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Identification and cardiotropic actions of sulfakinin peptides in the American lobster *Homarus americanus*

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Summary

In arthropods, a group of peptides possessing a –Y$_{(SO3H)}$GHM/LRFamide carboxy-terminal motif have been collectively termed the sulfakinins. Sulfakinin isoforms have been identified from numerous insect species. In contrast, members of this peptide family have thus far been isolated from just two crustaceans, the penaeid shrimp *Penaeus monodon* and *Litopenaeus vannamei*. Here, we report the identification of a cDNA encoding prepro-sulfakinin from the American lobster *Homarus americanus*. Two sulfakinin-like sequences were identified within the open-reading frame of the cDNA. Based on modifications predicted by peptide modeling programs, and on homology to the known isoforms of sulfakinin, particularly those from shrimp, the mature *H. americanus* sulfakinins were hypothesized to be pEFDEY$_{(SO3H)}$GHMRFamide (Hoa-SK I) and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide (Hoa-SK II). Hoa-SK I is identical to one of the previously identified shrimp sulfakinins, while Hoa-SK II is a novel isoform. Exogenous application of either synthetic Hoa-SK I or Hoa-SK II to the isolated lobster heart increased both the frequency and amplitude of spontaneous heart contractions. In preparations in which spontaneous contractions were irregular, both peptides increased the regularity of the heartbeat. Our study provides the first molecular characterization of a sulfakinin-encoding cDNA from a crustacean, as well as the first demonstration of bioactivity for native sulfakinins in this group of arthropods.

Key words: cDNA, neurohormone, pEFDEY$_{(SO3H)}$GHMRFamide, GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide, heart, sulfakinin, *Homarus americanus*, expressed sequence tag (EST), neuromodulation, cardiac ganglion.

Introduction

In addition to providing important commercial fisheries, decapod crustaceans have long served as model organisms for a number of biological fields, including endocrinology and neurobiology. For example, studies done on the sinus gland of the land crab *Gecarcinus lateralis* provided the first formal demonstration of neurosecretion in any animal (Bliss, 1951; Passano, 1951). Likewise, the neural circuits contained within the crustacean stomatogastric and cardiac nervous systems have served as two of the premier models for investigating the generation, maintenance and modulation of rhythmic behavior at the cellular and systems levels (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Cooke, 2002). Despite their commercial and biological importance, relatively little is known about these animals at the molecular level. This lack of information is due, in part, to the scarcity of nucleotide sequence data for them; e.g. prior to 2006, less than 100 nucleotide sequences were known from the American lobster *Homarus americanus*, although it has been the subject of extensive physiological investigations (Towle and Smith, 2006).

To help facilitate gene-based studies of crustacean biology, expressed sequence tags (ESTs) have recently been produced for the normalized cDNA libraries of several decapod species, including *H. americanus* (Towle and Smith, 2006). As the tissues used to construct some of the libraries include neural and endocrine tissues, the ESTs generated from them provide useful tools to search for putative cDNAs encoding peptide precursor proteins. With the identification of such transcripts, not only can the amino acid sequences of the encoded peptide hormones be deduced, but their distributions and regulation within neural and endocrine systems can also be investigated. To this end, we have recently begun database mining of several *H. americanus* EST

collections for prepro-hormone transcripts, particularly those that encode neuropeptides that are likely present in the nervous system in low abundance and/or ones with modifications that make them difficult to identify using other methods.

One family of peptides that has long proved challenging to identify in decapod crustaceans is the sulfakinins, a group of peptides containing the carboxy (C)-terminal motif –$Y_{(SO3H)}$GHM/LRFamide. First using biochemical methods, and more recently *via* molecular techniques, a number of sulfakinin isoforms have been identified from insects (Nachman et al., 1986a; Nachman et al., 1986b; Nichols et al., 1988; Veenstra, 1989; Schoofs et al., 1990; Fonagy et al., 1992; Nichols, 1992; Duve et al., 1995; Maestro et al., 2001) (NCBI accession number AY341429; NCBI accession number AY758365) (Table 1). In contrast, sulfakinin isoforms have thus far been biochemically isolated and characterized from just two crustaceans, the penaeid shrimp *Penaeus monodon* and *Litopenaeus vannamei* (Johnsen et al., 2000; Torfs et al., 2002) (Table 1). For each shrimp species, over a thousand central nervous systems (CNSs) [1030 for *P. monodon* (Johnsen et al., 2000) and 3500 for *L. vannamei* (Torfs et al., 2002)] were needed to isolate and characterize the native sulfakinins, pQFDE$Y_{(SO3H)}$GHMRFamide and AGGSGGVGGE$Y_{(SO3H)}$D$DY_{(SO3H)}$GHLRFamide.

Undoubtedly, large tissue pools were necessary for these identifications due, in part, to the fact that the shrimp CNS possesses a very small number of sulfakinin-containing neurons [10 or fewer in *P. monodon* (Johnsen et al., 2000), a distribution shared with insects (Duve et al., 1994; Davis et al., 1996; Nichols and Lim, 1996; East et al., 1997)]. As such, large tissue samples are impractical to collect from *H. americanus*, we have taken advantage of *H. americanus* ESTs to identify and sequence a cDNA encoding prepro-sulfakinin from this species. Based on modifications suggested by peptide modeling

Table 1. *Sequences of sulfakinin isoforms that have been identified in a variety of species to date*

Phylogenetic grouping/ Species	Sequence of short isoform	Name	Reference	Sequence of long isoform	Name	Reference
Crustacea/Decapoda						
Homarus americanus	pEFDEYGHMRFa	Hoa-SK I	1	GGGEYDDYGHLRFa	Hoa-SK II	1
Penaeus monodon	pQFDEYGHMRFa	Pem-SK I	2	AGGSGGVGGEYDDYGHLRFa	Pem-SK II	2
Litopenaeus vannamei	pQFDEYGHMRFa	Pev-SK II	3	AGGSGGVGGEYDDYGHLRFa	Pev-SK I	
Insecta/Blattodea						
Leucophaea maderae	pQSDDYGHMRFa	Lem-SK II	4	EQFEDYGHMRFa	Lem-SK I	5
Periplaneta americana	pQSDDYGHMRFa	Pea-SK II	6	EQFDDYGHMRFa	Pea-SK I	6
Blattella germanica	ND			EQFDDYGHMRFa	Blg-SK	7
Insecta/Diptera						
Drosophila melanogaster	FDDYGHMRFa	Drm-SK I	8,9	GGDDQFDDYGHMRFa	Drm-SK II	8
Neobellieria bullata	FDDYGHMRFa	Neb-SK I	10	XXEEQFDDYGHMRFa	Neb-SK II	10
Calliphora vomitoria	FDDYGHMRFa	Cav-SK I	11	GGEEQFDDYGHMRFa	Cav-SK II	11
Lucilia cuprina	FDDYGHMRFa	Luc-SK I	11	GGEEQFDDYGHMRFa	Luc-SK II	11
Anopheles gambiae	FDDYGHMRFa	Ang-SK I	12	GGEGDQFDDYGHMRFa	Ang-SK II	12
Anopheles maculatus	FDDYGHMRFa	Ang-SK I	13	GGEGDQFDDYGHMRFa	Anm-SK II	13
Insecta/Orthoptera						
Locusta migratoria	pQLASDDYGHMRFa	Lom-SK	14	ND		
Urochordata						
Ciona intestinalis	NYYGWMDFa	Cionin	15			
Chordata						
Multiple species	DYMGWMDFa	CCK-8				

SKs are grouped into the long and short isoforms, as both have been identified in nearly all species examined. None of the sulfates are shown, so that the peptides align to show the homologies more clearly.

