulfakinins and subsequent investigations suggest, based on the bioactivities observed and high degree of structure conservation, these peptides play important role(s) in animal biology. The data we present here expands the understanding of these structurally-related peptides and the complexity of the mechanisms associated with their roles in biology. In order to decipher the physiological functions of the sulfakinins and the underlying molecular events, research needs to focus on the family of naturally-occurring peptides, both sulfated and nonsulfated forms.

6. ACKNOWLEDGEMENTS

We thank Professor Anna Di Cosmo for her support. Research done in the Nichols' laboratory was funded by the National Institutes of Health, the National Science Foundation, and The American Heart Association, Midwest Affiliate.

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Key Words: Cardiovascular, Cholecystokinin, Drosulfakinin, DSK I, DSK II, FMRFamide

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