Modulation of Rhythmic Motor Activity by Pyrokinin Peptides

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ABSTRACT

Pyrokinin (PK) peptides localize to the central and peripheral nervous systems of arthropods, but their actions in the CNS have yet to be studied in any species. Here, we identify PK peptide family members in the crab *Cancer borealis* and characterize their actions on the gastric mill (chewing) and pyloric (filtering) motor circuits in the stomatogastric ganglion (STG). We identified PK-like immunolabeling in the STG neuropil, in projection neuron inputs to this ganglion, and in the neuroendocrine pericardial organs. By combining MALDI mass spectrometry (MS) and ESI tandem MS techniques, we identified the amino acid sequences of two *C. borealis* pyrokinins (CabPK-I, CabPK-II). Both CabPKs contain the PK family-specific carboxy-terminal amino acid sequence (FXPRLamide). PK superfusion to the isolated STG had little influence on the pyloric rhythm but excited many gastric mill neurons and consistently activated the gastric mill rhythm. Both CabPKs had comparable actions in the STG, and these actions were equivalent to those of Pevpyrokinin (shrimp) and Leucopyrokinin (cockroach). The PK-elicited gastric mill rhythm usually occurred without activation of the projection neuron MCN1. MCN1, which does not contain CabPKs, effectively drives the gastric mill rhythm and at such times is also a gastric mill central pattern generator (CPG) neuron. Because the PK-elicited gastric mill rhythm is independent of MCN1, the underlying core CPG of this rhythm is different from the one responsible for the MCN1-elicited rhythm. Thus neuromodulation, which commonly alters motor circuit output without changing the core CPG, can also change the composition of this core circuit.
Key Words: Stomatogastric Ganglion, Gastric Mill Rhythm, Pyloric Rhythm, Central Pattern Generator, Mass Spectrometry.
INTRODUCTION

Neuromodulation enables individual neuronal networks to generate a repertoire of motor patterns (Marder and Bucher 2001; Marder et al. 2005). Neuromodulators gain access to these motor networks via neuronal and/or neuroendocrine release. This neuronal release often occurs from the terminals of projection neurons or sensory inputs (Beenhakker and Nusbaum 2004; Blitz et al. 2004; Christie et al. 2004; Billimoria et al. 2005; Koh and Weiss 2005; Proekt et al. 2005). Ultimately, to fully understand motor network operation in the unrestrained animal, it will be necessary to identify and characterize all the neuromodulators that have access to that network. Thus far, only a subset of the modulatory inputs has been so studied in any single model system.

One small motor system where a full characterization of all sources of neuromodulation is potentially achievable is the stomatogastric nervous system (STNS) of the crab *Cancer borealis* (Nusbaum and Beenhakker 2002; Marder and Bucher 2006). Many modulatory influences to this system are identified, with each one having distinct actions (Nusbaum et al. 2001; Marder and Bucher 2006). The STNS, which consists of four ganglia plus their connecting and peripheral nerves, contains a set of interacting central pattern generating (CPG) circuits underlying various aspects of feeding. The two best characterized of these CPGs, the gastric mill (chewing) and pyloric (filtering) circuits, are located in the stomatogastric ganglion (STG). These CPGs are modulated by projection neuron inputs originating in the neighboring, paired commissural ganglia (CoGs) and unpaired oesophageal ganglion (OG) (Nusbaum et al. 2001; Marder and Bucher 2006). They also receive hormonal modulation from several
In this paper, we report the identity and influence of pyrokinin (PK) peptides in the *C. borealis* STG. The PK peptide family occurs in the central and peripheral nervous system of many arthropods, and its actions on peripheral tissues are well-studied (Holman et al. 1986; Schoofs et al. 1991; Predel et al. 1997; Veelaert et al. 1997; Torfs et al. 2001; Choi et al. 2001; Zdárek et al. 2002; Clynen et al. 2003; Choi and Jurenka 2004). However, its actions have yet to be determined in the CNS of any species. Here we localize and identify the amino acid sequences of *Cancer borealis* pyrokinins I and II (CabPK-I, CabPK-II) and show that these PKs excite all gastric mill circuit neurons and elicit the gastric mill rhythm in the isolated STG. Whereas many previously identified modulators elicit and modify the pyloric rhythm, this is the first neuromodulator in *C. borealis* whose application to the isolated STG activates the gastric mill rhythm. The CabPK-elicited gastric mill rhythm occurs in the absence of activity in modulatory commissural neuron 1 (MCN1), a projection neuron that also elicits the gastric mill rhythm. When driving the gastric mill rhythm, MCN1 is also a central pattern generator neuron for this rhythm (Coleman and Nusbaum 1994; Coleman et al. 1995). These results therefore indicate that the CPGs underlying these two gastric mill rhythms are distinct.

Some of these data were published in abstract form (Hertzberg et al. 2003).
MATERIALS AND METHODS

Animals. Male Jonah crabs (*Cancer borealis*) were obtained from Commercial Lobster and Seafood, Boston, MA and the Marine Biological Laboratory, Woods Hole, MA. Before experimentation, the crabs were housed in commercial tanks containing recirculating, aerated artificial seawater (10°C). Before dissection, the crabs were cold-anesthetized by packing them in ice for at least 30 minutes. The foregut was then removed and maintained in chilled physiological saline while the STNS was dissected away from the foregut.

Solutions. The STNS was maintained in physiological saline containing (in mM): 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, 10 Trizma base, and 5 maleic acid (pH 7.4–7.6). CabPK-I and CabPK-II (Biotechnology Center, University of Wisconsin, Madison, WI), Pevpyrokinin-2 (PPK-2: ADFAFSRPLamide; Protein Chemistry Laboratory, University of Pennsylvania School of Medicine, Philadelphia, PA) and Leucopyrokinin (LPK: pETSFTPRLamide; Bachem, King of Prussia, PA; Protein Chemistry Laboratory, University of Pennsylvania School of Medicine, Philadelphia, PA) were prepared in physiological saline immediately before use. Picrotoxin (PTX: 10⁻⁵ M; Sigma-Aldrich, Inc., St. Louis, MO) was prepared in physiological saline, with stirring for at least 40 minutes, and superfused to suppress glutamatergic inhibitory synaptic transmission in the STG (Marder and Paupardin-Tritsch 1978; Marder 1987). PTX suppresses the synaptic actions of most gastric mill neurons, including those of the reciprocally inhibitory lateral gastric (LG) neuron and interneuron 1 (Int1) (Marder and Paupardin-
Tritsch 1978). Int1 and the LG neuron are key gastric mill CPG neurons (Coleman et al. 1995; Bartos et al. 1999).

**Antiserum development.** Synthetic PPK-1 (DFAFSPRLamide) was coupled, through the free carboxyl group of its N-terminal aspartate residue, to bovine thyroglobulin using 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO). This procedure increased the likelihood that the resulting antiserum generated against thyroglobulin-bound PPK-1 would be directed against the C-terminal portion of the PK peptide, which is conserved across the different members of this peptide family.

After overnight incubation, the water-soluble isourea, which is released as a byproduct of the conjugation reaction, and the excess reagent were separated from the hapten-carrier complex by dialysis. The complex was then dissolved in distilled water and emulsified with an equal amount of Freunds complete adjuvant and injected subcutaneously into New Zealand white rabbits. A second, third and fourth boost was given, using Freunds incomplete adjuvant, 2, 4 and 6 weeks respectively after the initial immunization.

**Dot immunobinding assay.** The antiserum obtained after the final bleed was characterized using Dot Immunobinding Assay (DIA) according to Salzet et al. (1997). Briefly, 1 µl of each of several synthetic peptides was spotted onto a nitrocellulose membrane (0.45 µm pore size) in a dilution series ranging from 100 pg/dot up to 1 µg/dot. These peptides included PPK-1, PPK-2, Pev-kinin-6 (AFSPWAamide; Torfs et
al., 1999), Cancer borealis tachykinin-related peptide-Ia (CabTRP-Ia: APSGFLGMRamide; Christie et al., 1997; Nieto et al. 1998) and Pev-Sulfakinin-2 (Pev-SK 2: pQFDEY(SO3H)GHMRFamide; Torfs et al. 2002). Membranes were baked for 30 min at 110°C, blocked with skimmed milk in 50 mM Tris buffered saline (TBS) to reduce background staining (1 hr gentle agitation at room temperature) and subsequently incubated overnight with the primary antiserum in a dilution of 1/1000 in TBS. Membranes were washed several times and incubated with horseradish peroxidase-linked goat anti-rabbit IgG at 1/500 dilution for 2 hours at ambient temperature. Immunoreactive spots were visualized by incubation with a 0.3% 3,3’ diamino-benzidine (DAB)/H₂O₂ solution.