[1]This study; [2](Johnsen et al., 2000); [3](Torfs et al., 2002); [4](Nachman et al., 1986a); [5](Nachman et al., 1986b); [6](Veenstra, 1989); [7](Maestro et al., 2001); [8](Nichols et al., 1988); [9](Nichols, 1992); [10](Fonagy et al., 1992); [11](Duve et al., 1995); [12]NCBI accession number AY341429; [13]NCBI accession number AY758365; [14](Schoofs et al., 1990); [15](Johnsen and Rehfeld, 1990).

The origin of the N-terminal pyro-residue in the short SK isoforms of shrimp was originally given as being derived from glutamine rather than glutamic acid (as our molecular analysis has shown is the case for the Hoa-SK I), though there is no data to prove this assignment currently.

The sulfation state of a number of the tyrosine (Y) residues is unknown/unclear, although it appears likely that all tryrosines in each of these sequences is sulfated.

Blg-SK was placed within the long isoform grouping as it is the only sulfakinin thus far isolated from *B. germanica* and is identical to the long isoform of the *P. americana*.

XX in Neb-SK II represent two residues whose identity was undetermined.

Lom-SK was placed into the short isoform grouping as it is blocked by a pyro-residue at its N terminus, which thus far has been seen only in the short SK isoforms.

Table 2. *Primers used in sequencing the EST containing the sulfakinin mRNA*

Forward primer (5′ to 3′)	Reverse primers
TGGTGAGGCGAGTGTTTACAA	AAGTCTGACTCACAGCGACCAACA
ACGAAACAAACCGTGGGAGGAA	CACCATGCATAACACACCGAGGTT
TGTGCCATAACACACCAGGAGG	GTGCAAATATAGATCTAATATATCAGGCTT
CCACTGAACAGGTATTTACTACATCA	GGTATTTACTACATCACCTCCAAT
CACATCAACACGTGTTAACACAGGTC	

Both forward and reverse primers are listed in the order used, starting from the center of the sequence and moving towards both the 3′ and the 5′ ends.

programs and on homology to the known sulfakinins, particularly those from shrimp (Johnsen et al., 2000; Torfs et al., 2002), the putative mature forms of the *H. americanus* sulfakinins were predicted and the peptides synthesized. Exogenous application of each of the predicted peptides to the lobster heart produced dramatic increases in both contraction amplitude and frequency, suggesting that the hypothesized post-translational modifications were correct and demonstrating that each isoform is bioactive on the heart. Taken collectively, our data provide the first molecular characterization of a sulfakinin-encoding cDNA from a crustacean and demonstrate, for the first time, that native sulfakinins are biologically active in this group of arthropods. Some of these data have appeared previously in abstract form (Brennan et al., 2006).

Materials and methods

Animals

American lobsters *Homarus americanus* Milne-Edwards were purchased from local (Maine) suppliers. All animals were housed in flow-through or recirculating natural seawater aquaria at 10–12°C.

cDNA library construction, normalization, sequencing and EST submission

The construction and normalization of the *H. americanus* cDNA library used in this study were described in detail in a previous report (Towle and Smith, 2006). In brief, multiple tissues (including the supraoesophageal ganglion, commonly referred to as the brain) from four individuals were collected, total RNA samples were prepared individually from each tissue, checked for quality, then pooled for construction and normalization of a cDNA library by Invitrogen Corporation (Carlsbad, CA, USA). Plasmids were isolated and inserts single-pass sequenced from their 5′ end using SP6 primer (5′-ATTTAGGTGACACTATAG-3′) at the Marine DNA Sequencing and Analysis Facility at Mount Desert Island Biological Laboratory (Salisbury Cove, ME, USA). Sequence traces were processed for submission to dbEST (National Center for Biotechnology Information; Bethesda, MD, USA) using the trace2dbest component of PartiGene software (University of Edinburgh, Edinburgh, Scotland, UK). Before submission, all ESTs were subjected to blastx analysis [i.e. translated nucleotide sequence *versus* protein sequence (Altschul et al., 1997)] and annotated accordingly. A single EST (NCBI accession number CN952349) with significant homology to a sulfakinin precursor from the blowfly *Calliphora vomitoria* (NCBI accession number Q7M3V5) (Duve et al., 1995) was identified during this analysis.

cDNA sequence analysis

To characterize the *H. americanus* cDNA clone (Ha_mx0_58h07) identified by blastx analysis, a sample of the bacteria (*Escherichia coli*) possessing the insert-containing vector was cultured overnight in LB-medium at 37°C. Plasmid containing the cDNA was subsequently isolated using a Purelink™ Quick Plasmid Miniprep kit (Invitrogen). The vector insert was then sequenced on an ABI 3100 16-capillary sequencer (Applied Biosystems Incorporated, Foster City, California, USA) using both vector- and insert-specific forward and reverse sequencing primers (Integrated DNA Technologies, Inc., Coralville, IA, USA; Table 2). The sequence trace files resulting from each round of sequencing were analyzed using Chromas 2.31 software (Technelysium Pty Ltd, Tewantin, Queensland, Australia), and the high quality nucleotide sequences were aligned using SeqMan 2.6 software (DNASTAR Inc., Madison, WI, USA).

Nucleotide translation and structural analysis of the deduced amino acid sequence

Translation of the full-length nucleotide sequence of cDNA clone Ha_mx0_58h07 was accomplished using the online program WWW Nucleotide Translation (BioInformatics & Molecular Analysis Section [BIMAS], National Institutes of Health, Bethesda, MD, USA; http://bimas.dcrt.nih.gov/molbio/translate/). Signal peptide and signal peptide cleavage prediction was done *via* the online program SignalP 3.0 using both Neural Networks and Hidden Markov Models algorithms (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark; http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). Prohormone cleavage sites were predicted based on the information presented in several recent reviews (Veenstra, 2000; Fricker, 2005). Prediction of the sulfation state of tyrosine residues was done using the online program Sulfinator (Swiss Institute of Bioinformatics, Geneva, Switzerland; http://www.expasy.org/tools/sulfinator/) (Monigatti et al., 2002), as well as through homology to known sulfakinin

sequences from shrimp (Johnsen et al., 2000; Torfs et al., 2002). Likewise, other post-translational modifications (i.e. cyclization of N-terminal glutamic acid residues and C-terminal amidations) were predicted by homology to known sulfakinin sequences, particularly those isolated from shrimp (Johnsen et al., 2000; Torfs et al., 2002).

