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THE EFFECTS OF SDRNFLRFamide AND TNRNFLRFamide ON THE MOTOR PATTERNS OF THE STOMATOGASTRIC GANGLION OF THE CRAB *CANCER BOREALIS*

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Summary

TNRNFLRFamide was isolated and sequenced from the stomatogastric nervous system of the crab *Cancer borealis* by reverse-phase high performance liquid chromatography followed by automated Edman degradation. An SDRNFLRFamide-like peptide that exactly co-migrated with SDRNFLRFamide was also observed. The effects of TNRNFLRFamide and SDRNFLRFamide on the gastric and pyloric rhythms of the stomatogastric nervous system of the crab *Cancer borealis* were studied. Both peptides activated pyloric rhythms in quiescent preparations in a dose-dependent manner with a threshold between 10^{-11} and 10^{-10} mol l^{-1}. Both peptides increased the pyloric rhythm frequency of preparations showing moderate activity levels and had relatively little effect on preparations that showed strong pyloric rhythms prior to peptide application. Both peptides evoked gastric mill activity in preparations without existing gastric rhythms. The activation of the gastric rhythm is associated with activation of oscillatory properties in the dorsal gastric neurone. The induction of gastric rhythms by these peptides was accompanied by switches from pyloric-timed activity to gastric-timed activity by several stomatogastric ganglion neurones. Application of these peptides provides direct experimental control of circuit modification in the stomatogastric nervous system.

Introduction

The crustacean stomatogastric nervous system has been extremely valuable for the study of the mechanisms by which modulatory amines and peptides can influence the output of neural circuits and behaviour (Selverston and Moulins, 1987; Harris-Warrick and Marder, 1991). Although the stomatogastric ganglion (STG) contains only 30 neurones, at least 12–15 substances are found in the neural inputs to the STG and are

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thought to act as neurally released modulators of the motor patterns produced by the STG (Marder, 1987; Marder and Weimann, 1992).

Antibodies raised against the molluscan neuropeptide FMRFamide clearly indicated the presence of FMRFamide-like immunoreactive inputs to the crab and lobster STG (Hooper and Marder, 1984; Marder and Hooper, 1985; Callaway *et al.* 1987; Marder *et al.* 1987). This observation strongly suggested that FMRFamide or related peptides might function as modulators of the motor patterns produced by the STG. However, early physiological studies showed that FMRFamide itself was active at only relatively high concentrations (Hooper and Marder, 1984; Marder and Hooper, 1985). Moreover, preliminary biochemical characterizations of the FMRFamide-like peptides in *Panulirus interruptus* and *C. borealis* suggested that the immunoreactivity found in these species was likely to be extended FLRFamide-like peptides such as TNRNFLRFamide and SDRNFLRFamide that have been purified and sequenced from *Homarus americanus* (Trimmer *et al.* 1987). We now show that TNRNFLRFamide and SDRNFLRFamide are physiologically active at low concentrations on the motor patterns of the STG, and that TNRNFLRFamide and an SDRNFLRFamide-like peptide are among the FMRFamide-like peptides found in the *C. borealis* stomatogastric nervous system.

In vitro preparations containing the single STG, the oesophageal ganglion (OG) and the paired commissural ganglia (CG) routinely generate robust fictive motor patterns that resemble those recorded from semi-intact and behaving animals (Hartline and Maynard, 1975; Rezer and Moulins, 1983; Turrigiano and Heinzel, 1992; Heinzel *et al.* 1993). However, when impulse traffic in the stomatogastric nerve (stn) is blocked, the fictive motor patterns generated by the isolated STG either stop completely or decrease in frequency (Russell, 1979; Moulins and Cournils, 1982; Eisen and Marder, 1982; Heinzel and Selverston, 1988).

Many of the modulatory substances that are found in projections to the STG strongly activate motor patterns of the STG. For example, proctolin activates rhythmic pyloric and gastric mill activity patterns (Hooper and Marder, 1984; Marder *et al.* 1986; Heinzel and Selverston, 1988). Dopamine, octopamine and serotonin can activate strong pyloric rhythms (Selverston and Miller, 1980; Beltz *et al.* 1984; Flamm and Harris-Warrick, 1986*a*). Cholecystokinin-like (CCK-like) peptides can activate gastric mill rhythms (Turrigiano and Selverston, 1989, 1990), red pigment concentrating hormone (RPCH) activates pyloric (Nusbaum and Marder, 1988) and cardiac sac (Dickinson and Marder, 1989) rhythms, and muscarinic agonists activate both pyloric and gastric rhythms (Marder and Paupardin-Tritsch, 1978; Marder and Hooper, 1985; Elson and Selverston, 1992).

The STG of the crab, *C. borealis*, is particularly interesting because many of the motor neurones can change their activity patterns from being characteristic of pyloric-timed activity to being characteristic of gastric-timed activity (Weimann *et al.* 1990, 1991). Because the central pattern-generating networks are formed among the motor neurones, these switches of activity play major roles in organizing the pattern-generating circuits (Weimann, 1992). To understand better the mechanisms underlying circuit reconfiguration, it is crucial to understand how different modulatory substances act on the networks of the STG. In this paper, we demonstrate that SDRNFLRFamide and

TNRNFLRFamide reliably activate gastric motor patterns in the crab. We describe fictive gastric rhythms in the crab and provide the basis for future work in which these peptides can be used experimentally to control switching from pyloric-timed to gastric-timed activity.