As reported in Torfs (2001), DIA analysis of the anti-PPK-1 antiserum readily stained PPK-1 peptide at peptide levels down to 1 ng/dot and stained PPK-2 peptide at levels down to 0.1 μg/dot (lowest level tested). In contrast, this antiserum did not stain dots containing Pev-Kinin-6, CabTRP-Ia or Pev-SK 2 at levels up to 1 μg/dot (Torfs 2001). Tissue sections were also processed using the anti-PPK-1A antiserum, at a dilution of 1/1000, with labelling visualized with the peroxidase anti-peroxidase (PAP) method (VandeSande and Dierickx 1976). As above, DAB was used as the chromogenic agent.

**Tissue Preparation.** The CNS (brain, suboesophageal ganglion, thoracic ganglia and ventral nerve cord) of the adult white shrimp Penaeus vannamei (obtained from the Centro Nacional de Acuicultura e Investigaciones Marinas, Ecuador) was dissected
under physiological saline and transferred to Bouins Hollande's (10%) sublimate fixative. After 18-24 hours of fixation, the CNS was rinsed with distilled water (12 hrs), dehydrated in an ethanol series (70%, 95% and 100% for two times 1 hr each), cleared in Histosol plus and embedded in Paraplast. Alternating sections of 4 μm were made with a LKB Historange microtome using glass knives.

**Whole-mount immunocytochemistry.** Whole-mount immunocytochemistry was performed using standard techniques for the *C. borealis* STNS (Blitz et al. 1999; Christie et al. 2004; Saideman et al., 2006). Briefly, tissue was fixed for 4-24 hr with either 4% paraformaldehyde or 4% EDAC in 0.1 M sodium phosphate (*P*: pH 7.3) or Bouin's fixative (71% saturated picric acid, 24% of 40% formalin, 5% glacial acetic acid). Preparations were then rinsed five times at 1 hour intervals in 0.1 M *P* with 0.3% Triton X-100 (*P*-Triton, pH 7.3). Tissue was subsequently incubated with primary antiserum, diluted to final concentration with *P*-Triton, for 24-48 hours. PK-like immunoreactivity (PK-LI) was examined with the polyclonal anti-PPK antiserum (anti-PPK-1A) at a final dilution of 1:300 – 1:500. The tissue was then rinsed as above with *P*-Triton and incubated for 15-24 hours with goat anti-rabbit secondary antisera conjugated to Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes Inc, Eugene, OR). The secondary antisera were used at a final dilution of 1:300 in *P*-Triton. After incubation with secondary antisera, preparations were rinsed five times at 1 hour intervals with *P*. They were then mounted between a glass slide and coverslip, using a solution of 80% glycerol and 20% 20 mM Na₂CO₃ (pH 9.0). Digital images were taken with a Leica
DMRB microscope and Leica DC 350FX digital camera system (Leica Microsystems, Bannockburn, IL). Images were acquired with Image-Pro Express software (Media Cybernetics, Silver Spring, MD).

Preadsorption control experiments were conducted to confirm the specificity of anti-PK immunolabeling. The polyclonal antibody (1:500) was preincubated for 4 hours at room temperature with PPK (10^{-5} M) or an unrelated neuropeptide, corazonin (10^{-4} M: pQTFQYSRGWTNamide; Bachem, King of Prussia, PA). Corazonin localizes to the POs in *C. borealis* (Li et al. 2003). In some additional preparations, we omitted either the preadsorbing peptide or the primary antiserum but otherwise processed the wholemounts as above for preadsorption controls.

**Tissue extraction.** Supraoesophageal ganglion (brain) tissue and CoGs were pooled from approximately 100 animals (*C. borealis*) and two separate extractions were performed on each pool of ganglia. Tissues were placed in a 0.1 ml or 1.0 ml tissue grinder (Wheaton, Inc., Millville, NJ) with 100 μl acidified methanol (90:9:1 methanol: glacial acetic acid: deionized water, v/v/v) storage buffer. The tissue was homogenized completely, after which the extraction liquid was transferred to a 1.5-ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) and centrifuged at 13,200 x g for 10 minutes in an Eppendorf 5415 D microcentrifuge (Brinkmann Instruments Inc., Westbury, NY). The supernatant was retained and the resulting pellet re-extracted and re-spun. Supernatants were combined and concentrated to near dryness with a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL). Finally,
a minimal amount (100-150 µl) of resuspension solution (deionized water w/0.1% formic acid) was added to the extract. This resuspended extract was vortexed and briefly centrifuged, with the supernatant used for HPLC separation followed by immuno-dot-blot assay and matrix-assisted laser desorption/ionization (MALDI)–Fourier transform mass spectrometry (FTMS) screening or liquid chromatography (LC) tandem mass spectrometry (MS/MS) analysis of the immunopositive HPLC fractions.

**Reverse-Phase HPLC Separations.** HPLC separations were performed using a Rainin Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument Inc., Woburn, MA). The mobile phases used include: (Solution A) deionized water containing 0.1% formic acid, and (Solution B) acetonitrile (HPLC grade, Fisher Scientific) containing 0.1% formic acid. Twenty microliters of extract were injected onto a Macrosphere C18 column (2.1 mm i.d. x 250 mm length, 5 µm particle size; Alltech Assoc. Inc., Deerfield, IL). Two HPLC experiments were performed on aliquots of the extract. The first separation was a 60 minute gradient of 5%-95% Solution B. The second was a shallower gradient across 120 minutes, which allowed a higher resolution separation. To obtain more peptide in each fraction and to facilitate identification, the second gradient was performed on 6 separate injections of brain extract and fractions were pooled for a final volume of 1.2 ml. Fractions were automatically collected every minute using a Rainin Dynamax FC-4 fraction collector.

**Immuno-dot-blot assay.** The HPLC fractions were assayed for immunoreactivity to
anti-PPK antiserum. We used 30 μl from each fraction and added 6 μl of 1 mg/ml bovine serum albumin (BSA, RIA grade, Fluka, Switzerland) in PBS, after which each fraction was concentrated to dryness in a SpeedVac. Each dried fraction was resuspended in 6 μl of deionized water, spotted in 1 μl aliquots onto a nitrocellulose membrane (Schleicher & Schuell Bioscience Inc., Keene, NH) and dried in a 60°C oven for 1 hour. The membrane was then fixed for 16 hours at 37°C with 2% glutaraldehyde (EM grade, Ted Pella Inc., Redding, CA) vapor in a sealed container. A 2% glutaraldehyde solution in PBS was subsequently added to the membrane and incubated for 1 hour to complete the fixation. The blots were washed six times for 15 minutes each in PBS, blocked with blotto (5% non-fat dry milk, 0.1% Triton X-100, 0.001% Thimerosal in PBS) for 30 minutes, and then incubated with PPK antiserum diluted 1:500,000 in blotto for 16 hours at 4°C. A 10% blotto solution was used, three times for 15 minutes, to rinse unbound antibody from the blots. Secondary goat anti-rabbit antiserum coupled to horseradish peroxidase at a dilution of 1:500 in blotto was then incubated with the blot for 1 hour. Again the unbound antibodies were removed by three rinses in 10% blotto for 15 minutes. Bound antibodies were visualized by adding the blot to a solution of 0.006% H_2O_2, 0.03% 3,3′-diaminobenzidine 4-HCl, 0.5% CoCl_2 in PBS for 4-5 minutes. The membrane was washed thoroughly in water and dried at room temperature. Immunoreactive fractions were identified by a reddish-brown staining at the location that the fraction had been added to the membrane (Sithigorngul et al. 1991, 2003).
Direct tissue sample preparation. For direct tissue mass spectrometric analysis, samples were prepared as described by Kutz et al. (2004). Briefly, dissected and desheathed tissue was rinsed in acidified methanol. Tissue was desalted in dilute MALDI matrix consisting of 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) (MP Biomedicals Inc., Irvine, CA) prepared in deionized water. A spot containing 0.3 µl saturated DHB [150 mg/ml in 50:50 v/v water: purge trap-grade methanol (Fisher Scientific)] was placed on one facet of the IonSpec MALDI sample probe. Before the DHB crystallized fully, the tissue was placed carefully on the facet and an additional 0.3-µl drop of saturated DHB was placed on top of the tissue to help affix the tissue to the target. The saturated DHB was allowed to crystallize at room temperature.