Production of synthetic peptides

pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide were synthesized on an ABI Pioneer peptide synthesizer (Applied Biosystems Inc.) using standard Fmoc chemistry at the Biotechnology Center of the University of Wisconsin-Madison (Madison, WI, USA). Fmoc-Tyr(SO_3-OH) sodium salt was purchased from Chem-Impex (Wood Dale, IL, USA). The remaining Fmoc-amino acids, including pyroglutamine, were purchased from Novabiochem (San Diego, CA, USA). For the synthesis of pEFDEY$_{(SO3H)}$GHMRFamide, a 60 min extended coupling time was used to couple the sulfotyrosine residue and double 30 min couplings were used for the three N-terminal amino acids following the sulfotyrosine. Coupling times were 30 min for the first five C-terminal amino acids. For the synthesis of GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide, a 60 min extended coupling time was used for both sulfotyrosine couplings and double 30 min couplings were used for the two aspartic acid couplings following the first sulfotyrosine and for all four N-terminal residues following the second sulfotyrosine. Coupling times were 30 min for the first five C-terminal amino acids.

Following the completion of all coupling reactions, pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide were cleaved and deprotected for 90 min in thioanisole: ethanedithiol: trifluoroacetic acid (TFA) (5: 2.5: 92.5). The resin was then filtered off and the cleavage solution for each synthesis was dripped into 10 ml of cold *t*-butylmethyl ether to precipitate the peptide. The resulting precipitates were washed and centrifuged three times with additional volumes of ether. The ether precipitates were then dried by vacuum. To avoid acid hydrolysis of the sulfate groups, the crude peptides were dissolved in water:ammonium hydroxide (100:1) to neutralize the residual TFA.

Crude pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide were purified on a Beckman System Gold HPLC system (Fullerton, CA, USA) using a preparative C-18 reverse phase column (Dynamax 250×21.4 mm; Varian, Palo Alto, CA, USA). Solvent A was 0.1% TFA/water and Solvent B was 0.08% TFA/90% acetonitrile. The gradient program was 0% B (0–8 min), 0–7% B (8–11 min), 11–26% B (11–77 min), with a flow rate was 16 ml min^{-1}. Each collected fraction (9.6 ml) was neutralized immediately by addition of 20 μl of concentrated ammonium hydroxide to prevent the hydrolytic loss of sulfate groups. The final purities of pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide were 94% and 90%, respectively.

Cardiac physiology

To determine the effects of the sulfakinins on the neurogenic heart of *H. americanus*, lobsters were cold-anaesthetized by packing them in ice for 30–60 min, after which the posterior dorsal region of the thoracic carapace that lies directly over the heart, as well as the underlying cardiac tissue, was removed. This dissected region was pinned through the carapace to the bottom of a small Sylgard 184 (KR Anderson, Santa Clara, CA, USA)-lined dish. The dorsal part of the heart remained attached to the carapace, so that the extent to which it was stretched was identical to that in the intact animal. The posterior artery was cannulated with a short piece of polyethylene tubing drawn out to fit the artery, and was continuously perfused with 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, 5.00 Hepes, pH 7.4 (Bucher et al., 2003)] cooled with a Warner Instruments CL-100 bipolar temperature control system (Hamden, CT, USA). Because isolated hearts continue to contract only when adequately stretched (Cooke, 2002), flow rate through the heart was kept at approximately 2 ml min^{-1}. Under our recording conditions, stable heart activity could be recorded for at least 8 h. A second perfusion line was directed across the top of the heart to help maintain temperature, which was monitored continuously and kept between 10–12°C.

To record heart contractions, the anterior arteries were tied off with a human hair and attached to a Grass FT03 force-displacement transducer (Astro-Med, Inc., West Warwick, RI, USA) at an angle of approximately 30°. The output of the transducer was amplified *via* a Brownlee 410 instrumentation amplifier (San Jose, CA, USA), and recorded onto a PC computer using a Micro 1401 data acquisition board and Spike2 version 5 software (Cambridge Electronic Design Limited, Cambridge, UK). Both heart rate and contraction amplitude were measured using the built-in functions of Spike2. Data were further analyzed and graphed using Prism4 software (GraphPad Software, Inc., San Diego, CA, USA).

Preparations were allowed to stabilize for 1–2 h before the first application of sulfakinin. Both Hoa-SK I and Hoa-SK II were dissolved in deionized water at a concentration of 10^{-3} mol l^{-1}, and kept as a frozen stock solution at –20°C for use in physiological experiments. Control experiments to compare the effects of frozen stock peptide and freshly dissolved peptide showed that both sulfakinin isoforms were stable when frozen in aqueous solution (data not shown). Peptides were diluted in chilled physiological saline to a final concentration of 10^{-6} mol l^{-1} just before use. Both Hoa-SK I and II were applied to most preparations, in random order, with at least 1 h of wash in control saline between peptides, to allow for comparisons within the same preparation. No order effect was seen (data not shown).

Results

Nucleotide sequence of a putative Homarus americanus prepro-sulfakinin cDNA

Using the blastx algorithm (i.e. translated nucleotide sequence *versus* protein sequence), a *H. americanus* EST

(accession number CN952349) with homology to blowfly *C. vomitoria* prepro-sulfakinin (NCBI accession number Q7M3V5) (Duve et al., 1995) was identified. Using a combination of vector- and insert-specific forward- and reverse-sequencing primers (Table 2), a 1523 base pair (bp), putative full-length cDNA was sequenced (NCBI accession number EF418605). As is shown in Fig. 1, this full-length clone consisted of a 42 bp 5′-untranslated region (UTR), a 363 bp open-reading frame (ORF), as well as a 1118 bp 3′-UTR containing two AATAAA polyadenylation signal sequences located 12 and 658 bps upstream of a 79 bp poly-A tail.

Structural analysis of deduced Hoa-prepro-sulfakinin

Translation of the ORF of cDNA clone Ha_mx0_58h07 predicted a 120 amino acid (aa) prepro-peptide (Figs 1 and 2). SignalP 3.0 analysis (Bendtsen et al., 2004) of this amino acid sequence using both Neural Networks and Hidden Markov Models algorithms identified the first 24 amino acids of the prepro-hormone as a signal peptide, with a cleavage site between Ser[24] and Ala[25] (Figs 1 and 2). Processing between these residues would produce a 96 aa pro-sulfakinin that contains two Lys-Arg and two Arg-X_n-Arg (where X is a variable amino acid and n is either 2 or 6) processing sites (Veenstra, 2000; Fricker, 2005) (Figs 1 and 2). Among the peptides predicted to be cleaved from the pro-peptide *via* proteolytic processing are EFDEYGHMRFG (Figs 1 and 2) and GGGEYDDYGHLRFG (Figs 1 and 2), both of which contain the core sequence –YGHM/LRF that is the hallmark of the sulfakinin family (Table 1). In addition to the sulfakinins, three other peptides, collectively termed sulfakinin precursor-related peptides (SPRPs), are also predicted to be liberated from the pro-hormone: APARPSSLARVLAPVV (SPRP I; Figs 1 and 2, Table 1), QRLEESHLPPALVEELVQDFEDP-ELLDFHDAAG (SPRP II; Figs 1 and 2, Table 1) and SLTHSDQHHHHDTTVN (SPRP III; Figs 1 and 2, Table 1).