Materials and methods

Animals and saline

All experiments were performed on male *Cancer borealis* purchased from Neptune Lobster and Seafood Co. (Boston, MA) and held in aerated saltwater aquaria at 13˚C until used. Physiological experiments used 104 animals weighing between 250 and 800g. Biochemical purifications were made from two extractions of 35 and 101 animals. Physiological saline had the following composition: NaCl, 440mmol l^{-1}; KCl, 11.3mmol l^{-1}; $CaCl_2$, 13.3mmol l^{-1}; $MgCl_2$, 26.3mmol l^{-1}; Trizma base, 11.0mmol l^{-1}; maleic acid, 5.2mmol l^{-1}; pH7.4–7.5. SDRNFLRFamide and TNRNFLRFamide were purchased from American Peptide.

Physiology

Physiological recordings were made as described by Weimann *et al.* (1991). Monopolar extracellular pin electrodes were used in all experiments. Intracellular electrodes were filled with 2mol l^{-1} potassium acetate and 10mmol l^{-1} KCl and typically had tip resistances of 20 MΩ. Most intracellular signals were amplified with Getting amplifiers. For the single-electrode current-clamp experiments on the dorsal gastric (DG) neurone, the electrodes were filled with 0.6mol l^{-1} K_2SO_4/20mmol l^{-1} KCl and signals were recorded with an AXOCLAMP 2A (Axon Instruments). Data were collected on a Gould TA4000 thermal array recorder. A Computerscope system (RC Electronics) was used for on-line data analysis.

Impulse traffic in the stn was reversibly blocked by placing a Vaseline well around the desheathed stn, and then replacing the saline inside the well with isotonic sucrose (750mmol l^{-1}). Peptides were bath-applied by means of a switching port on the inflow of the continuously flowing superfusion system (10–15 ml min^{-1}). Bath volume was approximately 10ml. The saline temperature was held at 12.5˚C by means of a Peltier cooling system, and was continuously monitored with a thermoelectric probe in the bath.

The activity phases were measured from the chart paper using a Jandel Scientific digitizer and the phase angles were calculated using a program written by M. O'Neil.

Peptide extraction, purification and sequencing

Brains (*N*=35) or stomatogastric nervous systems (*N*=101) from rock crabs, *Cancer borealis*, were dissected under cold physiological saline, immediately immersed in acidified methanol solution (90% methanol, 9% glacial acetic acid, 1% water, 1 mmol l^{-1} dithiothreitol) and stored in a conventional freezer (−20˚C) until extracted. Tissues were homogenized in a large volume of acidified methanol solution in a straight-walled glass grinder and then sonicated over dry ice/acetone with a Branson sonifier 250.

The resulting slurry was spun in an Eppendorf microcentrifuge (model 5414) for 10min, the supernatant reserved, and the pellet re-extracted with methanol and respun. Supernatants were combined and concentrated to approximately 1ml under vacuum with a Speed Vac concentrator. The extract was then loaded on a C18 Sep-Pac cartridge (Waters) which had been pre-equilibrated with methanol, desalted with at least 10ml of water and eluted with 5ml of methanol containing 0.1% trifluoroacetic acid (TFA). The eluate was concentrated almost to dryness in a Speed Vac concentrator and water was added to achieve an appropriate volume (500–1000 μl).

FMRFamide-like peptides were purified from the extract with a four-step reverse-phase high pressure liquid chromatography (rpHPLC) process similar to that described by Evans *et al.* (1991). Gradients of water and acetonitrile with either 0.1% TFA or heptafluorobutyric acid (HFBA) were used as solvents. For steps 1–3, a Whatman Partisil 5 ODS-3 column (C18, 5 μm particle size, 250mm×4.7mm) was used with a Ranin Rabbit HPLC machine. For step 4, an Aquapore OD-300 column (C18, 250mm×1 mm) was used with an Applied Biosystems micro separation system HPLC machine. Step 1: flow 1mlmin^{-1}; gradient 10%–85% acetonitrile over 65min; fractions 1ml; counterion TFA. Step 2: flow 1mlmin^{-1}, gradient 10%–40% acetonitrile over 60min; fractions 1 ml; counterion TFA; samples were oxidized by incubation in 1.5% hydrogen peroxide for at least 4h prior to step 2. Step 3: flow 1mlmin^{-1}; gradient 14%–38% acetonitrile over 58min; fractions 1ml; counterion HFBA. Step 3′: flow 1mlmin^{-1}; gradient 10%–32% acetonitrile over 68min; fractions 1ml; counterion HFBA. Step 4: flow 0.09mlmin^{-1}; gradient 0%–40% acetonitrile over 75min; fractions collected corresponded to ultraviolet absorbance peaks (214nm); counterion TFA. Fractions containing FMRFamide-like immunoreactivity were detected by radioimmunoassay (RIA) with antibody 671c as described by Marder *et al.* (1987), combined as necessary, and evaporated under vacuum to achieve the desired volume for the next step of purification and sequencing.

Sequencing of purified peptides was carried out at the Emory University Microchemical Facility by Dr Jan Pohl using an automated Edman degradation on an Applied Biosystems model 477A protein sequencer with on-line phenyltiohydantion (PTH) analyzer. In each sequence, contaminating amino acids detected by the PTH analyzer were at significantly lower levels than was the designated amino acid in the sequence, and Dr Pohl approached each sequence unaware of the expected result.