MALDI FTMS. MALDI FTMS experiments were performed on an IonSpec Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0-Tesla actively shielded superconducting magnet. The FTMS instrument contained an external ion source utilizing a quadrupole ion guide to transfer the ions to the ion cyclotron resonance (ICR) cell, which was differentially pumped. The sample probe was a 10-faceted stainless steel target. A 337 nm nitrogen laser (Laser Science, Inc., Franklin, MA) was used for ionization/desorption. All mass spectra were collected in positive ion mode using the in-cell accumulation method. The latter method was written using IonSpec version 7.0 software, as described previously (Kutz et al. 2004). The pulse sequence involved the use of seven laser ionization/desorption events, each being optimized for ions at m/z 1000 to be transported down the quadrupole. This method
was used to increase the ion concentration before detection. Five pulse sequences were signal averaged to improve the signal to noise ratio further. The instrument was externally calibrated using Substance P and several matrix ions. Additionally, the mass spectra were internally calibrated to known neuropeptides.

**Capillary LC-ESI-QTOF-MS/MS.** Nanoscale LC QTOF MS/MS was performed using a Waters capillary LC system coupled to a QTOF Micro mass spectrometer (Waters Corp., Milford, MA). Chromatographic separations were performed on a C18 reverse phase capillary column (75 µm internal diameter × 150 mm length, 3 µm particle size; Micro-Tech Scientific Inc.). The mobile phases used were: (Solution A) deionized H$_2$O with 5% acetonitrile and 0.1% formic acid; (Solution B) acetonitrile with 5% deionized H$_2$O and 0.1% formic acid; (Solution C) deionized H$_2$O with 0.1% formic acid. A 1.4 µl aliquot of PK immuno-positive *Cancer borealis* brain HPLC fraction was injected and loaded onto the trap column (PepMap™ C18; 300 µm column internal diameter × 1 mm, 5 µm particle size; LC Packings, Sunnyvale, CA) using mobile phase C at a flow rate of 30 µl/min for 3 min. After this procedure, the stream select module was switched to a position at which the trap column became in-line with the analytical capillary column, and a linear gradient of mobile phases A and B was initiated. A splitter was added between the mobile phase mixer and the stream select module to reduce the flow rate from 12 µl/min to 200 nl/min.

The nanoflow ESI source conditions were set as follows: capillary voltage 3800 V, sample cone voltage 40 V, extraction cone voltage 1 V, source temperature 120°C,
cone gas (N₂) 13 l/hr. For the reference spray, the same settings were used except that the sample cone voltage was set at 10 V and reference scans were performed every 10 sec. A data-dependent acquisition was employed for the MS survey scan and the selection of precursor ions and subsequent MS/MS of the selected parent ions. The MS scan range was from m/z 100-2000 and the MS/MS scan was from m/z 50-2000. The MS/MS de novo sequencing was performed with a combination of manual sequencing and automatic sequencing by PepSeq software (Waters Corp.).

**Electrophysiology.** Electrophysiological experiments were performed using standard techniques for this system (Beenhakker and Nusbaum 2004). The isolated STNS was pinned down in a Sylgard 184 (KR Anderson, Santa Clara, CA)-lined Petri dish (Fig. 1). All preparations were superfused continuously (7-12 ml/min) with *C. borealis* physiological saline (10-12°C). Each extracellular recording was made by pressing a stainless steel wire electrode into the Sylgard alongside the nerve and isolating that area with petroleum jelly (Vaseline: Lab Safety Supply Inc., Janesville, WI). The second electrode was connected to ground by insertion into the Sylgard in the main bath compartment. To facilitate intracellular recordings, the desheathed ganglia were viewed with light transmitted through a dark-field condenser (Nikon, Tokyo, Japan).

Intracellular recordings of STG somata were made using microelectrodes (15-30 MΩ) filled with 4 M K-acetate plus 20 mM KCl or 0.6 M K₂SO₄ plus 10 mM KCl. Intracellular current injection was performed using Axoclamp 2 amplifiers (Molecular Dynamics, Foster City, CA) in single-electrode discontinuous current-clamp (DCC) mode.
rates during DCC were 2-3 kHz.

Individual STNS neurons were identified by their axonal pathways, activity patterns and interactions with other neurons (Beenhakker and Nusbaum 2004). Data were collected directly onto chart recorder (Model MT-95000 or Everest: Astromed Corp., West Warwick, RI), and simultaneously digitized (about 5 KHz) and collected onto a PC computer using data acquisition/analysis tools (Spike2, Cambridge Electronic Design, Cambridge, UK).

To study neuromodulatory inputs in isolation, the CoGs were cut away by bisecting the inferior oesophageal nerves (ions) and superior oesophageal nerves (sons) to remove modulatory input from the CoG projection neurons. The axon of each MCN1, which projects through the ipsilateral ion, was stimulated extracellularly (tonic stimulation: 4-10 Hz) using a Grass Telefactor S88 stimulator, via a stimulus isolation unit (SIU5: Astro-Med/Grass Telefactor, West Warwick, RI). In most experiments, CabPK-I, CabPK-II, PPK or LPK were superfused continuously across the isolated STG at 10⁻⁶ M concentration. These four PK peptides had identical actions so, except where noted, the data resulting from their application were pooled.

Data analysis was facilitated with a custom-written program for Spike2 that determines the activity levels and phase relationships of neurons (freely available at http://www.neurobiologie.de/). Unless otherwise stated, each datum in a data set was derived by determining the average of 10 consecutive impulse bursts. Briefly, burst duration was defined as the duration between the onset of the first and last action potential in an impulse burst. The firing rate of a neuron was defined as the number of
action potentials minus 1, divided by the burst duration. The cycle frequency of the pyloric rhythm was determined by calculating the inverse of the period between the onsets of 2 successive pyloric dilator (PD) neuron bursts. Gastric mill cycle period was determined as the duration between successive impulse bursts in the LG neuron.

Figures were made from Spike2 files incorporated into Adobe Ilustrator (Adobe, San Jose, CA) and Powerpoint graphics programs (Microsoft, Seattle, WA). Statistical analyses (two-tailed Student’s t-test or one-way repeated measures ANOVA) were performed with SigmaStat 3.0 and SigmaPlot 8.0 (SPSS, Chicago, IL). Data are expressed as means ± SD.
RESULTS

Pyrokinin-like Immunolabeling in the STNS and Pericardial Organs

We identified PK-LI in the C. borealis STNS (Fig. 1). Specifically, there was consistently PK-LI in the STG neuropil but not in any STG somata (n=25) (Fig. 1). There was also PK-LI in what appeared to be 4 or 5 individual axons that entered the STG from the stomatogastric nerve (stn), but there was no PK-LI in any peripheral nerves projecting from the STG (Fig. 1). The PK-LI within the STG neuropil thus appeared to originate from projection neurons in neighboring ganglia. In C. borealis, nearly all the projection neurons that innervate the STG originate in the CoGs (Coleman et al. 1992).

Consistent with the possibility that PK-LI-containing projection neurons in the CoGs innervated the STG, there was also PK-LI in approximately 2 axons within each son and in a subset of neuronal somata in each CoG (n=21) (Fig. 1). As many as 20 PK-LI somata were counted in individual CoGs (mean: 8 ± 4 somata, n=20), and there was consistently PK-LI in the CoG neuropil. No PK-LI was observed in either the ions or the OG (n=27). Finally, there was also PK-LI in the neuropil region at the anterior end of the stn, near its junction with the sons and oesophageal nerve (n=20) (Fig. 1).

PK-LI was also found in the POs, a major neurohemal site that releases neuromodulators into the pericardial cavity (Skiebe 2001; Fu et al. 2005). The PK-LI labeling was sparsely distributed in axon fibers and varicosities throughout the POs (not shown). This immunolabeling occurred consistently across preparations (n=12/15).

To increase the likelihood that the PK-LI represented immunolabeling of antibody
binding to PK peptides, we performed preadsorption controls. Specifically, pre-incubating the PPK antiserum with PPK \((10^{-5} \text{ M})\) abolished PK-LI in STNS wholemount preparations \((n=5)\). Omitting the primary antiserum also abolished labeling \((n=7)\). In contrast, wholemounts in which the PPK antiserum was pre-incubated with the neuropeptide corazonin \((10^{-4} \text{ M})\) exhibited PK-LI that was comparable to standard PK-LI without any pre-incubation, such as that shown in Figure 1 \((n=5)\). Although we did not perform preadsorption controls with every neuropeptide that has been localized to this system, it is noteworthy that the PK-LI distribution in the STNS was distinct from that of previously identified neuropeptides. For example, the absence of PK-LI in the OG distinguished it from immunolabeling for proctolin, RPCH, allatostatins, orcokinins and FLRFamides \((\text{Marder et al. 1987; Nusbaum and Marder 1988, 1989a; Skiebe and Schneider 1994; Li et al. 2002})\), while the lack of PK-LI in the ions separated it from CabTRP Ia \((\text{Blitz et al. 1995})\). Similarly, like corazonin, immunolabeling for the peptides CCAP and PevPK is evident in the POs but not in the STNS \((\text{Christie et al., 1995; Saideman et al. 2006})\).

