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PARTIAL PURIFICATION, TISSUE DISTRIBUTION AND MODULATORY ACTIVITY OF A CRUSTACEAN CHOLECYSTOKININ-LIKE PEPTIDE

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Summary

Reversed-phase chromatography was used to separate several forms of cholecystokinin-like peptides (CCKLP) from the pericardial organs (PCOs) of the spiny lobster *Panulirus interruptus.* Fast protein liquid chromatography of PCOs, stomatogastric ganglia (STGs) and eyestalks revealed five peaks of CCKLP (peaks *A–E*) that were common to all three tissues, as well as two additional peaks (peaks *F* and *G*) in the STG. Peaks *A–E* were present in the hemolymph of fed, but not starved, lobsters. The bioactivity of peaks *A–E* was tested on the gastric mill rhythm of the isolated STG. Only peak *E* elicited activity. The effects of peak *E* included activating the gastric mill rhythm in quiescent preparations and strengthening existing rhythms in a dose-dependent manner. Further purification of peak *E* by high performance liquid chromatography resolved this peak into two immunoreactive peaks, one of which retained its bioactivity. The effects of peak *E* were blocked by the CCK antagonist proglumide. These results are consistent with a role for peak *E* in the feeding-induced activation of the gastric mill.

Introduction

The STG has been a useful system for elucidating the mechanisms by which neuromodulators act on central neural circuits (Harris-Warrick and Marder, 1991). This ganglion contains approximately 30 identified neurons that produce two rhythmic motor patterns, the pyloric and the gastric mill rhythms (Selverston and Moulins, 1987). The pyloric rhythm controls the filtering and pumping movements of the posterior region of the stomach, and the gastric mill rhythm controls the movements of three teeth within the stomach (Turrigiano and Heinzel, 1992). The expression of these rhythms is dependent upon descending modulatory inputs from higher ganglia. Blocking this input causes the rhythms to slow or become quiescent, and rhythmicity can then be strengthened or re-established by bath-applying a number of modulatory substances (Flamm and Harris-Warrick, 1986; Hooper and Marder, 1987; Heinzel and Selverston, 1988; Nusbaum and

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Marder, 1988; Turrigiano and Selverston, 1989). Many of these substances, including a crustacean cholecystokinin-like peptide (C-CCKLP) (Turrigiano and Selverston, 1989, 1991; Van Wormhoudt and Dircksen, 1990), have been identified immunocytochemically in neurons and fibers that project to and terminate within the neuropil of the STG.

Cholecystokinin (CCK)-like peptides have been localized to the nervous systems of a wide range of vertebrate and invertebrate species (Dockray *et al.* 1981, 1985; Osborne *et al.* 1981; Larson and Vigna, 1983; Marley *et al.* 1984; Rehfeld *et al.* 1985; Nachman *et al.* 1986; Favrel *et al.* 1987; Nichols *et al.* 1988). In mammals, CCK and related peptides are released into the blood following ingestion, where they influence several feeding-related behaviors, including gut motility and the induction of satiety. Several invertebrate CCK-related peptides have now been sequenced, including the leucosulfakinins (Nachman *et al.* 1986), the drosulfakinins (DSKs) I and II (Nichols *et al.* 1988; Nichols, 1992) and cionin (Johnsen and Rehfeld, 1990). A family of peptides has been isolated from the gut of the lobster *Nephrops norvegicus* using an antiserum directed against CCK. Unlike the other CCK-related peptides that have been isolated, these peptides do not contain a tyrosyl residue and are likely to represent a separate family of peptides (Favrel *et al.* 1991).

C-CCKLPs play an important role in the modulation of the gastric mill. C-CCKLP is contained within input fibers to the STG and can be released directly into the ganglion by electrical stimulation of these fibers. Application of mammalian CCK and related peptides to the isolated STG can initiate activity in a quiescent gastric mill as well as intensify an existing gastric rhythm (Turrigiano and Selverston, 1989), suggesting that C-CCKLP acts as an endogenous modulator of the gastric mill rhythm.

In addition to the apparent local release of C-CCKLP described above, this peptide has a second neurohormonal function in the control of the gastric mill. C-CCKLP is present within a major crustacean neuroendocrine organ, the paired pericardial organs (PCOs). There are detectable levels of C-CCKLP in the haemolymph, and these levels increase following feeding (Van Wormhoudt *et al.* 1989; Turrigiano and Selverston, 1990). In intact lobsters, this feeding-induced elevation in C-CCKLP is correlated with activation of the gastric mill. Injection of CCK into the hemolymph of intact lobsters also activates the gastric mill (Turrigiano and Selverston, 1990). Finally, injection of the CCK antagonist proglumide into the hemolymph inhibits feeding-induced gastric mill activity, suggesting that feeding-induced release of C-CCKLP is necessary for gastric mill activation (Turrigiano and Selverston, 1990).

It is unclear why there should be both a direct modulatory projection and a neurohormonal pathway by which CCKLPs have access to the STG. One possibility is that the CCKLP contained in the STG input fibers is distinct from the CCKLP that is released into the hemolymph. The antisera used in both this and previous studies of Crustacea cannot distinguish between the many forms of CCK/gastrins (Favrel *et al.* 1987; Turrigiano and Selverston, 1991), and so the CCKLP identified by these antisera may represent a heterogeneous population of peptides. Thus, in this paper, we use reversed-phase chromatography to isolate and compare the CCKLP in the STG, the PCOs, the eyestalks and the hemolymph.

While mammalian CCK can modulate the output of the gastric mill network, it requires

high concentrations of peptide (Turrigiano and Selverston, 1989, 1990), suggesting that the endogenous peptide is similar to, but distinct from, the mammalian forms. This also raises the possibility that the physiological effects of mammalian CCK are not identical to the effects of the endogenous peptide. For this reason, we wished to determine the effects of the endogenous peptide on the output of the gastric mill network. The results presented here suggest that there are several species of C-CCKLP in the *P. interruptus* nervous system and neuroendocrine organs, five of which are common to all the tissues examined, and two that are contained only in the STG. Of the species of CCKLP that are common to all three tissues, only one, peak *E*, modulates the output of the gastric mill network. Peak *E* is present in the STG, the PCOs and in the hemolymph of fed lobsters, a distribution consistent with a role for this peptide in the modulation of the gastric mill motor pattern.

Materials and methods

Animals

Specimens of the California spiny lobster *Panulirus interruptus* were obtained from a local fisherman and kept without food in a running sea water aquarium at 13–15 ˚C until use. Animals of both sexes were used.

Saline composition

P. interruptus physiological saline consisted of (in $mmol\,l^{-1}$): NaCl, 479; KCl, 12.7; $CaCl_2$, 13.7, $MgSO_4$, 10; Na_2SO_4, 3.9; glucose, 2; Hepes, 5; Tes, 5; pH 7.4.

Tissue extractions

Organs were dissected in ice-cold saline, placed into polypropylene tubes, and frozen immediately on methanol/dry ice. Tissue was stored at −70 ˚C until used. Eyestalks were split open and the optic lobes, optic peduncles and X organs were removed. The hearts with attached PCOs were dissected away from the pericardial cavity. In some experiments, the PCOs were dissected away from the hearts prior to extraction, while in others hearts and PCOs were extracted together. Yields were similar in both cases. Samples of hemolymph were withdrawn from the dorsal heart sinus directly into 2 vols of ice-cold methanol with $5\,mmol\,l^{-1}$ phenylmethylsulfonyl fluoride (PMSF); samples were vortexed, microfuged, and the supernatant was withdrawn, dried in a Speed Vac concentrator and stored at −70 ˚C until used.