Results

Isolation and sequence of RFamide peptides from the STN

Trimmer *et al.* (1987) isolated and sequenced SDRNFLRFamide and TNRNFLRFamide from the nervous system of the lobster *Homarus americanus*. Marder *et al.* (1987) measured the amounts of chromatographically (rpHPLC) and immunologically similar peptides in extracts from ganglia of the rock crab *C. borealis*. To determine whether authentic SDRNFLRFamide and TNRNFLRFamide are present in the stomatogastric nervous system of *C. borealis*, we employed a four-step rpHPLC purification procedure, similar to that previously employed to isolate FMRFamide-

like peptides from central nervous system (CNS) extracts of the leech *Hirudo medicinalis* (Evans *et al.* 1991). Extracts of crab tissues were made in acidified methanol (see Materials and methods), desalted and then applied to rpHPLC. Fractions from one rpHPLC step, which co-migrated with synthetic SDRNFLRFamide and TNRNFLRFamide and contained significant levels of immunoreactivity, as determined by an FMRFamide peptide RIA (Marder *et al.* 1987), were passed to the subsequent rpHPLC step. Synthetic SDRNFLRFamide and TNRNFLRFamide migrated very close to one another in each rpHPLC step, with SDRNFLRFamide always having a slightly longer retention time than TNRNFLRFamide (Fig. 1Di–iii).

We used an extract of 35 crab brains to develop our purification procedure and were able to isolate and sequence TNRNFLRF (Table 1). Because the sequenced peptide co-migrated exactly with synthetic TNRNFLRFamide in our last rpHPLC step (data not shown), and because the RIA that we used to detect the peptide is highly specific for amidated peptides (Marder *et al.* 1987), we conclude that the brain of *C. borealis* contains authentic TNRNFLRFamide.

Extracts from 101 stomatogastric nervous systems (each containing one stomatogastric ganglion, one oesophageal ganglion and two commissural ganglia) were processed with the same purification scheme. In step 2 of the procedure, two immunoreactive fractions that roughly co-migrated with SDRNFLRFamide and TNRNFLRFamide were independently processed, one with step 3 and one with step 3′.

The step 2 fraction processed with step 3 yielded two immunoreactive fractions that co-migrated with SDRNFLRFamide and TNRNFLRFamide. These fractions were designated fractions 50 and 51, respectively, and were processed independently in step 4 (Fig. 1A,B). Fraction 50 (Fig. 1) yielded a single immunoreactive peak in step 4 (Fig. 1A) that exactly co-migrated with TNRNFLRFamide (Fig. 1Di) and was subsequently sequenced as TNRNFLRF (Table 1). Fraction 51 (Fig. 1B) yielded two immunoreactive peaks in step 4, which co-migrated exactly with SDRNFLRFamide and TNRNFLRFamide (Fig. 1Di–iii). The yield of pure peptides was not high enough to obtain sequence data.

The step 2 fraction processed with step 3′ yielded an immunoreactive fraction that co-migrated with SDRNFLRFamide and TNRNFLRFamide. This fraction was designated fraction 71 and was processed independently in step 4 (Fig. 1C). Fraction 71 (Fig. 1C) yielded two immunoreactive peaks in step 4, one of which co-migrated exactly with TNRNFLRFamide (Fig. 1Di) and was subsequently sequenced as TNRNFLRF (Table 1). The other immunoreactive peak had a shorter retention time than either SDRNFLRFamide or TNRNFLRFamide and the yield of pure peptide was not high enough to obtain sequence data.

The purified peptides sequenced as TNRNFLRF co-migrated exactly with TNRNFLRFamide and were detected by our RIA, which is specific for amidated peptides (Marder *et al.* 1987). Therefore, we conclude that the stomatogastric nervous system of *C. borealis* contains authentic TNRNFLRFamide. Moreover, because we were able to detect an immunoreactive peptide that co-migrated exactly with SDRNFLRFamide, we conclude that SDRNFLRFamide is also likely to be present.

Activation of the pyloric rhythm by SDRNFLRFamide and TNRNFLRFamide

Bath application of SDRNFLRFamide and TNRNFLRFamide can activate pyloric and gastric rhythms in preparations in which neither rhythm is being expressed. The schematic drawing shown in Fig. 2A illustrates the experimental recording sites and conditions used to collect the data shown in Fig. 2B,C. The STG was isolated from