**Immonoassay screening for pyrokinin-like peptides in *C. borealis* tissue extract**

PK peptides share the conserved C-terminal pentapeptide sequence FXPRLamide, where X is a variable amino acid \((\text{Torfs et al. 2001})\). The PK antiserum that we used binds with this conserved sequence. Using this antiserum, we performed immuno-dot blots on tissue extracts and HPLC fractions from these extracts. To assess the specificity of labeling in these experiments, we determined the relative ability of
several PK peptides, as well as the unrelated peptide Pevkinin-2 (DFSAWAamide) (Torfs et al. 1999; Saideman et al. 2006), to generate an immunopositive result with this method. The dot blots containing Pevkinin-2 (1.4 X 10^{-6} M) exhibited no immunoreactivity to the PK antiserum (n=4). In contrast, LPK (1.1 X 10^{-6} M; n=3), PPK-2 (9.8 X 10^{-7} M; n=17), CabPK-I (9.5 X 10^{-7} M; n=1) and CabPK-II (9.7 X 10^{-7} M; n=1) each showed immunostaining. Immunostaining was considerably weaker with LPK than with the other three peptides.

We performed immunoaffinity-based dot blots on tissue extracts from the *C. borealis* brain (n=6), thoracic ganglion (n=1), CoG (n=2), STG (n=1) and PO (n=1). We obtained positive results from every sample of tissue extract tested, with immunostaining appearing strongest for the brain extracts (data not shown). We also used this dot blot screening assay to identify several HPLC fractions containing PK peptide candidates for further characterization. Specifically, we screened for and identified immunopositive HPLC fractions from *C. borealis* extracts of brain (n=6) and CoG tissue (n=1). MALDI FTMS profiling was then used to reveal the peptide content of the immunoreactive fractions (brain, Fig. 2A; CoG, Fig. 2B). The immunoreactive species eluted at the same retention time for both brain and CoG extracts, suggesting that the same peptides occurred in both locations. However, due to the complexity of the tissue extracts, for both tissues multiple peptide peaks co-eluted and thus were detected in individual HPLC fractions. By determining the masses in common between the two HPLC fractions, a list of candidate masses was composed for further sequencing.
De novo sequencing of CabPK-I and CabPK-II by QTOF MS/MS

Given the complexity of the peptide profiles observed in the HPLC fractions, capillary Liquid Chromatography (LC) coupled with electrospray ionization (ESI) quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) was performed on the immunoreactive HPLC fractions to further separate peptide mixtures and obtain peptide sequence information (Fu and Li 2005; Fu et al. 2005). Using this approach, two novel CabPKs were de novo sequenced. These CabPK peptides had molecular masses of 1050.69 and 1036.65 Da. The MS/MS fragmentation spectrum of the first peptide (molecular mass 1050.69) from brain extract is shown in Figure 3A. Upon automatic sequencing by PepSeq software, a sequence of TNFAFSPRLamide was determined. Although the b5-b8 ions were absent, a complete series of intense y ions enabled the assignment of the peptide sequence. In the low mass region, immonium ions m/z 70.069, 86.09, 120.101 indicated the presence of proline, leucine/isoleucine and phenylalanine.

A peptide standard with the proposed sequence was synthesized and subjected to LC ESI QTOF tandem MS analysis. The MS/MS fragmentation spectrum of the synthetic peptide is shown below the comparable spectrum from brain extract in Figure 3A. The nearly identical fragmentation patterns between TNFAFSPRLamide from C. borealis brain HPLC fraction and the synthetic peptide further confirmed the sequence assignment of the new peptide. Because the newly sequenced peptide shares the conserved C-terminal motif that defines the pyrokinin peptide family (FXPRLamide), we
designated this peptide as CabPK-I.

The MS/MS fragmentation spectrum of the second peptide (molecular mass of 1036.65 Da) is shown in Figure 3B. We noticed that, in this spectrum, a series of fragmentation ions such as \( m/z \) 384.3, 471.3, 618.4, 689.4 and 836.5 were the same as those observed in the MS/MS spectrum of CabPK-I, and in a comparable pattern. In the MS/MS spectrum of CabPK-I, these fragment ions were assigned as \( y_3 \) (384.3), \( y_4 \) (471.3), \( y_5 \) (618.4), \( y_6 \) (689.4) and \( y_7 \) (836.5), respectively. This observation suggested that the C-terminal sequence of this 1036.65 Da peptide shared the identical sequence of FAFSPRLamide with CabPK-I. Although the \( b_1 \) and \( b_2 \) ions were weak and the \( y_9 \) ion was missing, due to glycine in the sequence which generally produces less abundant fragment ions upon cleavage (Papayannopoulos 1995), several internal fragment ions including 115.091 (GG) enabled us to assign the N-terminal sequence as SGG. The second novel peptide, with the sequence SGGFAFSPRLamide, was thus designated as CabPK-II.

**Identification of CabPK-I and CabPK-II in the *C. borealis* STNS by MALDI FTMS**

With the sequences of CabPK-I and CabPK-II determined, direct tissue MALDI FTMS was used to map the distribution of these two novel crustacean pyrokinin peptides in the *C. borealis* STNS. MALDI FTMS spectra revealed peaks that were consistent with the presence of both peptides in the CoG (Fig. 4A) and STG (Fig. 4B). As expected, numerous peptides were detected in both tissues. Even though there was only weak immunostaining observed in the dot blot resulting from the extraction of 100
animals worth of STG, both CabPK-I ([M+H]+=1051.570) and CabPK-II ([M+H]+=1037.558) were detected in the STG mass profiling by MALDI FTMS. Using internal calibration, both peptides were determined with mass measurement accuracy (MMA) of 1.59 ppm and 5.17 ppm (n=3), respectively. (Note: In an effort to gain sensitivity to observe low abundance peptides, the ion cyclotron resonance cell can be overloaded with higher abundance peptides. This approach degrades slightly the MMA in complex spectra with a large concentration dynamic range.) Similarly, CabPKs were observed in the CoG with MMA of 0.124 ppm for CabPK-I and 2.30 ppm for CabPK-II. Finally, we also identified CabPK-II in the LC fraction of *C. borealis* PO extract. SORI-CID tandem mass spectrometric analysis was used to confirm the CabPK-II amino acid sequence from the HPLC fraction of the PO extract (data not shown). CabPK-I was not detected in the POs, suggesting that only CabPK-II was expressed in the POs. However, the lack of CabPK-I detection instead might have resulted from analyte suppression and/or a lower level of this isoform in the PO tissue.

**Pyrokinin actions on the pyloric rhythm are limited to its excitation of the gastro-pyloric neurons IC and VD**

In most isolated STGs, the influence of PK (10^{-6} M) superfusion on the pyloric rhythm was limited to an excitation of the inferior cardiac (IC) and ventricular dilator (VD) neurons (Fig. 5). These are the two pyloric neurons that also participate in the gastric mill rhythm (Weimann et al. 1991; Blitz and Nusbaum 1997). As is evident from Figure 6 and subsequent results (see below), all tested PKs, including CabPK-I,
CabPK-II, PPK and LPK, had comparable actions when each was superfused at 10^{-6} M. For example, none of these PKs elicited a consistent change in pyloric cycle frequency (Saline: 0.62 ± 0.22 Hz; PPK/LPK: 0.66 ± 0.25 Hz; CabPKI/II: 0.52 ± 0.13 Hz; n=10-18, ANOVA, p>0.05).

Because some neuromodulators influence the pyloric cycle frequency only when the pyloric rhythm cycles relatively slowly (<1.0 Hz) (Hooper and Marder 1987; Nusbaum and Marder 1989b), we also determined if the combined results reported above were skewed by the fact that relatively fast control rhythms were affected less than weakly cycling rhythms. We therefore plotted the pyloric cycle frequency during PK application as a function of its cycle frequency immediately before PK application for each preparation. Doing so revealed that none of the PKs altered the pyloric cycle frequency, regardless of the control frequency (Fig. 6A). Similarly, PK application did not alter the mean number of LP neuron spikes per burst across preparations (Saline: 3.6 ± 2.1; PPK/LPK: 5.1 ± 2.9; CabPKI/II: 2.8 ± 1.3; n=10-15, ANOVA, p>0.05), regardless of whether the pre-application level of LP activity was relatively weak or strong (Fig. 6B).