Tissue samples were extracted twice in ice-cold methanol with $5\,mmol\,l^{-1}$ PMSF, and once in $0.5\,mol\,l^{-1}$ acetic acid, with homogenization and sonication to disrupt the tissue; for each extraction step the extracts were microfuged and the supernatants withdrawn, dried in a Speed Vac concentrator and stored at −70 ˚C until used. Samples were reconstituted in HPLC-grade H_2O just prior to use in fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) experiments (see below). In some experiments, tissue was extracted in the presence of the reducing agent dithiothreitol (DTT).

FPLC and HPLC

FPLC runs were performed on a preparative reversed-phase pepRPC HR 10/10 column

(C2/C18), using a Pharmacia FPLC system, with a flow rate of 1 ml min^{-1}. The A buffer was H_2O with 0.1 % trifluoroacetic acid (TFA) and the B buffer was 20 % H_2O and 80 % acetonitrile, with 0.1 % TFA. The absorbance at 280 nm was monitored using a Pharmacia ultraviolet monitor. Fractions of 1.3 ml were collected into microcentrifuge tubes and stored at −70 °C until use. HPLC runs were performed on a two-pump LKB HPLC system, using a C-18 Protein-Vydac column, with a flow rate of 1 ml min^{-1}. The A buffer was H_2O with 0.1 % TFA and the B buffer was 40 % H_2O and 60 % acetonitrile, with 0.1 % TFA. Absorbance was monitored at 226 nm. Fractions of 1 ml were collected and stored at −70 °C until used.

Standard peptides CCKNS (unsulfated CCK8, from Sigma), CCK ($CCK8SO_4$, from Sigma) and DSK I (DSK I and II were the kind gift of R. Nichols, Departments of Biological Chemistry and Biology, University of Michigan) were run on the FPLC system using the same conditions as for tissue extracts, and column fractions were dried and assayed by radioimmunoassay (RIA) to determine the elution time. Following standard runs, the column was extensively washed with 100 % acetonitrile, and a blank run was performed and assayed by RIA to ensure that no residual standards were eluting from the column.

For RIA, samples of each column fraction were dried in a Speed Vac concentrator and reconstituted in RIA buffer. For bioassay, samples of the desired column fractions were dried as above and reconstituted in *P. interruptus* saline.

Radioimmunoassay

Two radioimmunoassays using polyclonal antisera directed against CCK were used to detect CCK-like immunoreactivity. RIA1 has been previously described (Turrigiano and Selverston, 1991). This RIA is routinely sensitive to 0.2 fmol of CCK. It recognizes peptides that share a terminal 5 amino acid sequence with CCK (e.g. gastrins and caerulein peptide) about equally well, but does not recognize a number of structurally unrelated peptides (Turrigiano and Selverston, 1991), including the crustacean FMRFamide-like peptides TNRNFLRFamide and SDRNFLRFamide (the kind gift of E. Marder, Department of Biology, Brandeis University), and has about a 1:100 cross-reactivity with DSK II (Fig. 1) and DSK I (data not shown). RIA2 has been previously described (Favrel *et al.* 1987) and is similar to RIA1 in specificity and sensitivity. Both RIAs detected peaks of CCKLI in the same positions in the FPLC column runs and gave similar values for the amount of C-CCKLP present.

For each assay, a standard curve was constructed using CCK8, in the range 0–100 fmol; all points were run in duplicate. The amount of CCK-like material in tissue extracts and column fractions was estimated by determining the amount of CCK needed to produce the same inhibition of binding; values are therefore expressed as CCK mole equivalents (CCKE) or CCK fmol equivalents.

Bioactivity of column fractions

The stomatogastric nervous system, including the STG, the esophageal ganglion and the paired commissural ganglia (CGs) and attached nerves, was dissected for physiology as previously described (Selverston and Moulins, 1987). Extracellular recordings from gastric mill neurons were obtained using standard recording techniques (Mulloney and

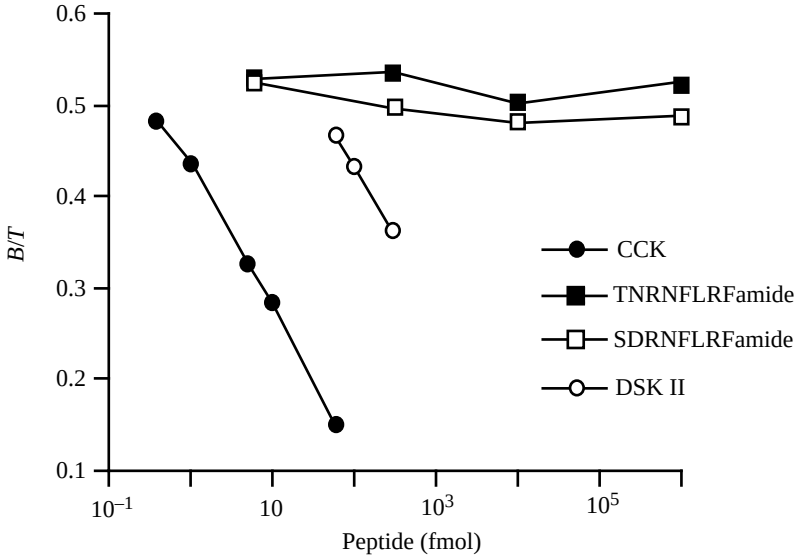


Fig. 1. RIA1, showing the inhibition of binding of labeled cholecystokinin (CCK) by unlabeled peptides (CCK, the crustacean FMRFamide-like peptides SDRNFLRFamide and TNRNFLRFamide and DSK II). *B*/*T* is the number of counts bound over the total number of counts.

Selverston, 1974). To detect excitatory effects of column fractions, vigorously cycling gastric mill rhythms were slowed and weakened by blocking inputs to the STG from one CG. Under these conditions, the gastric rhythm is typically slow and irregular and occasionally stops cycling, while the pyloric rhythm remains vigorous.

A 25–50 μl Vaseline well was constructed around the desheathed STG. Column fractions were reconstituted in *P. interruptus* saline, at concentrations of 0.2–50 fmol equivalents ml^{-1}, and the pH was adjusted to 7.4 if necessary. Because there was not enough material in the column fractions to allow superfusion, solutions around the ganglion were exchanged by withdrawing and replacing the saline. Changing solutions produced a transient disruption of gastric mill activity that was over within 2 min. Recordings were made 10 min after change of solutions. Saline applied in the same manner as column fractions had no effect on gastric mill activity. For washes, saline was superfused over the STG for at least 30 min to ensure removal of material from the ganglion; the superfusion was then turned off for the duration of the wash, and for at least 10 min before wash activity was recorded. The temperature of the entire preparation was controlled by means of a circulating water bath and was maintained at 15 ˚C.