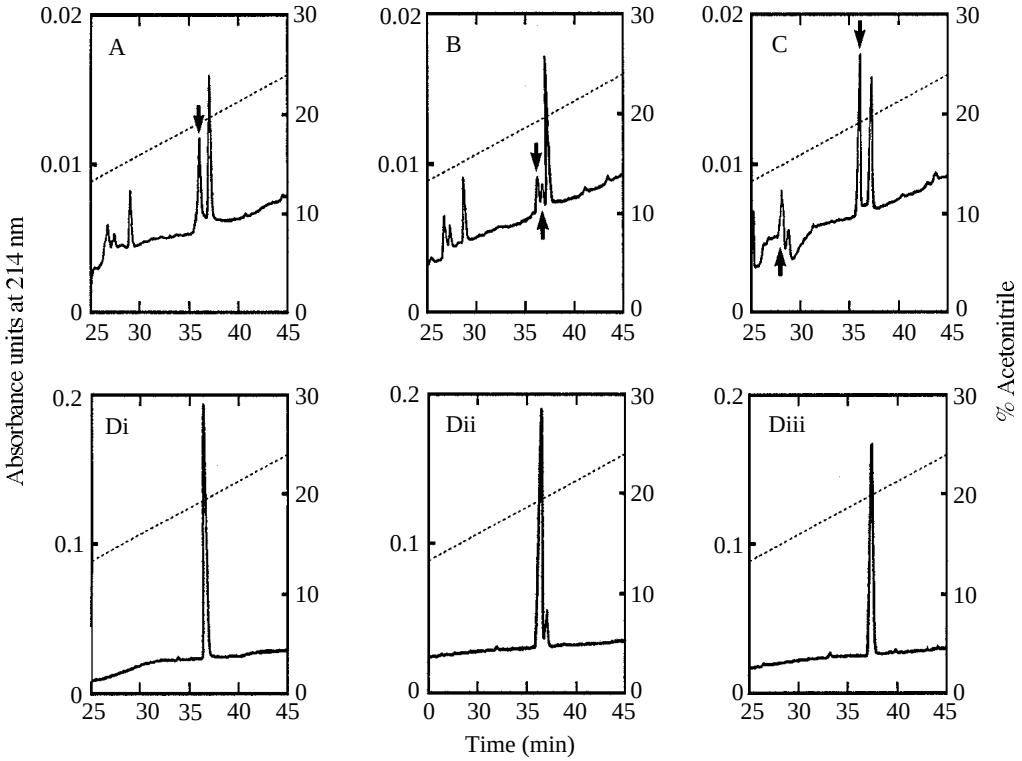

Fig. 1. Chromatograms of three independent peptide purification runs (A–C) on rpHPLC step 4 and of peptide standards, TNRNFLRFamide and SDRNFLRFamide, run on rpHPLC step 4 (Di–iii). (A) Fraction 50 from rpHPLC step 3. One immunoreactive peak (downward arrow) was detected, that co-migrated with TNRNFLRFamide and was sequenced as TNRNFLRF (see Table 1). (B) Fraction 51 from rpHPLC step 3. Two immunoreactive peaks (arrows) were detected; one (downward arrow) co-migrated with TNRNFLRFamide and the other (upward arrow) co-migrated with SDRNFLRFamide. (C) Fraction 71 from rpHPLC step 3′. Two immunoreactive peaks (arrows) were detected; one (downward arrow) co-migrated with TNRNFLRFamide and was sequenced as TNRNFLRF (see Table 1); the other (upward arrow) migrated at a retention time earlier than TNRNFLRFamide or SDRNFLRFamide. (D) 1nmol of synthetic TNRNFLRFamide (i) and 1nmol of synthetic TNRNFLRFamide plus 0.1nmol of synthetic SDRNFLRFamide (ii) and 1nmol of synthetic SDRNFLRFamide (iii) run on rpHPLC step 4 to determine their retention times. In each chromatogram, absorbance at 214nm (right vertical axis, solid line) and percentage acetonitrile in the rpHPLC gradient (left vertical axis, dashed line) are plotted *versus* time during the 75min gradient of 0%–40 % acetonitrile. In A, B and C, fractions were judged to be immunoreactive if they contained more than 200fmol of FMRFamide-like immunoreactivity in a 2 μl sample. The limits of detection of the RIAs were between 20 and 50fmol.

Table 1. *Sequence data from* Cancer borealis *FMRFamide-like peptides*

	Cycle number							
	1	2	3	4	5	6	7	8
Source	T	N	R	N	F	L	R	F
From brain extracts	17	20	9	45	45	42	19	11
From step 3, fraction 50, stomatogastric ganglion extracts	8	9	4	10	10	13	8	2
From step 3′, fraction 71, stomatogastric ganglion extracts	10	14	6	14	15	20	6	5

The identity and amount (in pmol) of the predominant amino acid detected in each sequence cycle for three peptides sequenced. In each case there was no detectable amino acid in cycle 9.

anterior ganglia inputs by placing sucrose in the Vaseline well, as indicated. An intracellular recording electrode was placed in the soma of the pyloric dilator (PD) motor neurone. Extracellular recording electrodes were placed on the nerves containing the axons of the other motor neurones of the stomatogastric nervous system. The dorsal ventricular nerve (dvn) is a large nerve that carries the axons of many of the motor neurones that participate in both the pyloric and gastric mill rhythms. The medial ventricular nerve (mvn) carries the axons of the ventricular dilator (VD) and inferior cardiac (IC) neurones. The dorsal gastric nerve (dgn) carries the axon of the DG motor neurone, an important component of the gastric mill network, as well as the axon of a sensory neurone.

Under control conditions (Fig. 2B), the PD neurone membrane potential was flat, the dvn recording showed some intermittent activity, the mvn was silent, and the dgn recording showed high-frequency activity in the small sensory unit. 90s after application of 10^{-7} mol l^{-1} SDRNFLRFamide (right-hand panel), the DG neurone started to fire in high-frequency bursts (seen as bursts of the large unit in the dgn trace). Increased firing in the dvn is due in part to activation of the lateral pyloric (LP) neurone, which evokes IPSPs that can be seen in the PD neurone trace before it starts to fire. As the PD neurone started to fire, the pyloric rhythm was expressed as the rapid and repeating sequence of activity seen in the dvn trace and the activity in the mvn trace. Fig. 2C shows that 10^{-7} mol l^{-1} TNRNFLRFamide produces the same general sequence of events, including the activation of the DG neurone and rhythmic pyloric activity.