In contrast to the lack of PK influence on pyloric cycle frequency and LP neuron activity, PK application consistently increased IC and VD neuron activity. Specifically, the number of spikes/burst in the IC neuron increased in the presence of all applied PKs (Saline: 1.2 ± 1.5; PPK/LPK: 4.3 ± 1.7; CabPKI/II: 5.5 ± 2.4; n=10-14, ANOVA, p<0.05). The same was the case for the VD neuron (Saline: 0.05 ± 0.2; PPK/LPK: 4.7 ± 1.9; CabPKI/II: 3.7 ± 2.2; n=9-14, ANOVA, p<0.05). In the isolated STG, the IC neuron was
either silent or weakly active during saline superfusion while the VD neuron was generally silent (e.g., Fig. 5). PK excitation of IC appeared to be equally effective whether it was silent or active before PK application (Fig. 6C). Similarly, PK superfusion consistently activated the VD neuron (Fig. 6D).

**Pyrokinins excite gastric mill neurons**

Superfusing any of the examined PKs in normal *C. borealis* saline routinely excited many gastric mill neurons, including the gastric mill protractor phase neurons LG (n=252), IC (n=205; Figs. 5, 6) and MG (n=12) and the retractor phase neurons Int1 (n=271), VD (n=218; Figs. 5, 6) and DG (n=264) (Saideman 2006). This PK-mediated excitation usually coincided with its activation of the gastric mill rhythm (see below). The two remaining types of gastric mill neurons, including the gastric mill (GM: n=17) protractor neuron and the anterior median (AM: n=5) retractor neuron, were never activated by PK peptide application when they were at their normal resting potential (~-60 mV), which is subthreshold for action potential generation. However, these two neurons each exhibited increased activity during PK superfusion if they were sufficiently depolarized to be suprathreshold when PK was applied (GM: n=17; AM: n=6) (data not shown). Thus, the activity of all gastric mill neurons was enhanced by PK peptides superfused in normal saline.

In addition to exciting the gastric mill neurons, PK superfusion also upregulated the intrinsic properties of some of these neurons. For example, the PKs unmasked the ability of the DG neuron (n=11) to generate a plateau potential (Fig. 7A). During PK
superfusion, a brief depolarizing current injection into DG evoked persisting spike activity that outlasted the current pulse (Fig. 7A). As is typical for plateau potentials (Russell and Hartline 1978; Kiehn 1991), injection of a brief but sufficiently strong hyperpolarizing current pulse prematurely suppressed these plateau responses in the DG neuron (Fig. 7). The duration of the DG plateaus tended to be longer in duration than its gastric mill-timed bursts. PK superfusion also elicited plateau potentials in the LG neuron (data not shown; see Saideman 2006).

PK superfusion in normal saline also unmasked intrinsic oscillatory properties in the DG neuron. The likelihood that these PK-elicited oscillations in DG were intrinsic to this neuron were supported by the fact that the injection of constant amplitude depolarizing current into this neuron during PK application increased the frequency of its rhythmic bursting (n=3) (Fig. 7B). The DG neuron response to depolarizing current injection also persisted during times when LG neuron activity was suppressed by hyperpolarizing current injection, which also suppressed the gastric mill rhythm (n=2). Further increases in the level of DG neuron depolarization reversibly switched its rhythmic bursting pattern to tonic activity (Fig. 7B). The conclusion that the PKs enabled intrinsic oscillatory activity in the DG neuron was further supported by the observation that PK (10^{-6} M) superfusion in PTX (10^{-5} M) saline either elicited (Fig. 8) or enhanced independently-occurring rhythmic bursting in this neuron. Comparable depolarizing current injections during normal saline superfusion did not elicit rhythmic bursting activity in this neuron (n=3).

To determine which gastric mill neurons were likely to be direct targets of PK
peptides, we applied PKs after suppressing all glutamatergic inhibition in the STG by superfusion of PTX ($10^{-5}$ M). Most STG neurons are glutamatergic and PTX suppresses their intra-circuit synapses (Marder and Paupardin-Tritsch 1978; Marder 1987). In fact, the non-glutamatergic STG neurons (the cholinergic VD, LPG, and 2 PD neurons) are largely follower motor neurons in *C. borealis* with modest (PD) or no (VD, LPG) evident transmitter-mediated synaptic actions within the STG, during superfusion with either normal saline or PTX saline (S.R. Saideman and M.P. Nusbaum, unpublished). Thus, when PTX was applied to the isolated STG, there were few active transmitter-mediated synapses remaining between the STG neurons, although electrical coupling persisted. Consequently, during PTX superfusion only the electrically coupled pyloric pacemaker ensemble (AB, PD, LPG) retained their pyloric-timed activity. For example, the gastric mill neurons that normally exhibited pyloric-timed activity (e.g., Int1, IC, VD) during normal saline superfusion lost that activity pattern during PTX superfusion and instead exhibited either tonic or intermittent activity (Fig. 8A). This was also the case for the MG neuron (n=4; not shown). Additionally, for some neurons such as IC, their level of spontaneous activity increased in PTX (Fig. 8A). As is evident in Figure 8, during PTX superfusion the gastric mill neuron activity patterns were largely unrelated to one another.

Many gastric mill neurons remained responsive to PK ($10^{-6}$ M) application during PTX superfusion, albeit with altered activity patterns relative to those in normal saline (Fig. 8). The VD neuron firing frequency was increased by PK application in PTX saline (PTX: $1.3 \pm 2.4$ Hz; PK/PTX: $3.4 \pm 2.5$ Hz, n=4, p<0.05). Under this condition, Int1 also
responded, reversibly, to PK application with a membrane potential depolarization and increased firing rate (PTX: 6.8 ± 3.1 Hz; PK/PTX: 9.9 ± 2.4, n=3, p<0.05). For example, in Figure 8A, the Int1 firing frequency increased from 6.7 ± 0.7 Hz in PTX alone to 9.5 ± 0.4 Hz when PK and PTX were co-applied. The Int1 firing frequency then returned to 4.0 ± 1.2 Hz when the preparation was returned to PTX alone. In contrast, IC neuron activity was not enhanced by PK application in PTX saline (PTX: 7.1 ± 2.3 Hz, PK/PTX: 6.3 ± 0.7 Hz, n=5, p>0.05).

Each of the other gastric mill neurons also remained responsive to PK (10^{-6} M) application during PTX superfusion, albeit with neuron type-specific responses. For example, while the protractor LG neuron tended to be subthreshold during PTX superfusion, the addition of a PK peptide consistently, and reversibly, elicited gastric mill rhythm-like bursting from this neuron (n=11) (Fig. 8A,B). This result was surprising, because there was no indication of PK-elicited intrinsic oscillatory activity in the LG neuron in normal saline (Saideman 2006). This rhythmic bursting of the LG neuron could not have been the result of persisting rhythmic synaptic inhibition from the retractor phase neuron Int1 because, although LG and Int1 are reciprocally inhibitory and normally burst in alternation during the gastric mill rhythm, both neurons make PTX-sensitive synapses and therefore fired independently during PTX superfusion (Fig. 8A).

It is also noteworthy that, despite the presence of electrical coupling between the protractor phase neurons (LG, GM, MG, IC), only the LG neuron responded to PK application in PTX with a rhythmic bursting pattern. At these times, the IC (Fig. 8A) and MG neurons fired tonically whereas the GM neurons remained silent (not shown).
Rhythmic bursting was also consistently, and reversibly, elicited in the DG neuron by PK application in the presence of PTX (n=12) (Fig. 8B). Note that at these times the DG and LG neuron bursts were independent of each other (Fig. 8B). The cycle period of the PK-elicited rhythmic bursting in the DG neuron was comparable during normal saline and PTX superfusion (PK/Normal Saline: 47.2 ± 8.6 sec; PK/PTX: 31.8 ± 16.6 sec; n=4, p>0.05).

Lastly, as was also the case during normal saline superfusion, the gastric mill (GM) and anterior median (AM) neurons were silent during PTX superfusion. Under these conditions PK application elicited no response from either neuron (GM: n=15; AM: n=3). We did not test whether these two neurons exhibited the same voltage-dependent excitatory response to PK application in PTX that occurred when this peptide was applied in normal saline. Thus, PK application increased the activity in at least 5 of the 8 types of gastric mill neurons, even when most intra-circuit synapses were suppressed.