Results

Tissue extractions

Immunocytochemistry has demonstrated that STGs, PCOs and eyestalks of *P. interruptus* display CCKLI (Turrigiano and Selverston, 1991). Of these three tissues,

crude extracts of PCOs contained the highest levels of CCKLI by about an order of magnitude (Table 1). Dilutions of tissue extracts produced inhibition of binding in parallel with the standard curve, as shown previously (Turrigiano and Selverston, 1991).

FPLC fractionation of tissue extracts

Reversed-phase fractionation of PCO tissue extracts revealed five distinct peaks of CCKLI, peaks *A*–*E* (Fig. 2A). While the elution time of peak *D* (fraction 44) is close to that of CCK (fraction 43), the other peaks have elution times distinct from CCK, CCKNS or DSK I. Using the same conditions but a different gradient, the elution time of these peaks was also found to differ from those of human gastrin I and caerulein peptide (data not shown). In six runs of six separate extractions (using 20–90 PCOs per extraction), peaks *A*, *B* and *E* were present in each experiment and peaks *C* and *D* were present in five of six experiments. No additional peaks were consistently present. Peaks *A*, *B* and *E* each account for about 25 % of the immunoreactivity running on the column, with peaks *C* and *D* accounting for the remainder.

In all column runs of *P. interruptus* tissue extracts, some immunoreactive material (approximately 20 % of the total) did not stick to the column, but eluted in the wash (Fig. 2). Drying down and re-running the wash did not result in significant recovery of this material. Extracting tissue in the presence of DTT appeared to decrease the proportion of material that failed to stick to the column, but did not result in the appearance of additional peaks of CCKLI. Approximately 50 % of the immunoreactive material in the crude extracts was recovered from the fractions running on the FPLC column.

Reversed-phase fractionation of eyestalks (*N*=2, 10 eyestalks per extraction) and STGs (*N*=3, 9–80 STGs per extraction) revealed five peaks of CCKLI eluting in the same position as peaks *A*–*E* in the PCOs (Fig. 2B,C). In addition, there were two more hydrophobic peaks in the extracts of the STGs, peaks *F* and *G* (Fig. 2C).

FPLC fractionation of hemolymph

Feeding produces on average a fourfold increase in the levels of CCKLI in the hemolymph of lobsters (Turrigiano and Selverston, 1990). One such experiment is shown in Fig. 3A; the hemolymph of this animal was fractionated by FPLC before feeding and

Table 1. *The amount of CCKE in extracts of PCOs, eyestalks and STGs determined by RIA1*

Organ	Equivalents of peak *E* fmol per organ
PCO (*N*=6)	180±80
Eyestalk (*N*=2)	20±8
STG (*N*=3)	17±11

Values are means ± S.E.M. for the number of experiments indicated.
PCO, pericardial organ; STG, stomatogastric ganglion.

1 h after feeding (Fig. 3B). Before feeding, there was no detectable CCKLI running on the column. One hour after feeding, peaks *B–E* were detectable; the small peak running in the position of peak *A* was below the level of reliable detection in this assay. Similar

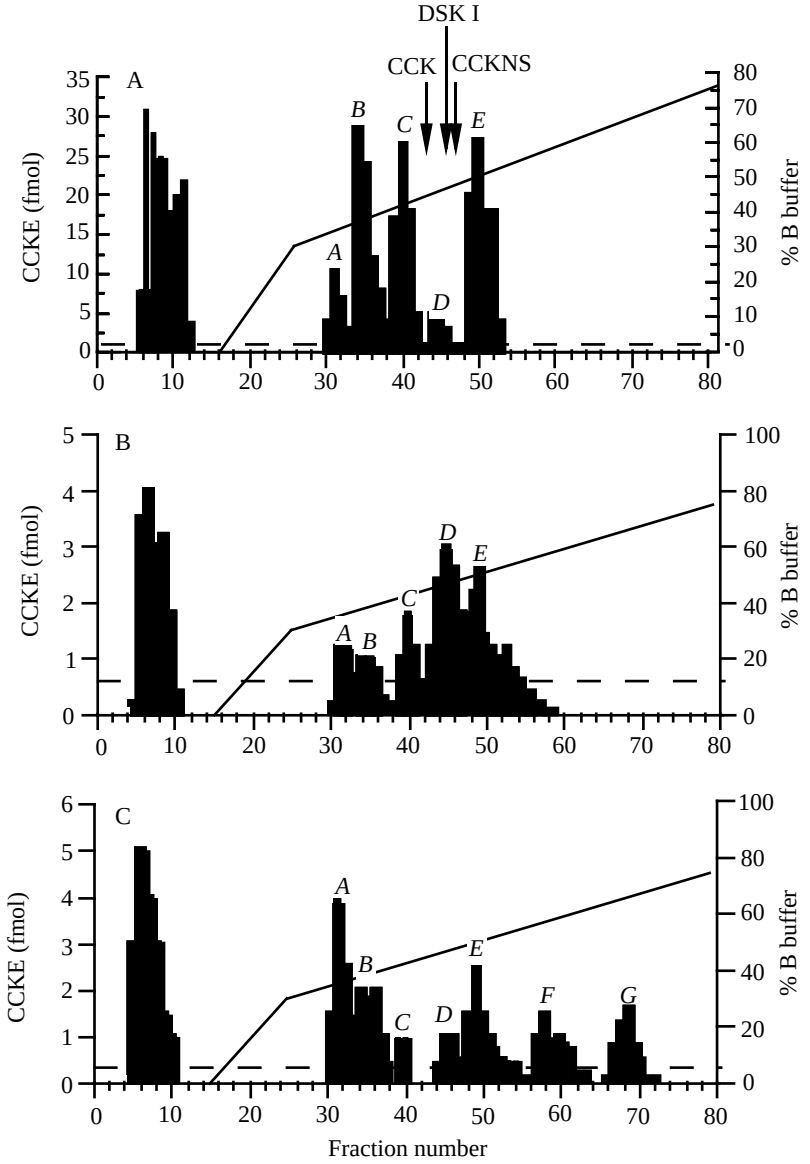


Fig. 2. Reversed-phase chromatography of extracts from the pericardial organs (A), eyestalks (B) and stomatogastric ganglia (C), using the FPLC system, and a B buffer of 80 % acetonitrile. The histograms represent the amount of CCK mole equivalents (CCKE) in each column fraction. The solid lines represent the percentage of B buffer and the broken lines mark the limits of detectability of the RIA.