The activation of the full pyloric rhythm by the peptides is illustrated more clearly in Fig. 3, in which the time bases are expanded to illustrate the alternation among the components of the pyloric rhythm. These recordings were taken from the same preparation as that shown in Fig. 2, but the peptide traces shown were later in the peptide applications than the transients shown in Fig. 2. Each set of recordings shows simultaneous intracellular recordings from the PD and LP neurones, which fire out of phase with each other. Under control saline conditions, the LP fired irregularly, with each action potential evoking unitary IPSPs in the PD neurone. After several minutes of peptide application, the LP and PD neurones fired alternating bursts of action potentials. The extracellular recording of the dvn additionally shows the activity of the pyloric (PY) neurones.

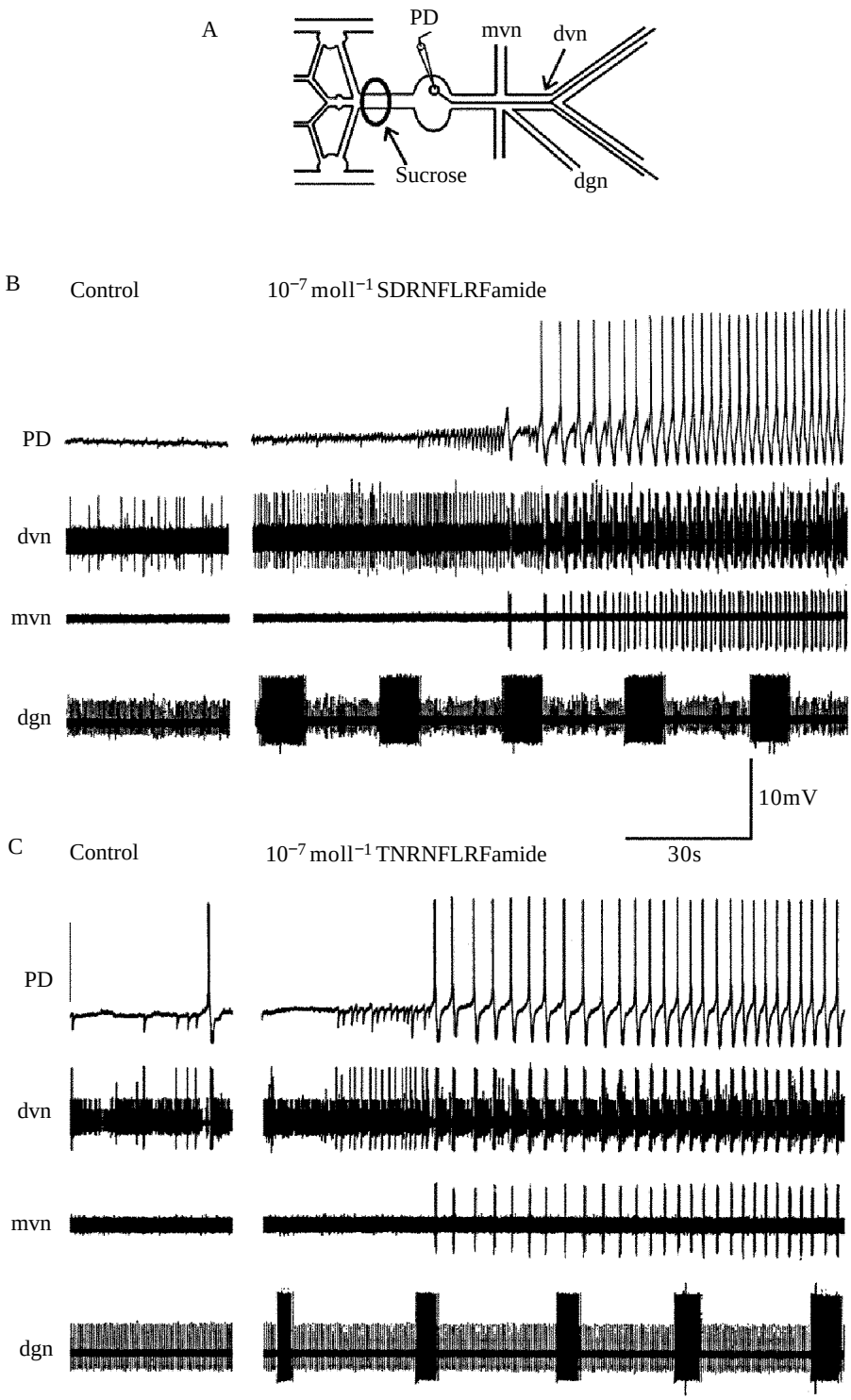
A
PD
mvn
dvn
Sucrose
dgn
B
Control
10⁻⁷ moll⁻¹ SDRNFLRFamide
PD
dvn
mvn
dgn
10mV
C
Control
10⁻⁷ moll⁻¹ TNRNFLRFamide
30s
PD
dvn
mvn
dgn

Fig. 2

In the preceding figures the stn was blocked, and no rhythmic activity was observed in the control situation. If the inputs from the other ganglia (paired CG and single OG) are left intact, a full pyloric rhythm oscillating at 1–2Hz is observed. Under these conditions, application of either peptide has relatively little effect on the pyloric rhythm, as measured by change in burst frequency, phase or burst duration or in the number of spikes per burst. Fig. 4 illustrates the state-dependence of SDRNFLRFamide's effects on the frequency of the pyloric rhythm. The diagonal line represents no frequency change resulting from the application of 10^{-7} mol l^{-1} SDRNFLRFamide. In preparations with starting pyloric frequencies greater than 1Hz, SDRNFLRFamide slowed the rhythm (filled circles below the line) or had little effect (open symbols near the line). In contrast, preparations that showed low initial frequencies (below 0.4Hz) were strongly activated by the peptide, as demonstrated by the points above the line in Fig. 4, and in the examples used in Figs 2 and 3.