**Pyrokinins activate the gastric mill rhythm**

PK peptide superfusion in normal saline effectively activated rhythmic neuronal activity from the gastric mill circuit. Specifically, superfusion of either CabPK-I (n=14), CabPK-II (n=13), PPK (n=31) or LPK (n=136) (10^{-6} M) to the isolated STG routinely and reversibly activated alternating bursting from the gastric mill CPG neurons LG and Int1 (Fig. 9). The cycle period for this rhythmic activity (16.8 ± 9.2 sec, n=50) was similar to that for gastric mill rhythms elicited by other pathways (Bartos et al. 1999; Beenhakker
and Nusbaum 2004; Wood et al. 2004). This gastric mill rhythm activity was consistently accompanied by appropriately coordinated bursting of the MG and IC protractor phase neurons and VD retractor phase neuron (n=191) (Figs. 9, 10). Neither the GM nor AM neurons participated in this PK-elicited rhythm (n=194). The retractor neuron DG was also consistently activated to generate rhythmic action potential bursts (n=168). However, the rhythmic DG bursts were often not time-locked with those of the other gastric mill neurons (n=157/168) (Fig. 9; see also Fig. 13). Although in Figure 9 there was a regular pattern in which there was a single DG burst for every two LG bursts, this was a rare occurrence (n=4/168). In some preparations (n=11/168), however, PK (10^{-6} M) application elicited a fully coordinated gastric mill rhythm. At these times, the DG neuron bursts were appropriately time-locked with those of the other gastric mill neurons in every gastric mill cycle (Fig. 11). The cycle period exhibited by these fully coordinated rhythms (16.2 ± 4.5 sec; n=9) was comparable to the cycle period occurring when the DG bursts were not coordinated with the other gastric mill neurons (p>0.05 ANOVA).

We also assayed the threshold concentration at which PK superfusion excited the gastric mill neurons and elicited rhythmic gastric mill activity. For these experiments, we exclusively used the native CabPK peptides. In general, the threshold CabPK concentration that either activated or increased the ongoing activity of the individual gastric mill neurons coincided with its ability to elicit rhythmic bursting from these neurons. Thus, we performed dose-response experiments in which we assessed the ability of the CabPKs to elicit rhythmic bursting from the LG and DG neurons. In
these experiments, LG bursting occurred in alternation with that of Int1, as typically occurs when this reciprocally inhibitory pair are co-activated (Coleman et al. 1995; Bartos et al. 1999). In contrast, the DG bursting was generally not time-locked with that of other gastric mill neurons on a cycle-by-cycle basis and appeared to burst independently. As shown in Figure 12, the effective threshold concentration for these CabPK actions was greater than $10^{-8}$ M. Moreover, the cycle period of both the LG and DG bursting was the same at $10^{-7}$ M and $10^{-6}$ M CabPK (Fig. 12). The same was true for the number of spikes per burst in the LG neuron but not in the DG neuron, where the number of spikes per burst was significantly higher at $10^{-6}$ M CabPK (Fig. 12).

**Pyrokinin excites the MCN1 axon proximal to the STG**

In some experiments (n=22/194), PK superfusion ($10^{-6}$ M) activated the projection neuron MCN1 (Fig. 13). In the subset of experiments where we monitored simultaneously the activity of each MCN1 (n=13/22), PK superfusion activated both of them in only 3 preparations. MCN1 was never activated by lower PK concentrations (n=9). This activation of MCN1 occurred despite the fact that the CoGs had been removed, thereby eliminating the MCN1 somata, their CoG arborizations and their normal spike initiation zones. Despite the absence of the CoGs, each MCN1 was readily identified in extracellular recordings (ion; Fig. 13) (Coleman and Nusbaum 1994; Bartos and Nusbaum 1997). In some experiments, MCN1 activity was further identified by the presence of unitary electrical EPSPs (eEPSPs) in the LG neuron that were time-locked with the action potentials recorded in the ion (see below) (Coleman et al. 1995).
In preparations where PK peptide activated MCN1, the latency to the start of its activity always lagged considerably relative to PK excitation of the gastric mill neurons. In general, the gastric mill neurons were excited within a few minutes (3.6 ± 0.9 min, n=22) after the start of PK superfusion, with a considerable fraction of this duration resulting from the time for the peptide to clear the superfusion line, enter the bath and reach an effective bath concentration. In contrast, PK activation of MCN1 exhibited a latency of 8.1 ± 4.6 min (n=22). Moreover, whereas PK excitation of the gastric mill neurons persisted well into the post-PK washout, PK activation of MCN1 was transient and usually terminated before the end of PK superfusion.

When activated by PK superfusion, MCN1 fired at a relatively low frequency (range: 3-15 Hz). Nonetheless, this firing frequency was sufficient to influence the gastric mill rhythm (Fig. 13). In preparations where PK application resulted in DG neuron bursting separately from the other gastric mill neurons, there were two different outcomes of subsequent PK activation of MCN1. In some experiments (n=15/22), the DG neuron continued to burst independently but its activity, as well as that of the other rhythmically active gastric mill neurons, was enhanced (Fig. 13A). In the other 7 preparations, subsequent activation of MCN1 switched the gastric mill pattern to one where the DG bursts became time-locked to the other gastric mill neurons (n=7/22) (Fig. 13B). This distinction did not correlate with the MCN1 firing frequency elicited by PK.

Interestingly, PK activation of MCN1 often occurred in one of the nerves anterior to the STG. As indicated above, this activation did not occur at the usual site of MCN1 excitation because the CoGs had been removed. Three lines of evidence supported the
hypothesis that pyrokinin activation of MCN1 did not occur in the STG. First, when MCN1 action potentials are initiated in the STG and propagate antidromically, they are rhythmically suppressed during each gastric mill protraction phase (Nusbaum et al. 1992; Coleman and Nusbaum 1994). This results from the fact that the LG neuron presynaptically inhibits the MCN1 terminals in the STG (Coleman and Nusbaum 1994). In contrast, when MCN1 action potentials initiate in the CoG, they propagate past the ion recording site before they enter the STG and are affected by the inhibitory synapse from LG (Coleman and Nusbaum, 1994). In most of our experiments where PK activated MCN1 along with rhythmic LG neuron bursting, MCN1 activity in the ion was tonic instead of being suppressed during each LG burst (n=11/15) (Fig. 13A,B). In only a few preparations (n=4/15) was MCN1 activity rhythmically suppressed during each LG burst, indicating that in these instances the MCN1 action potentials had been initiated within the STG (Fig. 13C).

A second indication that PK peptide application activated MCN1 anterior to the STG came from preparations MCN1 activity in the ion was tonic during rhythmic LG bursting. Specifically, at these times there was a measurable delay (26 ± 3 msec, n=4) from each PK-activated MCN1 action potential in the ion to the onset of the resulting eEPSP in the LG neuron (Fig. 13D). This latency was comparable to that previously measured when MCN1 was stimulated extracellularly in the ion (Coleman et al. 1995). In contrast, in preparations where the evidence supported PK activation of MCN1 spikes in the STG, each eEPSP in the LG neuron remained time-locked to a MCN1 spike in the ion but preceded that spike by 24 ± 5 msec (n=4) (Fig. 13D). Lastly, in one experiment
during PK activation of MCN1, we transected the ion on the stn side of the extracellular ion recording site. Because this ion had been transected between the ion recording site and the CoG at the start of the experiment, this second transection completely separated this region of ion from the rest of the STNS. Nonetheless, the MCN1 axon activity in this isolated ion was reversibly activated to fire tonically by PK superfusion.
DISCUSSION

By combining immuno-dot blot assays with mass spectrometry, we have identified the amino acid sequences of two PK peptides, termed CabPK-I (TNFAFSPRLamide) and CabPK-II (SGGFAFSPRLamide), in the brain and STNS of C. borealis. CabPK-II was also identified in the POs. In parallel, we characterized the influence of several PKs, including the CabPKs, on the pyloric and gastric mill motor circuits in the isolated C. borealis STG.

The combined MS results and PK-LI support the presence of CabPKs in the neuropil of both the STG and CoGs and in a subset of CoG neuronal somata. The lack of PK-LI in any STG somata suggested that the CabPK present in the STG occurs in the axon terminals of two or three bilaterally symmetric CabPK-containing CoG projection neurons. The occurrence of CabPK in the POs suggests that this peptide gains access to the STG circuits as a circulating hormone as well as via local release from modulatory projection neurons. There are a number of previously identified neuroactive peptides that share these access routes to the STG with the CabPKs (Marder et al. 2005; Marder and Bucher 2006).