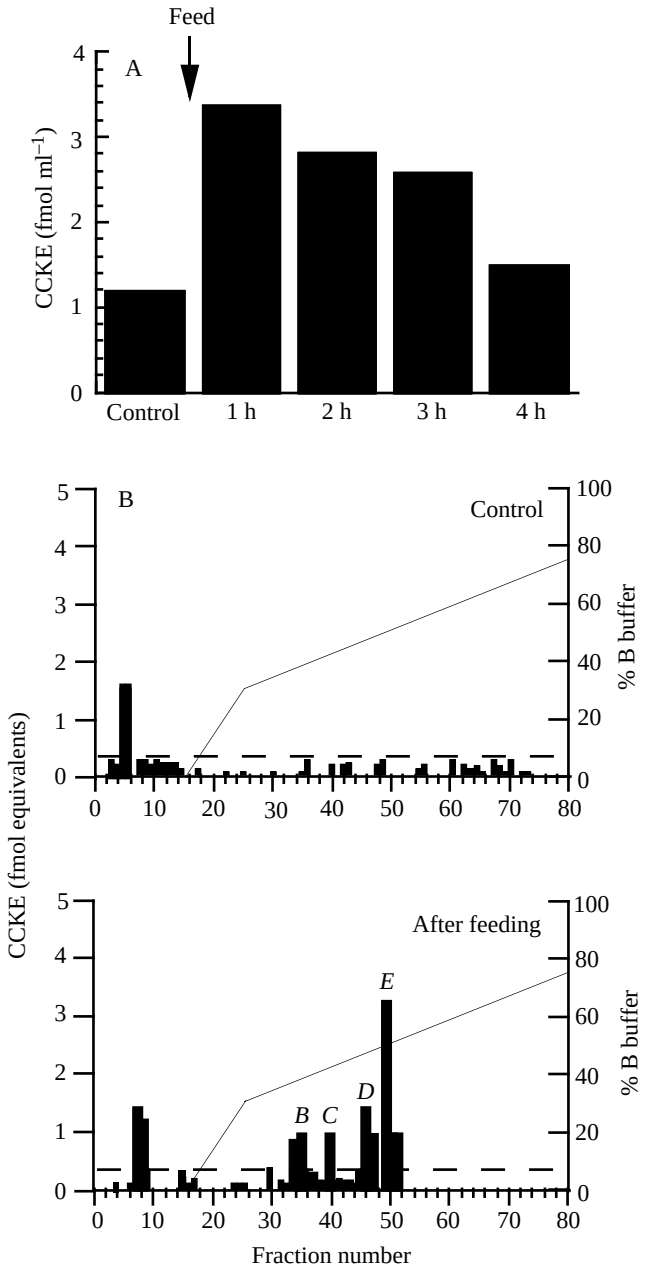


Fig. 3. CCKLP in hemolymph of starved and fed lobsters. (A) The amount of CCKE in the hemolymph of one lobster before (control) and at 1 h intervals after eating two smelt. (B) FPLC fractionation of 4 ml of hemolymph before and after feeding. The histograms represent the number of CCK mole equivalents (CCKE) in each column fraction. The solid line represents the gradient of B buffer and the broken line represents the limit of detectability of the RIA. Hemolymph was withdrawn from the dorsal heart sinus.

results were obtained in three separate experiments. Peaks *A–E* were each detectable 2/3 or 3/3 times, and no other peaks were detected.

Bioactivity of peaks A–E

To test the bioactivity of peaks *A–E*, extracts of the PCOs were run on FPLC, and the amount of CCKE in each fraction determined by RIA. The fractions corresponding to peaks *A–E* were then tested on the STG while monitoring gastric mill and pyloric activity. In three experiments each, peaks *A*, *B*, *C* and *D* had no effect on the activity of the STG when applied at concentrations of 10–50 fmol equivalents ml^{-1}. In one experiment, peak *A* was applied to both the STG and the attached desheathed commissural ganglia (CGs) and was observed to have mild excitatory effects.

In a total of 11 experiments, peak *E* had excitatory effects on the activity of the gastric mill network when applied to the STG at concentrations of 0.2–20 fmol equivalents ml^{-1}. When peak *E* was applied to a non-cycling preparation, it activated an intermittent gastric mill rhythm. When peak *E* was applied to a weakly cycling preparation, it decreased the period and increased the vigor of the gastric mill rhythm. For the six experiments in which peak *E* was applied to a cycling gastric mill rhythm, peak *E* decreased the period from 9.7±2.4 to 6.4±1.1 s. Peak *E* also increased the duty cycles of neurons LG and MG and delayed the phase of every gastric mill neuron relative to LG (Table 2).

Fig. 4 illustrates one such experiment. In control saline, the activity of the gastric mill (shown in the bottom three traces) was slow and weak. This activity was monitored by extracellular recordings of the LG neuron (on the lgn trace), the LPG neurons (on the lpgn trace) and the GM neurons (on the aln trace). Application of peak *E* to the STG induced strong and regular cycling of the gastric mill (Fig. 4B). This activity was characterized by strong LG activity. In 4/6 experiments in which peak *E* was applied at 20 fmol equivalents ml^{-1} to a weakly cycling gastric mill, LG was induced to fire in double bursts, with an initial high-frequency burst followed by a weaker burst.

Pyloric activity was monitored by extracellular recordings from the LP neuron (the large spikes on the lvn trace) and the PD neuron (on the pdn trace) (Fig. 4). While peak *E* had little direct effect on the pyloric rhythm, it did initiate periodic interruptions of pyloric activity, in phase with the initial strong LG burst. During these interruptions, the LP neuron was inhibited and the phase of the next PD burst was advanced.

The effects of peak *E* were slow to develop, taking 5–10 min to become maximal. When peak *E* was removed within 15 min of application, the effects were reversible within 1 h (Fig. 4). If peak *E* was left on the ganglion for more than 15 min, the effects could take hours to reverse, and in some cases did not reverse after 5 h or more after the wash.

The effects of peak *E* were dose-dependent (Fig. 5). Increasing concentrations of peak *E* produced a dose-dependent decrease in the period of the rhythm and a dose-dependent increase in the spike frequency of most of the gastric mill neurons, especially LG (Fig. 5, inset). Similar results were obtained in three separate preparations.

Further purification and bioactivity of peak E

To purify peak *E* further, the FPLC fractions corresponding to the peak immunoreactivity of peak *E* were pooled and run on HPLC using a Vydac C18 column

Table 2. *The effects of peak* E *on the duty cycle and phase of gastric mill neurons*

	Duty cycle		Phase	
Cell	Control	Peak *E*	Control	Peak *E*
LG	0.25±0.01	0.40±0.01*	0	0
MG	0.18±0.03	0.33±0.03*	–0.02±0.01	0.04±0.01*
LPG	0.84±0.03	0.66±0.10	0.22±0.05	0.31±0.03
DG	0.39±0.03	0.44±0.06	0.49±0.03	0.67±0.03*
GM	0.51±0.05	0.52±0.03	–0.05±0.02	0.06±0.01*

The duty cycle was defined as (burst duration)/(period); the phase was defined as (time at which the neuron began to fire relative to the beginning of the LG burst)/(period).

For each experiment the duty cycle and phase were averaged for six cycles of activity.

The values are the mean ± S.E.M. for six experiments; each neuron was represented in at least four experiments.

*Peak *E* significantly different from control, paired *t*-test ($P \leqslant 0.05$).

and a gradient of acetonitrile (Fig. 6). This column resolved peak *E* into two immunoreactive peaks (at fractions 47 and 50), neither of which corresponded to the maximum absorbance at 226 nm (at fraction 51).