The state-dependence of the peptide's actions described above makes it relatively difficult to generate dose–response curves, unless the preparation is relatively inactive before peptide application and activity returns to control levels during the washes between each application. However, in several experiments it was possible to generate full dose–response curves for the action of one or both peptides on the same preparation. The data from one of these is shown in Fig. 5. The sequence of peptide application started with the lowest concentration. There was a 45min wash between each application, during which the activity of the preparation returned to pre-application levels. The threshold concentration for any change in the activity of pyloric neurones was between 10^{-12} mol l^{-1} and 10^{-11} mol l^{-1}. At these low concentrations, some increases in firing rate of individual neurones were seen without activation of AB/PD rhythmicity (AB is the anterior burster neurone). As the concentrations were increased above 10^{-11} mol l^{-1}, the pyloric frequency increased (Fig. 5), but never exceeded 1.4Hz. Because of the state-dependence of the change in frequency produced by both peptides, we were not able reliably to measure the dose-dependence of TNRNFLRFamide and that of SDRNFLRFamide in the same preparation enough times to determine whether these peptides are significantly different in their action.

Measurement of the change in number of spikes per burst in individual neurones is sometimes a good indicator for the targets of peptide action. Table 2 shows the number of spikes per burst for the LP, IC and VD neurones. The IC and VD neurones showed a significant increase in the number of spikes per burst in the presence of 10^{-7} mol l^{-1} SDRNFLRFamide. This increase of a few spikes per burst may be a consequence of the increased pyloric frequency rather than of a direct action of the peptide on these neurones.

Fig. 2. (A) Schematic diagram of the experimental configuration. The stomatogastric ganglion (STG) was isolated by placing sucrose in a well around the stomatogastric nerve. (B) Simultaneous intracellular recordings from a PD neurone and extracellular recordings from the dorsal ventricular nerve (dvn), the medial ventricular nerve (mvn) and the dorsal gastric nerve (dgn) in control saline. Initiation of pyloric rhythm by SDRNFLRFamide. (C) Same as in B, in the presence of 10^{-7} mol l^{-1} TNRNFLRFamide. The most hyperpolarized membrane potentials of the PD neurone were −63mV in B and −64mV in C.

Although many neuromodulators produce significant changes in the phase of the pyloric rhythm (Hooper and Marder, 1987; Flamm and Harris-Warrick, 1986*a*), SDRNFLRFamide and TNRNFLRFamide do not seem to alter the phase relationships of