Prediction of mature sulfakinin and precursor-related peptide isoforms

Based on homology to known sulfakinin isoforms, particularly those from the penaeid shrimp *P. monodon* and *L. vannamei* (Johnsen et al., 2000; Torfs et al., 2002), it is likely that the immature *H. americanus* sulfakinins EFDEYGHMRFG and GGGEYDDYGHLRFG undergo significant post-translational modification prior to assuming their mature, bioactive isoforms (Fig. 2, Table 1). Specifically, the C-terminal glycine residue of each peptide is predicted to act as a donor for amidation in the mature peptides, and the N-terminal glutamic acid of EFDEYGHMRFG is predicted to become cyclized to pyroglutamic acid (Fig. 2). The tyrosine residue or residues contained within each of the predicted immature sulfakinins are also likely modified by the addition of sulfate groups; however, the two previous studies in shrimp (Johnsen et al., 2000; Torfs et al., 2002) provided conflicting pictures as to whether one or both of the tyrosines in GGGEYDDYGHLRFG are sulfated. In *P. monodon*, only Tyr[14] was identified as sulfated in AGGSGGVGGEYDDYGHLRFamide (Johnsen et

CAACAACATCGTGTACAGTGGTGAGGCGAGTGTTTACAAACC**ATG**AGGTGGACAAGCTGG
M R W T S W

ACCGCGGCGGTGCTGGTGGTGATGGCGGCCTTCATGTTGTCTGGGGGAGTGTCGGCTCCA
T A A V L V V M A A F M L S G G V S A P

GCCAGACCTTCCTCCCTAGCACGAGTTCTGGCCCCCGTGGTGAGACAAAGGTTAGAGGAG
A R P S S L A R V L A P V V R Q R L E E

AGCCACCTGCCGCCGGCGCTGGTGGAGGAACTGGTGCAGGACTTTGAAGACCCTGAACTA
S H L P P A L V E E L V Q D F E D P E L

CTGGACTTCCACGACGCGGCGGGCAAGAGGGAGTTTGACGAGTACGGTCACATGAGGTTT
L D F H D A A G K R E F D E Y G H M R F

GGTAAGCGAGGCGGCGGGGAGTACGACGACTACGGTCACTTGAGGTTTGGCAGAAGTCTG
G K R G G G E Y D D Y G H L R F G R S L

ACTCACAGCGACCAACACCACCACCACGACACAACCGTTAAC<u>TAA</u>ACACACACCTTTACA
T H S D Q H H H H D T T V N *

CGACAAGTTAATTAATTCGTGTGTGTGTGGCGCCAACGAAACAAACCGTGGGAGGAATTA
ATGTATTTTTGATAAAGTATATAGTCACAGGTTCTCAATATGATCTACACTACATTAGAA
CGTGTAAACATCAAACACGACGGGCTGCTTCACATGAGGATGGTGACGTAGTGTTGATAT
ATCTAAGGTGAAGTCATCAGTGTTTGGATCACCATGCATAACACACCGAGGTTTAGTTTA
ATAATGGTTTTATGTCGTGCAGCAACTGGACAATTGATGATGATAAATTGAATTACTTTT
CCCCAATTTCCCATCCCACAAACTATAACAATGTGCCATAACACACCAGGAGGTTAACTT
<u>AATAAA</u>AGTCAAAATCACAAATCCCCCCAAAAATATTTATATTCCAAAAAATACAATACT
ATATTTGTTGTTCTTTACAGCATAATATATACTAAGAAAACTCACATTATTATATTTCCA
CTGAACAGGTATTTACTACATCACCTCCAATTAAAAAAAAATAATAATTAAAAAACAAGA
AAAACAGACACATGGCTTTTACTGACCCCCCCCCCCCCCCTTCGACAAGTGTTCAAAGCA
TGGTGAACTTTTTCCTCTTATATGAGCAAAACTACAACAATATCACCATATTAAAAGCCT
GATATATTAGATCTATATTTGCACTCTATTGCTAACTAAGGTGTGTTTTGAAAGATACAA
TTAGAGTGTGTTGGTGCGTTTGTTTCACGTTGTATTATGATGATGATGATGACGTGTTTT
GTCGACTATGTTGTTCTTCCTGTTGTTATATATAAGATTTGTGGGTCCCTAATAATCTCA
ACACACGTACAGACACATCAACACGTGTTAACACAGGTCAAAGGTAATTCATGTTTCGTC
AAGGCCTGTCAAGACTTATGAATTTGTTAAGATGTGTCATAAAGTGTCAAGACGTGTCAC
AAGTCCTTAAGTGTAGTGTAGTGTCTTGTGTGAAGTGGCTGGGGCA<u>AATAAA</u>ACCTTATC
CTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAA

Fig. 1. Nucleotide and deduced amino acid sequences of *Homarus americanus* prepro-sulfakinin. Within the coding region of the cDNA (bold font), both the start (ATG) and stop (TAA) codons are underlined, as are the two polyadenylation signal sequences (AATAA) present in the 3′-UTR. The predicted amino acid sequence of the prepro-hormone signal peptide is shown in italics. Predicted cleavage sites within the preprohormone are shown in black. The amino acid sequence, including the C-terminal glycine residue which serves as a target for α-amidation, of *Homarus americanus* sulfakinin I (Hoa-SK I) is shown in blue, while that of *Homarus americanus* sulfakinin II (Hoa-SK II), including its C-terminal glycine, is shown in red. The amino acid sequences of three putatative *Homarus americanus* sulfakinin-precursor related peptides [Hoa-SPRPs I, II and III (named based on their relative positions within the prepro-hormone)] are shown in green (including the C-terminal glycine residue of Hoa-SPRP II). Within the amino acid sequence of the prepro-hormone, the position of the stop codon is denoted with an asterisk.