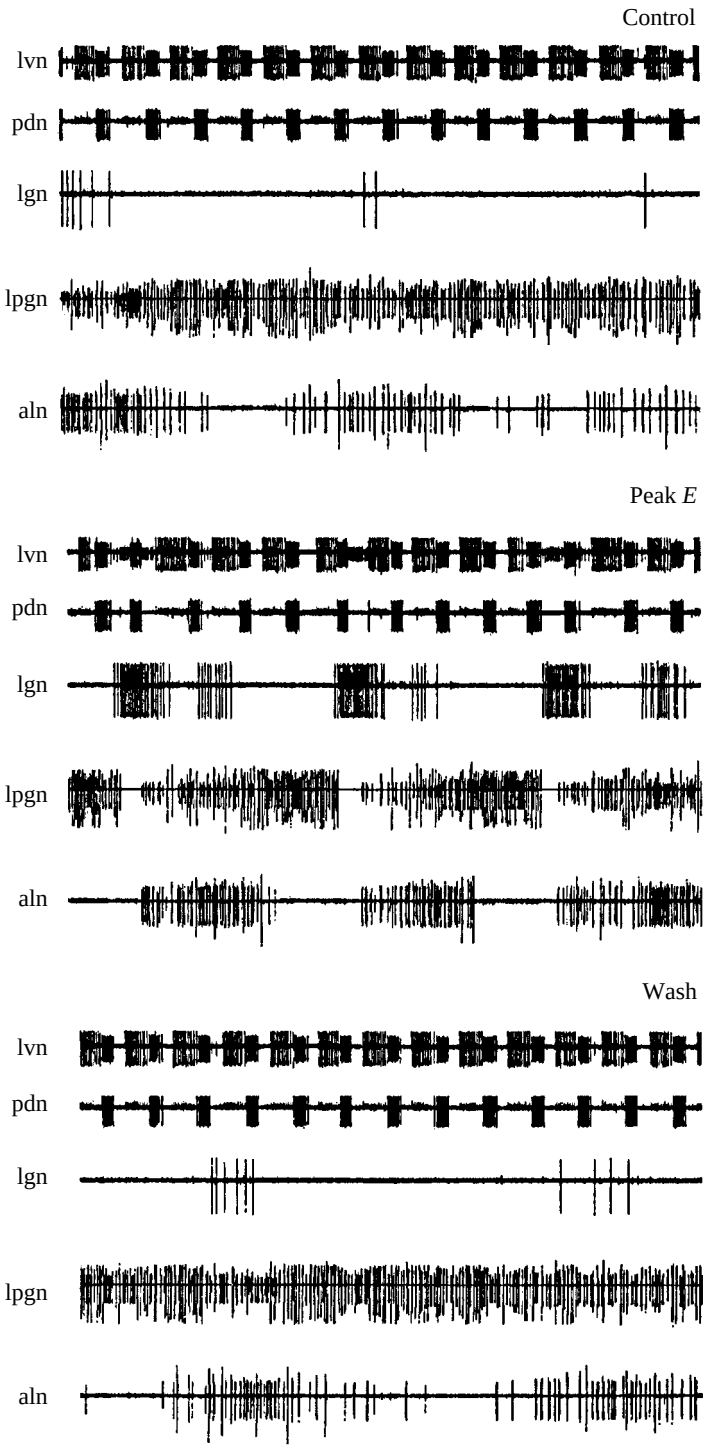
Both peaks were tested on the STG for bioactivity. Fraction 47 had no effect on the gastric mill rhythm (not shown), while fraction 50 retained the bioactivity of peak *E* (Fig. 7). Fraction 51 (the peak of absorbance) was less effective than fraction 50 at activating the gastric mill.

Peaks *A–D* were also run on the Vydac system. Each peak eluted as a single peak, at fractions 15, 23, 29 and 35, respectively (data not shown).

Proglumide blocks the effects of peak E

We tested the CCK antagonist proglumide for its ability to block the effects of peak *E* (Fig. 8). Peak *E* alone induced strong and regular gastric mill cycling, in which the LG and DG spike frequencies were greatly increased, and LG fired in double bursts with an initial strong burst followed by a second weak burst. In the presence of 10^{-6} mol l^{-1} proglumide, these effects were blocked. Similar results were obtained in three separate preparations. Proglumide alone had little or no effect on gastric mill activity. The effect of peak *E* on LG spike frequency at several doses with and without proglumide present is illustrated in Fig. 9A. Proglumide at 10^{-5} mol l^{-1} was able to block completely the effects of peak *E*. Proglumide was ineffective at doses below 10^{-7} mol l^{-1}, and at 10^{-5} mol l^{-1} the antagonism was complete (Fig. 9B).

Fig. 4. The effects of peak *E* on the gastric mill and pyloric rhythms. Pyloric activity was monitored by extracellular recordings of the LP neuron (the large unit on the lvn trace), and the PD neuron (on the pdn trace), and gastric mill activity was monitored with recordings of the LG neuron (on the lgn trace), the LPG neurons (on the lpgn trace) and the GM neurons (on the aln trace). Control, activity prior to application of peak *E*. Peak *E*, activity 10 min after application at 20 fmol equivalents ml^{-1}. Wash, activity after 90 min of washing.


Control
lvn
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lgn
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Peak *E*
lvn
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Wash
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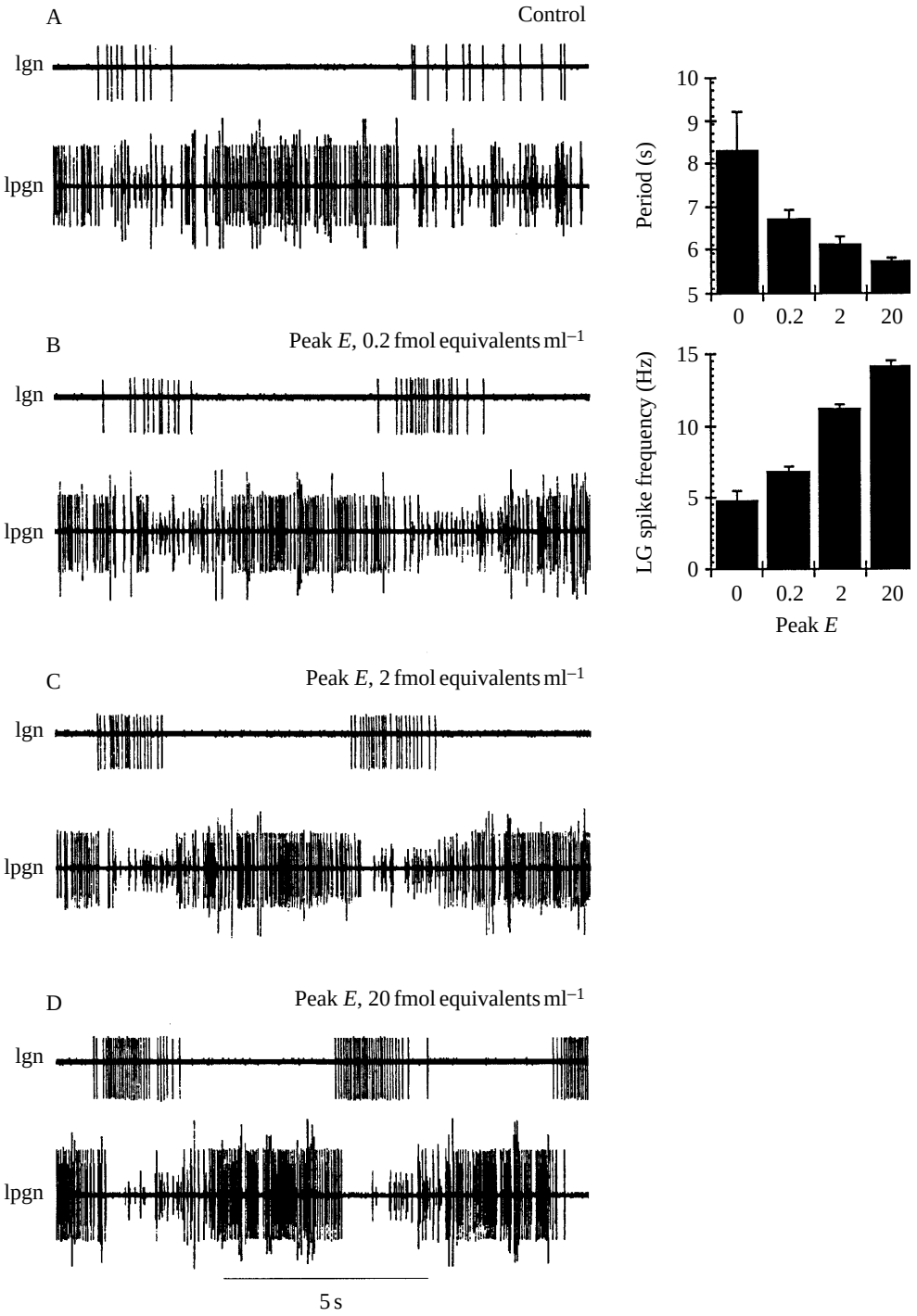
lpgn
aln