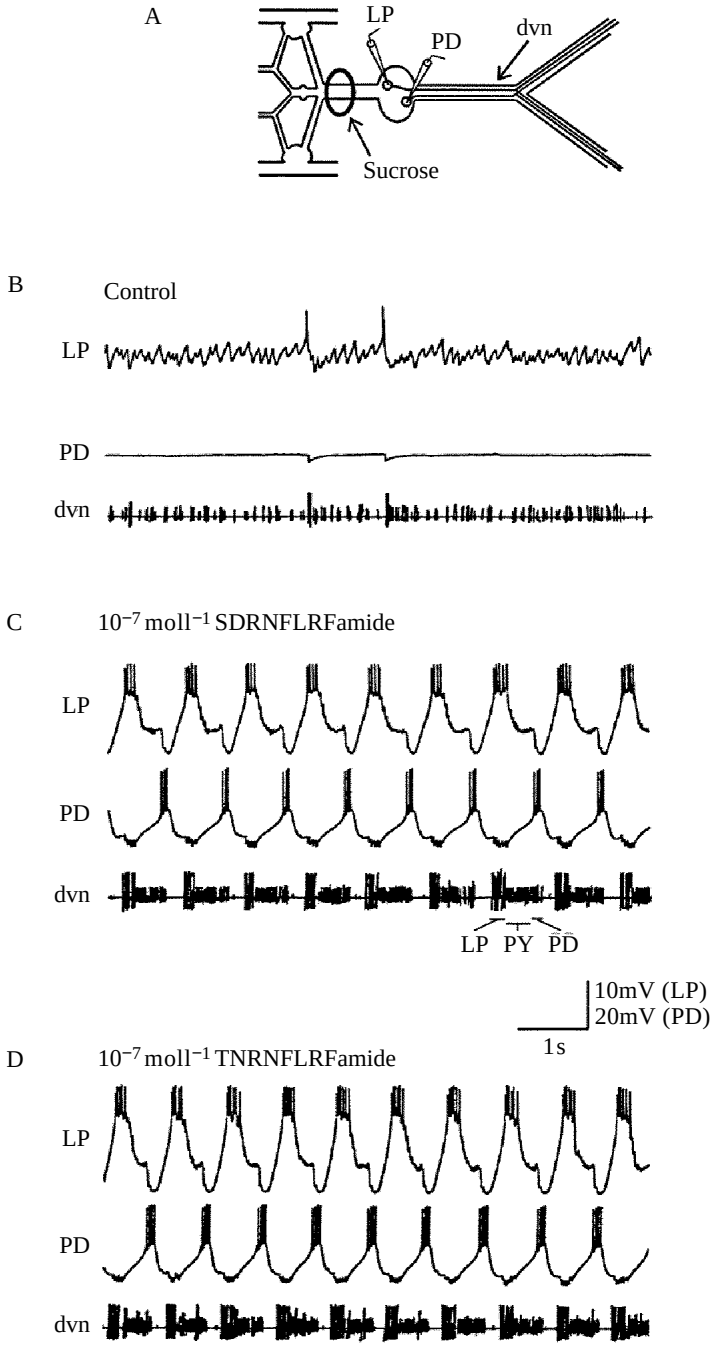

Fig. 3

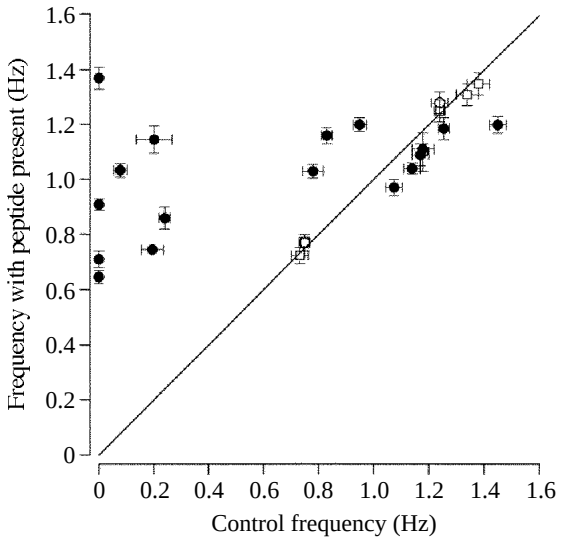

Fig. 4. State-dependence of the activation of the pyloric rhythm by 10^{-7} mol l^{-1} SDRNFLRFamide. Each point is the mean ± S.D. for 10 bursts in the presence of peptide. The *x*-axis shows the frequency of the pyloric rhythm recorded in saline just prior to peptide application. The *y*-axis shows the frequency recorded in the presence of peptide. The straight line indicates an unchanged frequency following peptide application. Each control frequency was compared to its peptide frequency in a *t*-test for differences beween two means. *P* values are: filled circles $P<0.001$; open circles $P<0.05$; open squares $P>0.05$.

the neurones in existing pyloric rhythms significantly (Fig. 6). There are five phases to the pyloric rhythm. The cycle starts with the AB/PD burst followed by a short quiescent period, then the LP neurone and finally the PY neurones fire. The IC and VD neurones have variable phases in relation to the AB/PD:LP:PY phases. The IC neurone fires before or during the LP burst, whereas the VD neurone fires within the PY burst. Applications of the peptides do not drastically alter this phase relationship (Fig. 6). The average phases from 19 preparations with initial frequencies between 0.8 and 1.3Hz in control saline are plotted. These are the typical phase relationships of the PD, LP, PY, IC and VD neurones. The AB neurone fired in phase with the PD neurones. After application of 10^{-7} mol l^{-1} SDRNFLRFamide, the phase relationships of the pyloric neurones changed only slightly (Fig. 6B). The onset of the IC and VD bursts occurred earlier, resulting in a longer burst duration for these two neurones. However, none of the changes in the onset and offset times was statistically different from control values.

Fig. 3. Activation of pyloric activity. (A) Schematic diagram of the experimental configuration. (B) Simultaneous intracellular recordings from the LP and PD neurones and an extracellular recording from the dvn in control saline. (C) Activation of pyloric rhythmicity by SDRNFLRFamide. LP and PD fire in antiphase and the PY neurones fire after the LP. (D) Activation of the pyloric rhythm by TNRNFLRFamide. The most hyperpolarized points of membrane potential were: (B) PD, −50mV; LP, −70mV; (C) PD, −63mV; LP, −75mV; (D) PD, −64mV; LP, −80mV.

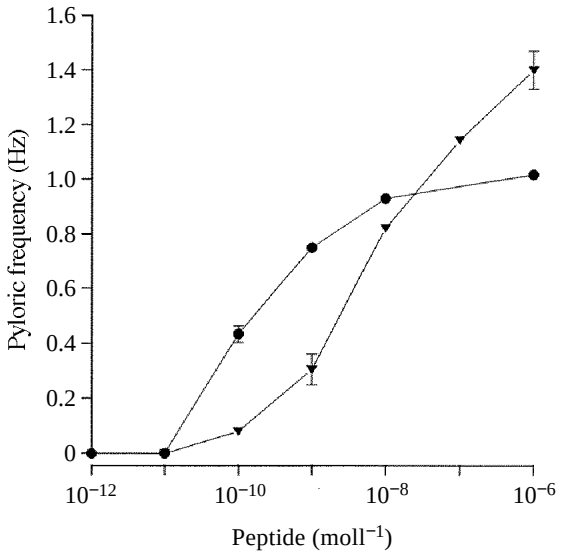

Fig. 5. Dose–response curve from one experiment showing the pyloric frequency (mean ± S.D. of 10 bursts) as a function of SDRNFLRFamide (triangles) and TNRNFLRFamide (circle) concentration.