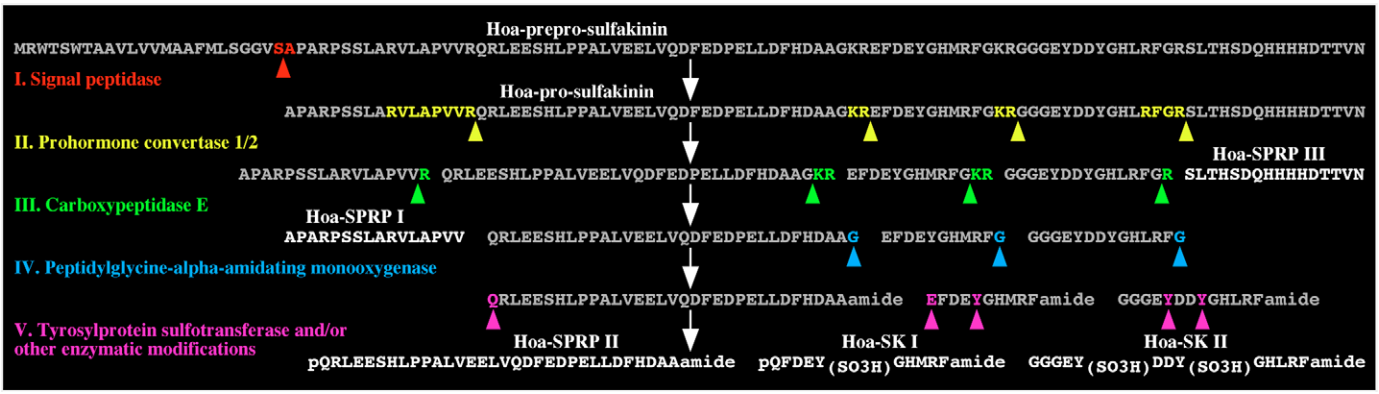

Fig. 2. Flow diagram showing the putative post-translational processing of sulfakinins and sulfakinin precursor-related peptides (SPRPs) from the deduced *Homarus americanus* prepro-sulfakinin. Translation of the nucleotide sequence of the cDNA encoding *H. americanus* prepro-sulfakinin predicts a 120 amino acid prepro-hormone (top sequence). The first 24 amino acids of the prepro-hormone are predicted to be a signal peptide (SignalP 3.0 analysis) (Bendtsen et al., 2004), with a cleavage site between Ser^{24} and Ala^{25} (red residues and arrowhead). Processing between these residues by signal peptidase would produce a 96 amino acid pro-sulfakinin (second sequence). *Via* homology to known insect pro-hormone cleavage sites (Veenstra, 2000), two Lys-Arg and two Arg-X_n-Arg (where X is a variable amino acid and n is either 0, 2, 4 or 6 residues) processing sites were identified in Hoa-pro-sulfakinin (yellow residues and arrowheads). Proteolytic processing by a prohormone convertase at these sites would liberate five peptides (third line of sequences); the basic residues on four of these are predicted to be the targets of carboxypeptidase action (green residues and arrowheads). In three of these four peptides, carboxypeptidase action would expose a glycine residue (fourth line of sequences), which likely serves as a target for α-amidation by peptidyl-amidating monooxygenase [blue residues and arrowheads; homology to known sulfakinin isoforms (e.g. Johnsen et al., 2000; Torfs et al., 2002)]. Action by this enzyme would result in the amidation of the carboxy termini of these three peptides (fifth line of sequences). Additional post-translational processing of tyrosine residues by tyrosylprotein sulfotransferase in two of the peptides is predicted to result in the addition of sulfate groups to them [purple residues and arrowheads; homology to known sulfakinin isoforms (e.g. Johnsen et al., 2000; Torfs et al., 2002) and prediction *via* Sulfinator software (Monigatti et al., 2002)]. Likewise, based on homology to known sulfakinin isoforms (e.g. Johnsen et al., 2000; Torfs et al., 2002), the amino (N)-terminal glutamic acid in one peptide and the N-terminal glutamine in another (purple residues and arrowheads) are hypothesized to undergo enzymatic or spontaneous cyclization, resulting in the formation of pyro-residues in the mature forms (final line of sequences). The five resulting peptides (two SKs and three SPRPs) are shown and labeled in white.

al., 2000), while in *L. vannamei* both Tyr^{11} and Tyr^{14} were identified as sulfated in this peptide (Torfs et al., 2002). Torfs et al. (Torfs et al., 2002) suggested that the discrepancy in the sulfation states of the peptides was due to the different methods used to assess the presence of this modification, i.e. MALDI-TOF MS and immunohistochemistry with sulfate-specific antisera [for *P. monodon* (Johnsen et al., 2000)] *versus* electrospray ionization MS and bioassay [for *L. vannamei* (Torfs et al., 2002)], with the latter combination of techniques providing a more accurate picture of the extent of tyrosine sulfation. Analysis of the *H. americanus* sulfakinin sequences using the Sulfinator software program (Monigatti et al., 2002) predicted all tyrosine residues in both isoforms are likely to be sulfated (E values <55 for each residue). Thus, we agree with the hypothesis put forth by Torfs et al. (Torfs et al., 2002) and predict the mature *H. americanus* sulfakinin isoforms to have the following structures: pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide (Fig. 2, Table 1). Based on standard convention, we named these peptides *Homarus americanus* sulfakinin I (Hoa-SK I) and *Homarus americanus* sulfakinin II (Hoa-SK II), respectively.

In addition to the sulfakinin isoforms, post-translational processing is also predicted for at least one of the encoded SPRPs, specifically Hoa-SPRP II. As with the sulfakinin isoforms, the C-terminal glycine residue is likely converted to an amide group, producing the peptide QRLEESHLPPALVEELVQDFEDPELLDFHDAAamide (Fig. 2, Table 1). It is also possible that the N-terminal glutamine in Hoa-SPRP II is enzymatically cyclized to pyroglutamic acid. Thus, the mature form of Hoa-SPRP may be either QRLEESHLPPALVEELVQDFEDPELLDFHDAAamide or pQRLEESHLPPALVEELVQDFEDPELLDFHDAAamide (Fig. 2, Table 1). No obvious motifs for post-translational processing are present in either Hoa-SPRP I or III and thus we predict their mature isoforms to be APARPSSLARVLAPVV and SLTHSDQHHHHDTTVN, respectively (Fig. 2, Table 1).

Physiological effects of Hoa-SK I and II on the heart

Based on our collective molecular and bioinformatic data, the mature forms of Hoa-SK I and Hoa-SK II were predicted and synthesized. To assess their potential bioactivity in *H. americanus*, we applied them to the isolated neurogenic heart, a known target of many circulating peptide hormones (Cooke, 2002). Under control conditions, both the heart rate and the specific pattern of cardiac contractions recorded in isolated *Homarus* hearts varied somewhat, with frequencies ranging from an average of approximately 0.2 Hz to 1.0 Hz. In approximately