The immuno-dot blot assay enabled us to screen HPLC fractions of complex tissue extracts, providing a more targeted discovery of endogenous CabPKs. The combination of immunoreactivity profiling with MALDI FTMS screening produced a short list of putative PK peptide candidates for further sequencing analysis. Despite the high chemical complexity of the samples, nanoscale LC ESI-QTOF MS/MS allowed us to fragment each individual peptide while eluting from nanoLC columns, leading to the
identification of each CabPK.

Pyrokinin peptide family members share an extended carboxy terminal sequence of FXPRLamide, with X representing a variable amino acid (Altstein 2004). The CabPKs are particularly closely related to PPK 1 (DFAFSPRLamide), sharing sequence identity for the 7 carboxy terminal amino acids (Torfs et al. 2001). The carboxy terminal appears to be the biologically active region of the PK peptides (Altstein 2004). This hypothesis is supported by our finding that the shrimp (PPK), cockroach (LPK) and crab (CabPKs) PKs had equivalent physiological actions in the C. borealis STG. These four peptides share the carboxy terminal amino acid sequence FXPRLamide but differ considerably at the amino terminal.

PKs localize to the peripheral and central nervous system of many insects and one previous crustacean, the white shrimp Penaeus vannamei (Torfs et al. 2001; Altstein 2004). PKs have many influences outside the CNS, including myotrop activity of the cockroach hindgut, contraction of the locust oviduct, melanization in moth larvae, egg diapause in silkworms, and stimulating sex pheromone biosynthesis in the moth (Nachman et al. 1986; Schoofs et al. 1991; Imai et al. 1991; Altstein et al. 1999; Altstein 2004). However, no previous studies have described the actions of any PK family member in the CNS of any species.

The PK actions in the STG were unusual in some respects relative to the actions of previously studied STG modulators. Nearly all previously studied peptide and small molecule modulators modify the pyloric rhythm, with each modulator having a unique influence on that rhythm (Marder and Thirumalai 2002; Nusbaum and Beenakker
In contrast, CabPK only influenced the two pyloric neurons that are shared with the gastric mill rhythm, and it excited all gastric mill neurons. Further, no previously studied modulator in *C. borealis* has activated the gastric mill rhythm in the isolated STG. The continued response of many gastric mill neurons during PK application in the presence of PTX suggested that these neurons were direct targets of CabPKs. However, it remains possible that these responses instead resulted from PK depolarization of the STG terminals of CoG projection neurons whose synaptic actions are not PTX-sensitive. This is because, although there were no CoGs present in these experiments, the STG terminals of these projection neurons can still be activated (Nusbaum et al. 1992).

Another unusual aspect of the PK actions was the relatively high concentration (>10^-8 M) for its threshold of action. Many previously characterized neuropeptides in the STG have thresholds of action that are several orders of magnitude lower (Skiebe 2001). The threshold concentration for CabPK action is not unreasonable for a neuronally released peptide with actions close to its release sites, such as within the STG neuropil. However, it is a surprisingly high concentration for a circulating hormone. It remains possible that our assay for CabPK actions on STG neurons was not sufficiently sensitive, resulting in only the apparent threshold of CabPK action. For example, when applied at lower concentrations, the PKs might not activate sufficient current to enable its target neurons to reach spike threshold, as was evidently the case for the GM and AM neurons. It is also possible that the determined threshold of PK action in the STG is accurate and that it results from the CabPKs being effectively
degraded and thereby inactivated by extracellular peptidase activity (Coleman et al. 1994; Wood and Nusbaum 2002; Nusbaum 2002). If the determined threshold of PK action in the STG is indeed accurate, then this would suggest that hormonally-released CabPK has no influence on the STG. Circulating levels of CabPK might nonetheless influence the output of the gastric mill and/or pyloric rhythms, at the level of their neuromuscular systems. The threshold of action for CabPK may be lower for such peripheral sites of action, and many of the same neuromodulators that influence the STG circuits also modulate their neuromuscular systems (Jorge-Rivera and Marder 1996; Jorge-Rivera et al. 1998; Messinger et al. 2005; Marder et al. 2005).

Many previous studies have established that bath application of a neuromodulator is a useful first step towards understanding its role in regulating neuronal circuit activity (Harris-Warrick et al. 1998; Marder and Thirumalai 2002; Marder et al. 2005). However, many neurons use co-transmitters (Dugue et al. 2005; Seal and Edwards 2006), including the projection neurons that innervate the STG (Blitz et al. 1999; Nusbaum et al. 2001; Christie et al. 2004). Thus, the actions of neuronally released CabPK are likely to occur in parallel with one or more co-transmitters. If CabPK is co-released with other transmitters, it may have distinct actions on the STG circuits relative to those resulting from its bath application (Nusbaum et al. 2001; Wood and Nusbaum 2002).

It remains to be determined why the DG neuron activity was not coordinated with the gastric mill rhythm in many experiments. During the MCN1-elicited gastric mill rhythm, DG can also burst independently but is regularly incorporated into the gastric
mill rhythm by having its excitatory drive from MCN1 removed during each LG burst. This results from LG-mediated presynaptic inhibition of MCN1 in the STG (Coleman and Nusbaum 1994). Because MCN1 did not participate in the PK-elicited gastric mill rhythm, a distinct and yet to be determined synaptic mechanism must be responsible for linking the DG neuron to the gastric mill rhythm in the subset of experiments where that occurred. This linkage might be strengthened when CabPK is neuronally-released, due to the actions of a co-released transmitter and/or to spatially non-uniform regulation of CabPK concentration by extracellular peptidase activity (Wood and Nusbaum 2002; Nusbaum 2002).

PK application also activated the projection neuron MCN1 in some preparations, despite the fact that its ganglion of origin (CoG) was not present in these experiments. Surprisingly, PK activation of MCN1 most commonly occurred anterior to the STG, within one of the nerves by which MCN1 projects to the STG. This site of action appeared to be within the ion. Extra-ganglionic receptors for neuromodulators, on axonal membrane, have been documented previously in the STNS (Meyrand et al. 1992; Bucher et al. 2003; Goillard et al. 2004). MCN1 itself elicits the gastric mill rhythm (Coleman and Nusbaum 1994), and the PK-elicited gastric mill rhythm was consistently strengthened when PK also activated MCN1. However, it was also clear that PK application effectively elicited the gastric mill rhythm independently of MCN1.

All previously characterized gastric mill rhythms involve the participation of MCN1 (Coleman and Nusbaum 1994; Beenhakker and Nusbaum 2004; Blitz et al. 2004; Christie et al. 2004; Wood et al. 2004). As mentioned above, a pivotal aspect of these
gastric mill rhythms is the LG neuron regulation of MCN1 transmitter release in the STG (Coleman and Nusbaum 1994; Coleman et al. 1995). Although we have yet to characterize the cellular and synaptic mechanisms underlying the CabPK-elicited gastric mill rhythm, it is clear that it does not require the participation of MCN1. Therefore, the core CPG circuit underlying the CabPK-elicited gastric mill rhythm is distinct from all previous versions of this rhythm. Although it is well-established that neuromodulation enables individual CPGs to elicit multiple neuronal activity patterns, the core CPG underlying these different activity patterns is generally preserved (Marder and Calabrese 1996; Stein et al. 1997; Marder and Bucher 2001; Marder et al. 2005). Recent studies of the respiratory CPG in the mammalian brainstem, however, support the hypothesis that the core CPG neurons can be distinct during different forms of respiration (Ramirez et al. 2004; Ramirez and Viemari 2005). Future studies will resolve the extent to which these gastric mill rhythms share underlying CPG components and cellular mechanisms for motor pattern generation.
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Wood DE, Manor Y, Nadim F, and Nusbaum MP. Intercircuit control via rhythmic


FIGURE LEGENDS

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Figure 2. MALDI FTMS spectra of pyrokinin-like immunoreactive HPLC fractions (n=6). (A) Cancer borealis brain fraction number 20, derived from HPLC separation of the extract from 100 supraoesophageal ganglia (brains), showing the presence of both CabPK-I (pentagon) and CabPK-II (star). (B) Cancer borealis CoG fraction number 20, from HPLC separation of the extract from 100 CoGs, showing the presence of both CabPK-I (pentagon) and CabPK-II (star). Spectra were calibrated to FDAFTTGFHGSH
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Figure 3. MS/MS fragmentation spectra of CabPK-I and CabPK-II (n=3). (A) MS/MS fragmentation spectra of CabPK-I, TNFAFSPRLamide (526.352+), eluted from LC MS/MS analysis of a (Top) synthetic CabPK-I peptide standard and (Bottom) PK immunopositive C. borealis brain HPLC fraction. Note the close match between the fragmentation patterns of the peptide isolated from the C. borealis brain HPLC fraction and the synthetic peptide standard, confirming the peptide sequence assignment. Major sequence-specific fragment ions (immonium ions P, L, F, b- and y-type ions) were identical in both spectra. (B) MS/MS fragmentation spectrum of CabPK-II, SGGFAFSPRLamide (519.332+), eluted from LC MS/MS analysis of the PK immunopositive C. borealis brain HPLC fraction.