Fig. 4


A
Control
lgn
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B
Peak E, 0.2 fmol equivalents ml−1
lgn
lpgn
C
Peak E, 2 fmol equivalents ml−1
lgn
lpgn
D
Peak E, 20 fmol equivalents ml−1
lgn
lpgn
5 s
Period (s)
10
9
8
7
6
5
0
0.2
2
20
LG spike frequency (Hz)
15
10
5
0
0
0.2
2
20
Peak E

Fig. 5

Fig. 5. Dose-dependency of the effects of peak E. (A) Control activity. (B) Activity 10 min after the application of 0.2 fmol equivalents ml^{-1} of peak *E*. (C) Activity 10 min after the application of 2 fmol equivalents ml^{-1} of peak *E*. (D) Activity 10 min after the application of 20 fmol equivalents ml^{-1} of peak *E*. Inset shows the period and the spike frequency of LG for the three doses of peak *E*. The numbers are the mean + S.E.M. for six cycles of activity. For each dose, peak *E* was applied for 10 min, the activity recorded and the preparation washed for at least 1 h or until activity returned to baseline, prior to application of the next dose.

Discussion

C-CCKLP is an important modulator of the gastric mill motor pattern of the spiny lobster, *P. interruptus* (Turrigiano and Selverston, 1989, 1990). Here we have described the partial purification of this peptide from the PCOs of *P. interruptus* and its physiological effects on the gastric mill rhythm.

Multiple forms of C-CCKLP

Reversed-phase chromatography has revealed that three structures that display CCKLI, the STG, the PCOs and the eyestalks (Turrigiano and Selverston, 1991), all contain several species of CCK-like peptide. Two sequential reversed-phase purification steps of PCO extracts revealed six immunoreactive peaks with distinct elution times. These six peaks may represent six distinct active peptides, or some of the peaks may represent precursor molecules or breakdown products of one or more of the other peaks. These peaks are common to the three tissues examined. An additional two peaks were observed in the STG extracts, suggesting that additional forms of C-CCKLP are present in the STG neuropil.

Recently, immunocytochemistry using two monoclonal antisera raised against CCK has produced staining patterns in the stomatogastric nervous system that are distinct from

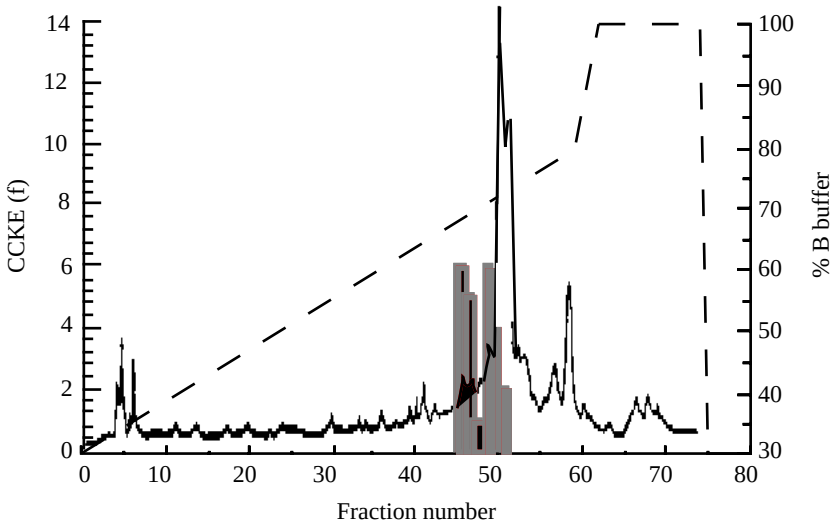

Fig. 6. HPLC fractionation of peak *E*, using a B buffer of 60 % acetonitrile. The histograms represent the amount of CCKE in each column fraction, the solid line represents the absorbance at 226 nm and the dashed line represents the gradient of B buffer.

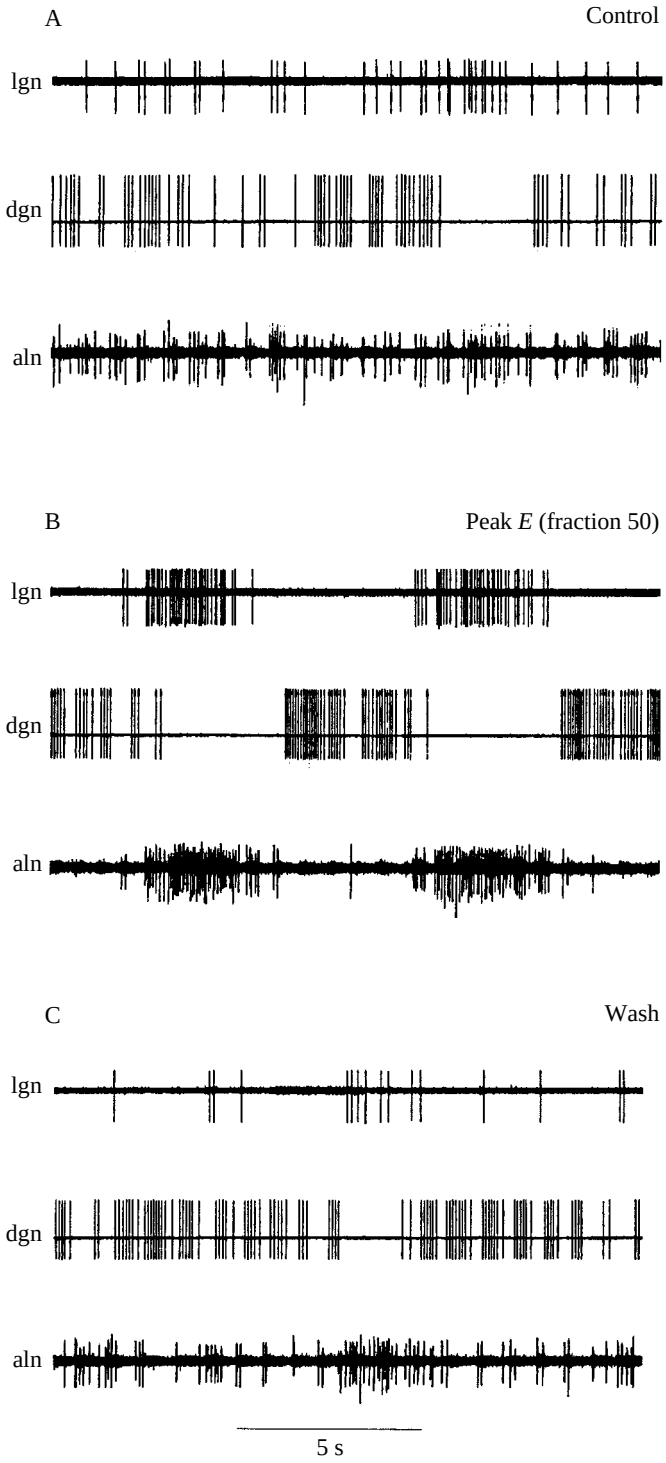
A
Control
lgn
dgn
aln
B
Peak E (fraction 50)
lgn
dgn
aln
C
Wash
lgn
dgn
aln
5 s