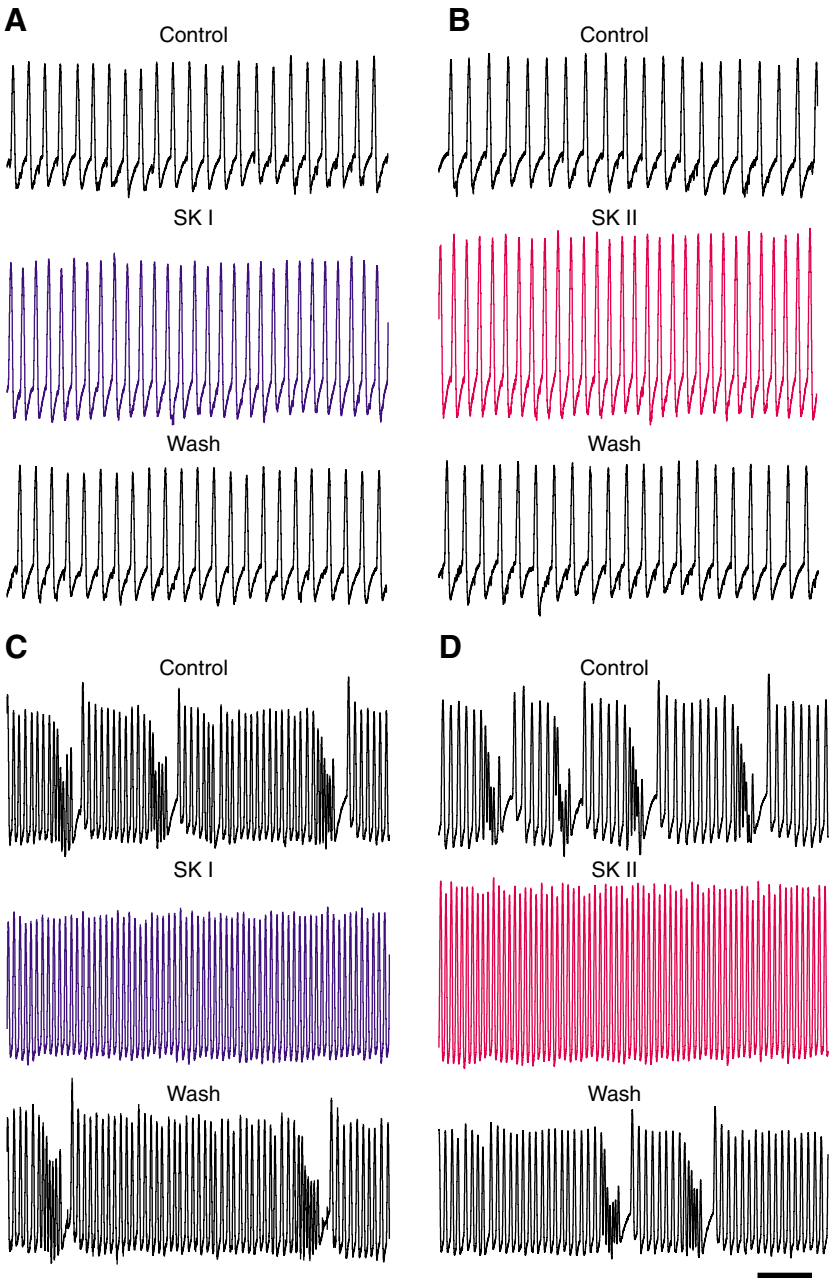

Fig. 3. Both Hoa-SK I and Hoa-SK II (10^{-6} mol l^{-1}) enhanced activity of the isolated heart. Shown are recordings from a force-displacement transducer in two preparations. (A,B) Recordings from a preparation in which the heartbeat was very regular. Each of the peptides evoked an increase in both frequency and amplitude of the heartbeat, although the effects were more pronounced with Hoa-SK II than with Hoa-SK I. (C,D) Recordings from a second preparation in which the heartbeat showed periodically irregularities. In addition to increasing the frequency and amplitude of heart contractions, Hoa-SK I and Hoa-SK II regularized heart beat frequency and amplitude when the heartbeat was initially irregular. Bar, 10 s for all recordings.

80% of preparations, the heartbeat was extremely regular (Fig. 3A,B), while in the remaining 20%, the heartbeat was regularly interrupted by a sequence (usually 4–6) of rapid and small amplitude beats followed by a long interbeat interval (Fig. 3C,D). In the examples shown in Fig. 3, it can be seen that upon application of either Hoa-SK I or Hoa-SK II, the heartbeat frequency increased in both types of preparations, as is particularly clear in the response to Hoa-SK II. When data from a number of preparations were pooled (Fig. 4), it was seen that exogenous application of both Hoa-SK I and Hoa-SK II (10^{-6} mol l^{-1}) was strongly cardioexcitatory, increasing the frequency of spontaneous contractions by 14.9±5.5% (± s.e.m.) and 19.4±5.7%, respectively. Calculated percent changes were significantly different from zero in both peptides (two-tailed single sample *t*-test: Hoa-SK I frequency, $P<0.05$; Hoa-SK II frequency, $P<0.01$; $N=11$ preparations for Hoa-SK I, $N=14$ preparations for Hoa-SK II). In addition, both Hoa-SK I and Hoa-SK II induced a regularization of the heartbeat in all preparations in which an irregular beat was present in control saline, as seen in Fig. 3C,D. In both peptides, regardless of the extent to which the heartbeat increased in frequency, the rapid beats and long interbeat intervals were eliminated in the presence of the peptide. The heartbeat again became irregular in the wash with physiological saline.

In addition to changes in the heartbeat frequency, both Hoa-SK I and Hoa-SK II caused significant increases in contraction amplitude of the heart, as can be seen in both the individual traces in Fig. 3B,D and in the pooled data in Fig. 4. Hoa-SK I increased amplitude by 15±4.4% (± s.e.m.) over control values, while Hoa-SK II caused an increase of 25.3±4.5% over control (single sample *t*-test; values significantly different from 0; $P<0.01$, $N=11$ preparations in Hoa-SK I and $N=14$ preparations in Hoa-SK II).

The effects of both Hoa-SK I and Hoa-SK II on heart contraction frequency were rapidly reversible, returning to control levels after approximately 15–20 min of superfusion with physiological saline (Fig. 5). The effects of the two peptides on contraction amplitude followed a very similar time course (compare Fig. 5A with 5C and 5B with 5D).

Discussion

The structural organization of Homarus americanus prepro-sulfakinin is similar to those of insects

Two decades ago, the first sulfated peptides from an invertebrate were identified (Nachman et al., 1986a; Nachman et al., 1986b): EQFDEDY$_{(SO3H)}$GHMRFamide and

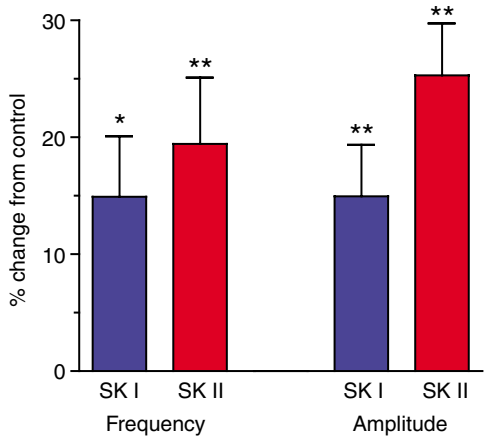

Fig. 4. Hoa-SK I and Hoa-SK II evoked increases in the frequency and amplitude of heart contractions. Both frequency and amplitude of cardiac contractions in the isolated heart increased by approximately 15% when the hearts were perfused with Hoa-SK I; frequency increased on average by 20% and amplitude by 25% when the hearts were perfused with Hoa-SK II. All changes were significantly different from 0 (two-tailed *t*-tests): **P*<0.05; ***P*<0.01; *N*=11 preparations for Hoa-SK I, *N*=14 preparations for Hoa-SK II. Values are means ± s.e.m.

pQSDDY$_{(SO3H)}$GHMRFamide from the cockroach *Leucophaea maderae*. This sulfation, in combination with their strong myotropic activity on the hindgut, resulted in these peptides being named sulfakinins (Nachman et al., 1986a; Nachman et al., 1986b). In the years that have followed, additional sulfakinins have been isolated biochemically and the prepro-hormones encoding members of this peptide family have been isolated. By far the majority of these peptides have been identified in insects (Nachman et al., 1986a; Nachman et al., 1986b; Nichols et al., 1988; Veenstra, 1989; Schoofs et al., 1990; Fonagy et al., 1992; Nichols, 1992; Duve et al., 1995; Maestro et al., 2001) (NCBI accession number AY341429; NCBI accession number AY758365) (Table 1). In addition, however, sulfakinins have been isolated from two species of penaeid shrimp, *Penaeus monodon* and *Litopenaeus vannamei* (Johnsen et al., 2000; Torfs et al., 2002) (Table 1), demonstrating the conservation of this peptide group across the arthropods. In the present study we report the identification of the first cDNA encoding sulfakinins from a crustacean, namely one from the American lobster *H. americanus*.