Figure 4. Direct tissue MALDI FTMS spectra of a single C. borealis (A) CoG and (B) STG. In both spectra, note the presence of both CabPK-I (pentagon) and CabPK-II (star). Spectra were calibrated to APQRNFLRFamide with a mass of 1147.64832. Repetitive acquisitions (n=3) were performed for each tissue sample.

Figure 5. Influence of CabPK-I on the pyloric rhythm in the isolated STG. Relative to its saline control, CabPK-I superfusion increased the pyloric-timed activity of the IC neuron and elicited pyloric-timed bursting in the VD neuron. Note that there was no obvious change in the PD and LP neuron activity, nor in the cycle frequency of the
pyloric rhythm (e.g., there were 6 PD neuron bursts in both conditions). The periodic change in the intensity of the IC and VD neuron bursts resulted from the concomitant activation of the gastric mill rhythm by CabPK-I superfusion (see below).

**Figure 6.** Pyrokinin peptide influences on the pyloric rhythm are limited to excitation of gastro-pyloric neurons. Scatter plots are shown for four pyloric rhythm parameters. Each data point represents the mean value (± SD) from at least 10 consecutive pyloric cycles in a single preparation during both saline (X-axis) and PK (10^{-6} M: Y-axis) superfusion. Clear symbols represent either PPK or LPK applications (n=14-18). Filled symbols represent either CabPK-I or CabPK-II applications (n=9). Data falling along the diagonal unity line represent equivalent values for the saline and peptide conditions. (A) Pyloric cycle frequency: Most values fall on or close to the unity line, regardless of the control pyloric cycle frequency and the identity of the applied PK peptide (n=27). There were few control values faster than 0.8 Hz because the pyloric rhythm rarely exceeded this value in the isolated STG during saline superfusion. (B) Number of LP neuron spikes/burst: Nearly all of the data points fall on or near the unity line, indicating that the PKs have little influence on LP neuron activity, regardless of its control level of activity (n=23). (C) Number of IC neuron spikes/burst: Whether the IC neuron was silent or active before PK application, its activity consistently increased in the presence of these peptides (n=23). (D) Number of VD neuron spikes/burst: The VD neuron was consistently silent during saline superfusion, but was consistently activated by the PK peptides (n=23).
Figure 7. Pyrokinin upregulates the intrinsic properties of the DG neuron. (A) Injection of a brief (0.3 sec), suprathreshold depolarizing current pulse into the DG neuron during PK superfusion elicited a brief action potential burst. After the end of the current injection, the DG neuron membrane potential remained depolarized and its activity persisted for another ~15 sec before repolarizing. In contrast, depolarizing the DG neuron to the same membrane potential (-37 mV) during saline superfusion elicited a brief burst of action potentials that terminated at the end of the current injection. (B) PK superfusion in normal saline elicits rhythmic oscillatory activity in the DG neuron. (Bottom Panel) During saline superfusion, the DG neuron was silent and exhibited a steady membrane potential. (Second Panel) During PK superfusion, the DG neuron was activated to burst rhythmically. (Third Panel) Injection of constant amplitude depolarizing current (+0.5 nA) into DG increased the frequency of its oscillatory activity. (Top Panel) Increased depolarization (+0.7 nA) caused the DG neuron to maintain a steady depolarized membrane potential at which it fired tonically for the duration of current injection. Oscillatory activity resumed at the end of this current injection. All four traces in Panel B are from the same DG neuron recording. Panels A and B are from separate experiments.

Figure 8. Pyrokinin superfusion continues to excite most gastric mill neurons when their synaptic interactions are reduced by picrotoxin (PTX) saline. (A) When the isolated STG was superfused with PTX, most intra-circuit synaptic actions were
suppressed. Note that the IC neuron and Int1 lost their rhythmic, pyloric-timed activity pattern and the rhythmic VD neuron bursts slowed and became irregular when normal saline was replaced by PTX saline. When PK was superfused in PTX saline, three (LG, Int1, VD) of the four recorded neurons exhibited increased activity. This included the onset of rhythmic bursting in the LG neuron that was unaffected by Int1 activity. (B) PK superfusion in PTX elicited independent rhythmic bursting in the DG and LG neurons. Panels A and B are from separate experiments.

**Figure 9.** Pyrokinin superfusion to the isolated STG elicits gastric mill rhythm activity but with the DG neuron bursting independently. (Left) During saline superfusion, there was no gastric mill rhythm. The DG neuron was bursting independently, at a slow and irregular rate. The IC neuron and Int1 were firing in pyloric-time, while the VD and LG neurons were silent. The pyloric rhythm is evident from the fast rhythmic bursting in the AB neuron. (Right) During PK superfusion, activity in all of the gastric mill neurons was increased. The LG and IC protractor neurons exhibited coordinated bursting that alternated with the coordinated bursts of the retractor neurons VD and Int1. DG neuron bursting was also enhanced but its cycle period was considerably slower than that of the other gastric mill neurons.

**Figure 10.** Pyrokinin superfusion elicits synchronous bursting in the gastric mill protractor neurons LG, IC and MG. (Left) During saline superfusion, the IC neuron fired pyloric-timed bursts while the MG and LG neurons were silent. The VD retractor neuron
(mvn recording) was also silent. (Right) During PK superfusion, coordinated rhythmic bursting occurred in these three protractor neurons. Note that the retractor phase neuron VD was also activated and fired in alternation with the protractor neurons.

**Figure 11.** Pyrokinin superfusion elicits a fully coordinated gastric mill rhythm. In a small number of preparations, PK superfusion elicited a fully coordinated gastric mill rhythm. Note that, in both panels, PK superfusion elicited synchronous bursting in the protractor LG and IC neurons that alternated with the bursting of the retractor DG and VD neurons. Panels A and B are from separate preparations.

**Figure 12.** Dose-response relationship for CabPKs on the gastric mill system. The threshold for activation of the gastric mill neurons LG and DG by superfusion of CabPK-I and CabPK-II was greater than $10^{-8}$ M. The zero (0) values for Saline and CabPK ($10^{-8}$ M) in each bar graph indicates that these neurons were never active under the indicated conditions. Symbols: *Different from saline control (p<0.001); #Different from $10^{-7}$ M CabPK (p<0.02). Legend for bar graphs- C, saline control. For all parameters, LG ($10^{-6}$ M), n=9; LG ($10^{-7}$ M), n=7; LG ($10^{-8}$ M), n=9; DG ($10^{-6}$ M), n=8; DG ($10^{-7}$ M), n=7; DG ($10^{-8}$ M), n=9.

**Figure 13.** Pyrokinin transiently activates the identified projection neuron MCN1 with its ganglion of origin removed. (A) (Left) During saline superfusion to the isolated STG, there was no spontaneous activity in either MCN1 (ion), DG or LG. (Middle) Soon after
the start of PK superfusion, rhythmic bursting began in the gastric mill neurons, albeit with the DG neuron exhibiting slower bursts that were uncoordinated with those of the LG neuron. At this time, MCN1 remained silent. (Right) Later, during the same PK application, tonic activity occurred in MCN1. During this time there was strengthened bursting in the DG and LG neurons, although their rhythmic bursting remained uncoordinated. (B) In a different preparation in which PK superfusion again initially elicited uncoordinated bursting between the DG and LG neurons, late activation of MCN1 by PK switched the gastric mill activity to that of a fully coordinated gastric mill rhythm. (C) A fully coordinated gastric mill rhythm, represented by the alternating bursting of the DG and LG neurons, was elicited by PK application in an isolated STG preparation in which MCN1 was also activated by this peptide. Note that, in contrast to Panel B, MCN1 activity was suppressed during each LG neuron burst. (D) PK (10^{-6} M)-activated MCN1 action potentials elicited constant latency EPSPs in the LG neuron that either (Left) followed each MCN1 action potential recorded in the ion, indicating orthodromic MCN1 activity, or (Right) preceded each of these MCN1 action potentials, indicating antidromic MCN1 activity. Each EPSP and MCN1 action potential represents the overlaid average of at least 10 individual, successive traces. Left and right panels are from different preparations.
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