Fig. 7

those produced by the antisera used in this study (Christie *et al.* 1991; A. E. Christie, G. G. Turrigiano, D. Baldwin, K. Graubard and E. Marder, in preparation), suggesting that there are additional forms of C-CCKLP to those described here.

Only one of the isolated forms, peak *E*, was able to modulate the output of the STG at the concentrations tested. Because we do not know the cross-reactivity of our antiserum with the endogenous peptides, we do not know the absolute concentrations of peptides used in these experiments. Our antiserum has about a 1:100 cross-reactivity with the *Drosophila* CCK-like peptides DSK I and II. A cross-reactivity between 1:10 and 1:1000 would give similar levels of C-CCKLP in the STG and PCOs to those reported for proctolin and crustacean FMRFamide-like peptides (Marder *et al.* 1986, 1987; Kobierski *et al.* 1987). If we assume cross-reactivities of 1:100 with the endogenous peptides, then the concentrations of each peptide used in our bioassay ranged from 2×10^{-11} to 2.5×10^{-9} mol l^{-1}. Estimated in this way, peak *E* was effective at concentrations as low as 2×10^{-11} mol l^{-1}. Even if the cross-reactivity is lower than estimated, this is still likely to be a much lower dose than the threshold dose of mammalian CCK, which is 10^{-6} mol l^{-1} (Turrigiano and Selverston, 1989).

If the cross-reactivity of our antiserum is different for the different forms of C-CCKLP, then it is possible that we were applying much lower concentrations of some forms than of others in our bioassay, and we cannot rule out the possibility that forms other than peak *E* are effective at modulating the gastric mill rhythm at higher concentrations. It is also probable that some forms have specific sites of action other than the gastric mill. While some members of the CCK family (CCK and caerulein peptide) are able to modulate the output of the STG, other members (the gastrins and DSK I and II) are ineffective, but are recognized by the antisera used in this study. CCK-related peptides extracted from the stomach of the lobster *Nephrops norvegicus* that do not have a tyrosyl residue are unable to modulate the output of the gastric mill (G. Turrigiano and A. Van Wormhoudt, unpublished observations), but are recognized by the antisera used in this study (Favrel *et al.* 1989, 1991). It is therefore possible that the different forms of CCKLP identified in PCO tissue extracts from *P. interruptus* represent a mixture of these peptide families.

C-CCKLP in hemolymph

Circulating C-CCKLP is involved in feeding-induced gastric mill activation (Turrigiano and Selverston, 1990). We therefore wished to know which forms of C-CCKLP were released into the hemolymph following feeding. Reversed-phase fractionation of the hemolymph of fed lobsters revealed that the immunoreactive peaks common to the PCOs, STG and eyestalks, are present in the hemolymph of fed, but not starved, lobsters. This is consistent with a hormonal role for peak *E* in feeding-induced activation of the gastric mill.

The sources of the peak *E* found in the hemolymph could include the PCOs and the eyestalks, which contain the neuroendocrine X organs. The average concentration of peak

Fig. 7. The effects of peak *E* on the gastric mill after two sequential purification steps on FPLC and HPLC. (A) Control activity of the gastric rhythm. (B) Activity of the gastric rhythm 10 min after application of the peak of immunoreactivity at fraction 50 of the HPLC column run shown in Fig. 6. (C) The activity of the gastric rhythm after 1 h of washing.

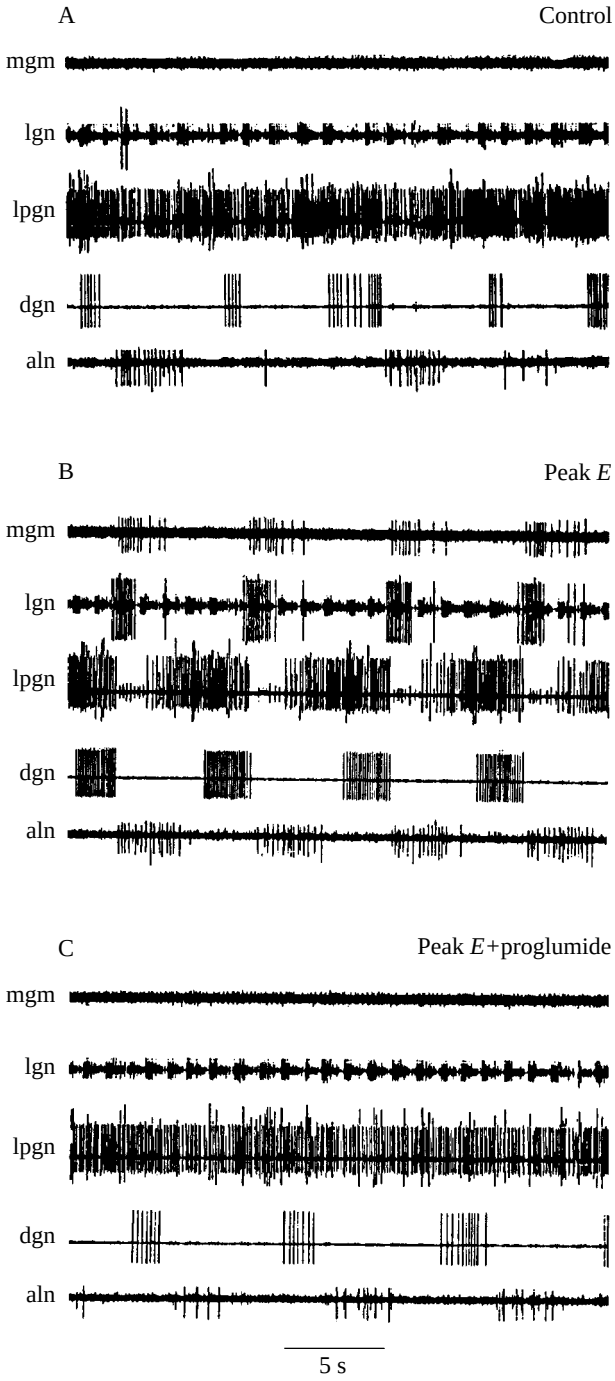

Fig. 8. Antagonism of the effects of peak *E* by proglumide. (A) Control activity of the gastric mill. (B) The effects of 20 fmol equivalents ml^{-1} of peak *E* alone. (C) The effects of 20 fmol equivalents ml^{-1} of peak *E* in the presence of 10^{-6} mol l^{-1} proglumide.