Comparisons of Hoa-prepro-sulfakinin to its insect homologs (Nichols et al., 1988; Duve et al., 1995) (NCBI accession number AY341429; NCBI accession number AY758365) show a number of conserved features. Specifically, all known prepro-hormones encode two distinct sulfakinin sequences, one short and one long, e.g. EFDEYGHMRF and GGGEYDDYGHLRF in *H. americanus* (this study) and FDDYGHMRF and GGEEQFDDYGHMRF in the blowflies *C. vomitoria* and *Lucilia cuprina* (Duve et al., 1995). In all species, the short form is preceded by the dibasic cleavage sequence KR and precedes the long form within the prepro-hormone. Likewise, in all known prepro-sulfakinins, the short form is separated from the long form by the amidating cleavage sequence GKR, with the long form being followed by the amidating cleavage sequence GR. Interestingly, while both the short and long isoforms are present in the same copy number within the prepro-hormones, in species in which the peptides have been biochemically isolated, there appears to be a much higher abundance of the shorter peptide (e.g. Nichols et al., 1988; Nichols, 1992; Duve et al., 1995; Johnsen et al., 2000; Torfs et al., 2002). Thus, it may be that prohormone convertase preferentially targets the dibasic cleavage sequence over the monobasic site, though differential degradation rates by peptidases may also play a role in controlling the relative abundance of the isoforms.

In addition to their encoded sulfakinin isoforms, all prepro-sulfakinins also possess other amino acid sequences that are predicted to be cleaved from them, producing a number of other peptides, termed here sulfakinin precursor-related peptides or SPRPs (Nichols et al., 1988; Duve et al., 1995) (NCBI accession number AY341429; NCBI accession number AY758365; this study). In *H. americanus*, these sequences are APARPSSLARVLAPVV,

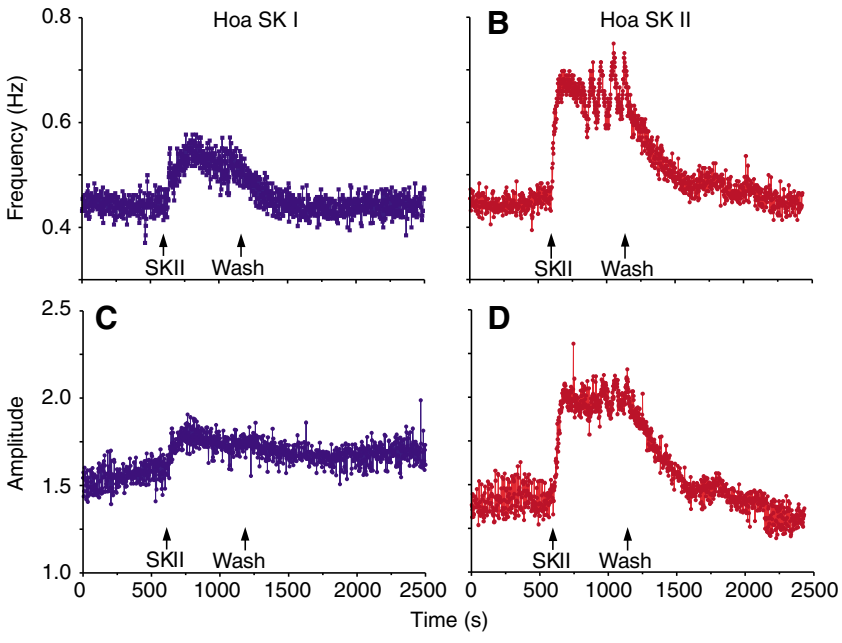

Fig. 5. The increases in both frequency and amplitude of heartbeat induced by Hoa-SK I (A,C) and Hoa-SK II (B,D) were rapid in onset, and relatively rapid in time to return to baseline. Shown are examples from a single, representative preparation, in which it can be seen that the peak effect was reached within less than 5 min. Wash-out took somewhat longer, but activity had returned to baseline within 10–12 min.

QRLEESHLPPALVEELVQDFEDPELLDFHDAAG and SLTHSDQHHHHDTTVN. Unlike the sulfakinin sequences, limited conservation is present in the SPRP sequences across species and no functional roles have been ascribed for these peptides in any species, if they are indeed bioactive.

Sulfakinin isoforms appear highly conserved both within and between taxa

As is the case in other species, two sulfakinins, one short and one long isoform, are encoded in the predicted *H. americanus* prepro-hormone. Based on bioinformatics and homology to known sulfakinin sequences (Table 1), we predicted the mature *H. americanus* structures to be pEFDEY$_{(SO3H)}$GHMRFamide (Hoa-SK I) and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide (Hoa-SK II). If our predictions are correct, Hoa-SK I would be identical to a previously described shrimp isoform, i.e. Pem-SK I/Pev-SK II (Johnsen et al., 2000; Torfs et al., 2002) (Table 1), while Hoa-SK II would be a novel sulfakinin family member (Table 1). It should be noted that the N-terminal pyro-residue of Pem/Pev-SK I is given as being derived from glutamine rather than glutamic acid (as we have shown here is the case for Hoa-SK I), though there are no data to prove unambiguously whether the cyclization is truly derived from this amino acid.

It is interesting to note that the short, but not the long, sulfakinin isoform is identically conserved between lobster and shrimp, which represent two distinct, and rather distantly related infraorders of the decapods (Johnsen et al., 2000; Torfs et al., 2002) (this study, Table 1). While the functional significance of a conserved short isoform and a variable long isoform is presently unknown, similar patterns of conservation have been noted in related insect taxa (Table 1). For example, FDDY$_{(SO3H)}$GHMRFamide has been identified or is predicted to be present in each of the dipteran (true fly) species thus far studied, whereas the longer sulfakinin isoform varies considerably between species (Nichols et al., 1988; Fonagy et al., 1992; Duve et al., 1995) (NCBI accession number AY341429; NCBI accession number AY758365) (Table 1). Similarly, pQSDDYGHMRFamide is shared by the two blattodean (cockroach) species thus far examined, while their extended isoforms differ (Nachman et al., 1986a; Nachman et al., 1986b; Veenstra, 1989) (Table 1). As additional studies are conducted on other crustacean and hexapod species, it will be interesting to see if this pattern of conservation holds as a rule or whether it is simply a function of not yet having a broad enough picture of the extant sulfakinin family members. If the former, extensive physiological investigations of sulfakinin actions on multiple tissues, as well as the identification and functional studies on the sulfakinin receptor(s) will be needed to elucidate the selective pressures resulting in the differential conservation of the short versus long sulfakinin isoforms.

Hoa-SK II, along with the long sulfakinin isoforms isolated previously from penaeid species (Johnsen et al., 2000; Torfs et al., 2002), possess several structural features that distinguish them from all other members of the sulfakinin family (Table 1). First, Hoa-SK II and Pem-SK II/Pev-SK I each end in –LRFamide rather than the usual –MRFamide C terminus. Moreover, these peptides contain two, rather than one, tyrosine residue, both of which are likely to be sulfated. Finally, the peptides contain glycine-rich N-termini, with the shrimp isoform exhibiting this feature to a greater extent than Hoa-SK II. The functional significance of these features is presently unknown, though as discussed below, they may well manifest themselves functionally in at least *H. americanus*, as Hoa-SK II appears to be a stronger modulator of the cardiac system than is Hoa-SK I.