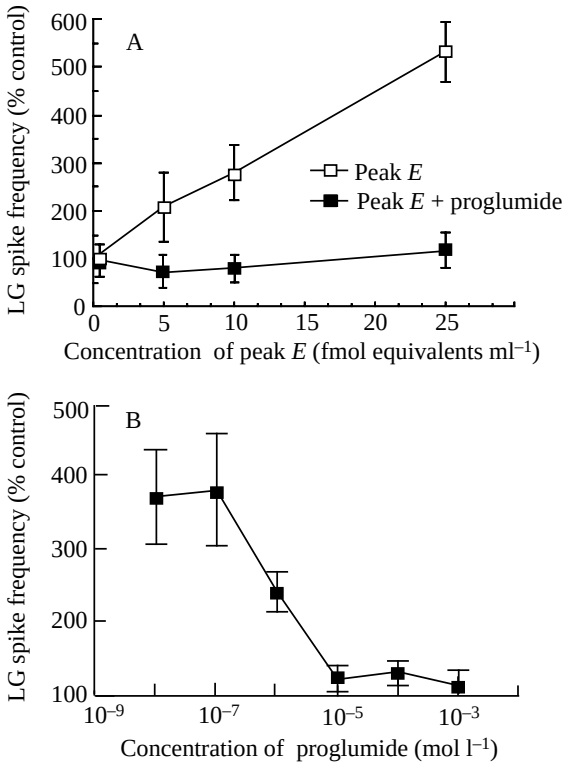

Fig. 9. Antagonism of the effects of peak *E* by proglumide. (A) LG spike frequency at three different doses of peak *E* in the presence and absence of 10^{-5} mol l^{-1} proglumide. (B) LG spike frequency in the presence of 20 fmol equivalents ml^{-1} of peak *E* at six different doses of proglumide. Values are mean ± S.E.M. for six consecutive cycles of activity and are expressed as the percentage of the control LG spike frequency.

E in the hemolymph of the dorsal heart sinus in the first hour after feeding was 0.3 fmol equivalents ml^{-1} (*N*=3), just above the threshold dose for gastric mill activation. The STG sits within an artery just anterior to the dorsal heart sinus, so it is likely to be exposed to concentrations of about this magnitude. We have measured about 30 fmol equivalents of peak *E* per PCO. Bleeding of medium-sized *P. interruptus* yielded approximately 30 ml of hemolymph, suggesting that approximately 30 % of the total amount of peak *E* in one PCO would have to be released to raise the hemolymph concentration to the measured level following feeding. It is probable that the local concentration of peak *E* in the dorsal heart sinus, where the hemolymph samples were taken, is higher than in the rest of the hemolymph, as the release sites of the PCOs are directly into this sinus. There also may be additional release sites that act in concert with the PCOs and eyestalks to release peak *E* following feeding.

C-CCKLP in the STG

Like the PCOs, the STGs contain peak *E*, indicating that there are two routes by which

peak *E* can influence the output of the gastric mill network. One route is a direct neuromodulatory projection into the STG neuropil, and the other is by way of the hemolymph. It is possible that these two sources act in concert to release peak *E* following feeding. Alternatively, the two sources may exert different effects on the gastric mill network, either as a result of co-release or co-localization of CCKLP with other modulators or as a result of restricted release of CCKLP onto specific neurons by the neuropilar inputs. While STG extracts contain two peaks of CCKLI not present in PCO extracts, we did not have sufficient material to determine whether these peaks were bioactive.

Modulation of the gastric mill by peak E

Like mammalian CCK, peak *E* is able to activate a quiescent gastric mill rhythm and to increase the frequency of a weakly cycling gastric rhythm in a dose-dependent manner. Also, like mammalian CCK, peak *E* has little or no effect on an existing pyloric rhythm (Turrigiano and Selverston, 1989). In this respect, mammalian CCK and peak *E* differ from other peptides that modulate the output of the STG, which have strong effects on the pyloric rhythm (Hooper and Marder, 1987; Nusbaum and Marder, 1988; Weimann *et al.* 1993). The effects of peak *E* on the gastric rhythm, though more dramatic, resemble the effects of CCK8, in that both increase the duty cycles and spike frequencies of neurons LG and DG when applied to a weakly cycling preparation (Turrigiano and Selverston, 1989). While CCK does not induce double LG bursts when bath-applied *in situ*, it does activate a pattern of chewing thought to involve LG double bursts when injected into intact animals (see below). It may be that CCK is too weak an agonist to induce LG double bursts *in vitro*, but that *in vivo* additional modulatory influences act in concert with the injected CCK to induce this pattern of activity in LG.

The most dramatic effects of peak *E* were on the activity of the LG neuron. Higher doses of peak *E* (estimated to be of the order of 10^{-9} mol l^{-1}) produced double bursts in neuron LG, characterized by an initial high-frequency burst followed by a shorter-duration low-frequency burst. It has been shown in another species of lobster that, when LG fires in such double bursts, the first component of the burst is due to the intrinsic bursting properties of LG, while the second component is due to synaptic input, and that the relative importance of these two components is under modulatory control (Nagy *et al.* 1988). This suggests that peak *E* is able to activate the conditional bursting properties of LG and that LG may be a direct target for C-CCKLP.

When LG fires in such double bursts, it is thought to result in a pattern of gastric mill behavior that has been termed ‘cut and squeeze’ chewing (Heinzel, 1988; Boyle *et al.* 1990; Turrigiano and Heinzel, 1992). This chewing pattern is characterized by double bites of the paired lateral teeth during each chewing cycle. When CCK is injected into intact lobsters, low doses initiate gastric mill activity in the ‘squeeze’ mode of chewing, in which LG fires only once per cycle, while higher doses result in predominantly ‘cut and squeeze’ chewing (Boyle *et al.* 1990; Turrigiano and Heinzel, 1992). The *in vitro* effects of peak *E* are consistent with the production of a pattern of chewing in the intact animal similar to that produced by CCK.

Although the C-CCKLP contained in peak *E* was not purified to homogeneity, the

ability of proglumide to block the effects of peak *E* suggests that it is the C-CCKLP in this fraction that is responsible for the observed physiological effects. We have shown previously that proglumide specifically blocks the effects of CCKLPs in this system (Turrigiano and Selverston, 1990).

We have shown that the C-CCKLP contained in peak *E* is present in the STG and in the neuroendocrine PCOs and is able to modulate the output of the gastric mill network. Peak *E* is released into the hemolymph following feeding at concentrations sufficiently high to activate the gastric mill. The effects of peak *E* are blocked by proglumide, as is the substance involved in feeding-induced gastric mill activation. These data are consistent with a role for peak *E* in the feeding-induced modulation of the gastric mill.

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