The sulfakinins appear to be multifunctional peptides

Physiological investigations in insects have shown the sulfakinins to be multifunctional. Their discovery in the cockroach *L. maderae* was based on the hindgut myotropic assay (Nachman et al., 1986a; Nachman et al., 1986b), and they appear to possess similar function in most (Schoofs et al., 1990; Predel et al., 1999) but not all species, i.e. the blowfly *C. vomitoria* (Duve et al., 1994; Duve et al., 1995). In the cockroach *P. americana*, the native sulfakinins have been shown to be cardioactive (Predel et al., 1999). While not demonstrated directly, the distributions of sulfakinin-like immunoreactivity in many insects, particularly the dipterans, suggests a neuromodulatory role for this peptide family in the CNS (Duve et al., 1994; Davis et al., 1996; Nichols and Lim, 1996; East et al., 1997). Additionally, sulfakinins have been shown to stimulate the release of α-amylase from the midgut of both the weevil *Rhynchophorus ferrugineus* and the moth *Opisina arenosella* (Nachman et al., 1997; Harshini et al., 2002), and have been shown to be potent inhibitors of food intake in the locust *Schistocerca gregaria*, the cockroach *Blatella germanica* and the blowfly *Phormia regina* (Wei et al., 2000; Maestro et al., 2001; Downer et al., 2007).

Prior to our study, the functional roles played by sulfakinins in crustaceans were unknown. As in the dipterans, mapping of the distribution of sulfakinin-like labeling in *P. monodon* suggested a neuromodulatory role for family members in the shrimp CNS (Johnsen et al., 2000). Both the stomatogastric nervous system and the cardiac ganglion system have served as excellent models for the study of neuromodulation in decapods. Although we are currently investigating the effects of sulfakinins on the pattern generators of the stomatogastric system, the effects on these pattern generators in preliminary experiments appear to be relatively weak, so we have chosen to focus in this study on the effects of sulfakinins on the heartbeat of the lobster. Thus, in this study, we have shown that both of the native isoforms of sulfakinin are potent modulators of heartbeat *in vitro*. Specifically, exogenous application of either peptide to the isolated heart increased both the frequency and amplitude of spontaneous heart contractions. Moreover, both peptides regularized the pattern of spontaneous contractions in preparations where the heartbeat was initially irregular. Irregularities in crustacean heartbeat frequency and amplitude have been noted in a number of previous studies, or are seen in the recordings shown in these studies (Kuramoto and Ebara, 1984; Kuramoto and Ebara, 1985; Mercier and

Russenes, 1992; Hokkanen, 2000). Kuramoto and Ebara (Kuramoto and Ebara, 1985) noted that both heartbeat frequency and the variability in heartbeat parameters are influenced by the perfusion pressure. Worden et al. (Worden et al., 2006) also noted that heartbeat frequency varies significantly as a function of temperature. In the present study, both perfusion pressure and temperature were held constant during each experiment, and the peptide alone was responsible for changing the irregular heartbeat to a regular one. We do not yet know what mechanisms may underlie either the initial irregularities or the regularization that occurred in response to sulfakinin application, but future examinations of the effects of the peptides on the output of the cardiac ganglion itself may help to elucidate these issues.

While both Hoa-SK I and Hoa-SK II exerted similar actions on the heart qualitatively, Hoa-SK II was more potent than Hoa-SK I on all examined effects. In terms of their ability to modulate heartbeat frequency, Hoa-SK II was approximately 5% more potent then Hoa-SK I in increasing the frequency of spontaneous contractions over controls. Likewise, it was approximately 10% more potent than Hoa-SK I in effecting an increase in contraction amplitude. At present, the mechanism(s) by which these quantitative differences are achieved is unknown. Moreover, we have not yet examined the thresholds for the effects of these peptides, which might also differ. These experiments, and experiments designed to determine whether the specific effects exerted by the sulfakinins are dose-dependent, are ongoing. Clearly the structures of the two peptides are distinct. Most notably, Hoa-SK II is a disulfated peptide whereas Hoa-SK I contains only a single sulfated tyrosine residue. Hoa-SK II also possesses a leucine for methionine substitution in its C terminus that is not present in Hoa-SK I, and Hoa-SK II is longer than Hoa-SK I: 13 *versus* 10 amino acids in total length, respectively. Any or all of these structural differences might result in differential affinities to bind to a common receptor. Alternatively, the two peptides may target distinct receptors, which results in the quantitatively distinct effects on the heart. It is also possible that the two peptides are differentially sensitive to peptidase actions within the heart tissue and that this results in Hoa-SK II being present at a higher absolute concentration at the receptor(s) than is Hoa-SK I.

Do the invertebrate sulfakinins and the vertebrate CCK/gastrins share a common ancestry?

As has been noted by many authors, the sulfakinins share structural similarities to members of the vertebrate cholecystokinin (CCK)/gastrin family of peptides (Table 1), as well as to cionin (Table 1), a disulfated peptide isolated from the protochordate tunicate *Ciona intestinalis* (Johnsen and Rehfeld, 1990). It is the belief of many that these structural similarities are the result of a common ancestry for the two groups of peptides (Nachman et al., 1986a; Nachman et al., 1986b; Nichols et al., 1988; Veenstra, 1989; Schoofs et al., 1990; Maestro et al., 2001; Torfs et al., 2001; Nachman et al., 2005). In addition to the observed sequence homologies, the hypothesis of a common ancestor for the CCK/gastrins and the sulfakinins is supported by the findings that both share a number of conserved functions: both groups are myoactive on the gut, induce the release of the enzyme amylase from the digestive system, and serve as satiety factors (Nachman et al., 1997; Wei et al., 2000; Maestro et al., 2001; Harashini et al., 2002; Downer et al., 2007). Moreover, studies of the effects of vertebrate CCK and of a partially purified CCK-like peptide in the spiny lobster showed that CCK itself can activate the neuronal pattern generators that control movements of the foregut in this species (Turrigiano and Selverston, 1989; Turrigiano and Selverston, 1990; Turrigiano et al., 1994). The threshold for CCK effects was quite high in these studies, suggesting that the native peptide is not authentic CCK, but a related peptide, such as a sulfakinin. One possibility is that the observed effects of CCK were due to the activation of sulfakinin receptors by CCK; however, this remains to be tested.

Analyses of the *D. melanogaster* genome also suggested that the *Drosophila* G-protein coupled receptors CG6857 and CG6881 and the vertebrate CCK/gastrin receptors CCKR and GASR diverged from a common ancestor (Hewes and Taghert, 2001). Moreover, the cloning and expression of CG6881 (also known as DSK-R1) in a mammalian cell line showed it to be the target of a variety of sulfakinin isoforms (Kubiak et al., 2002). It should be noted, however, that other authors have suggested that the convergent evolution of separate lineages, rather than a shared ancestry, is the source of the structural and functional homologies seen between the CCK/gastrins and the sulfakinins (Duve et al., 1994; Duve et al., 1995; Johnsen, 1998; Johnsen et al., 2000). It is clear that more studies, encompassing a broader sampling of species, like ours here, will be needed to address this issue fully.

Conclusion

In summary, we have identified and characterized a cDNA from the American lobster *H. americanus* encoding a prepro-sulfakinin peptide, which is the first description of a sulfakinin-encoding cDNA from any crustacean species. Using bioinformatics and homology to known sulfakinin isoforms, we predicted the structures of the mature sulfakinins encoded in the prepro-hormone and synthesized them. Exogenous application of either of the two native peptides to the isolated *H. americanus* heart induced both increased frequency and amplitude of the heartbeat and regularized the rhythm of contractions in preparations where periodic interruptions were initially present. Our identification of the native sulfakinins from the lobster opens the door for future functional studies of these peptides in *H. americanus*, and lays a foundation for future comparative studies of the sulfakinins and their actions in other crustacean species.

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