Activation of the gastric rhythm by SDRNFLRFamide and TNRNFLRFamide

When TNRNFLRFamide and SDRNFLRFamide are applied to preparations in which the inputs from anterior ganglia are intact, gastric rhythms are often initiated (37 out of 49 preparations) (Fig. 7). In the experiment shown in Fig. 7, the DG neurone (monitored in the dgn) was bursting weakly in control conditions, but the other gastric elements were not rhythmically active. The VD and IC neurones were firing in pyloric time (Fig. 7B). After application of 10^{-7} mol l^{-1} SDRNFLRFamide (Fig. 7C), the DG neurone started firing more intense and high-frequency bursts, and the lateral gastric (LG) and gastric mill (GM) neurones fired in antiphase with the DG neurone. SDRNFLRFamide also increased the frequency of the pyloric rhythm (see mvn trace). However, note that the activity patterns of the VD and IC neurones show not only fast bursts timed to the pyloric rhythm but also a slower rhythm, in time with the gastric rhythm. In fact, the VD neurone stopped firing entirely during the LG burst, and fired during the DG burst with pyloric-timed interruptions.

Table 2. *The number of spikes per burst for LP, IC and VD neurones*

	Control	10^{-7} mol l^{-1} SDRNFLRFamide
LP	5.8±0.7	7.2.±0.7
IC	2.3±0.4	3.7±0.4*
VD	0.6±0.2	2.1±0.3**

N=13; **P*<0.05; ***P*<0.01; Student's *t*-test.
Values are mean ± S.E.M.

The characteristic firing phases of the SDRNFLRFamide-evoked and TNRNFLRFamide-evoked gastric rhythms are illustrated in Fig. 8, which pools data from 19 preparations. Once again, note that the VD and IC neurones show periods of activity at specific gastric phases, but that they retain their phase relationships within the pyloric rhythm during the time that they are active.

Gastric rhythms activated by co-application of muscarinic agonists and peptides

Although SDRNFLRFamide and TNRNFLRFamide often initiated full and robust gastric rhythms, peptide application sometimes activated the DG neurone without full gastric activity in all the remaining neurones. However, co-application of 10^{-7} mol l^{-1} SDRNFLRFamide or TNRNFLRFamide with a low concentration (10^{-6} mol l^{-1}) of the muscarinic agonist pilocarpine produced robust gastric rhythms in 21 out of 25 preparations. Moreover, the gastric rhythms expressed in these cases tended to be more regular than those elicited by either peptide alone. The reliable activation caused by co-application of these agents allows us to describe in full the pattern of the gastric rhythm in crabs (Fig. 9).

In control saline (not shown) PD, VD, IC, interneurone 1 (INT1) and medial gastric

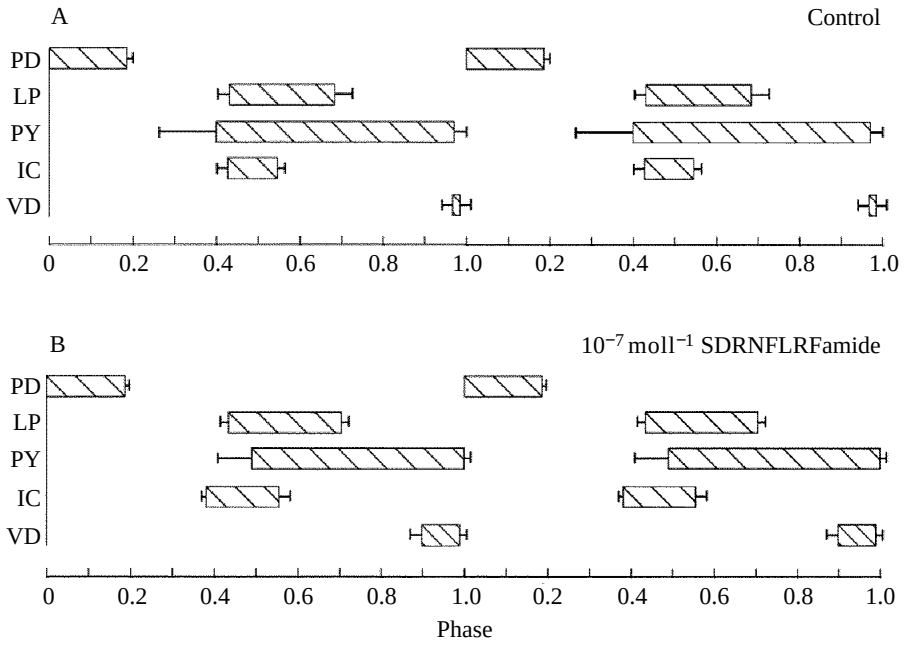

Fig. 6. (A) The effect of 10^{-7} mol l^{-1} SDRNFLRFamide on the phase of the neurones in the pyloric rhythm. 0 phase is the start of the PD neurone burst. Cycle period is the time between the onset of two successive PD bursts. Other phases were calculated as the time to the onset and termination of the neurone's activity divided by the cycle period. These data are means ± S.D. from 19 preparations with existing pyloric activity in control saline. Two cycles of activity are plotted. The onset and offset of spiking in each neurone showed no significant difference between the control value and the value during peptide application (Student's *t*-test, $P>0.05$).

(MG) neurones were firing in pyloric time while LG, GM and DG were silent. Application of SDRNFLRFamide (10^{-7} mol l^{-1}) and pilocarpine (10^{-6} mol l^{-1}) initiated gastric activity in which INT1, MG, VD and IC fired in gastric-timed bursts (Fig. 9). The

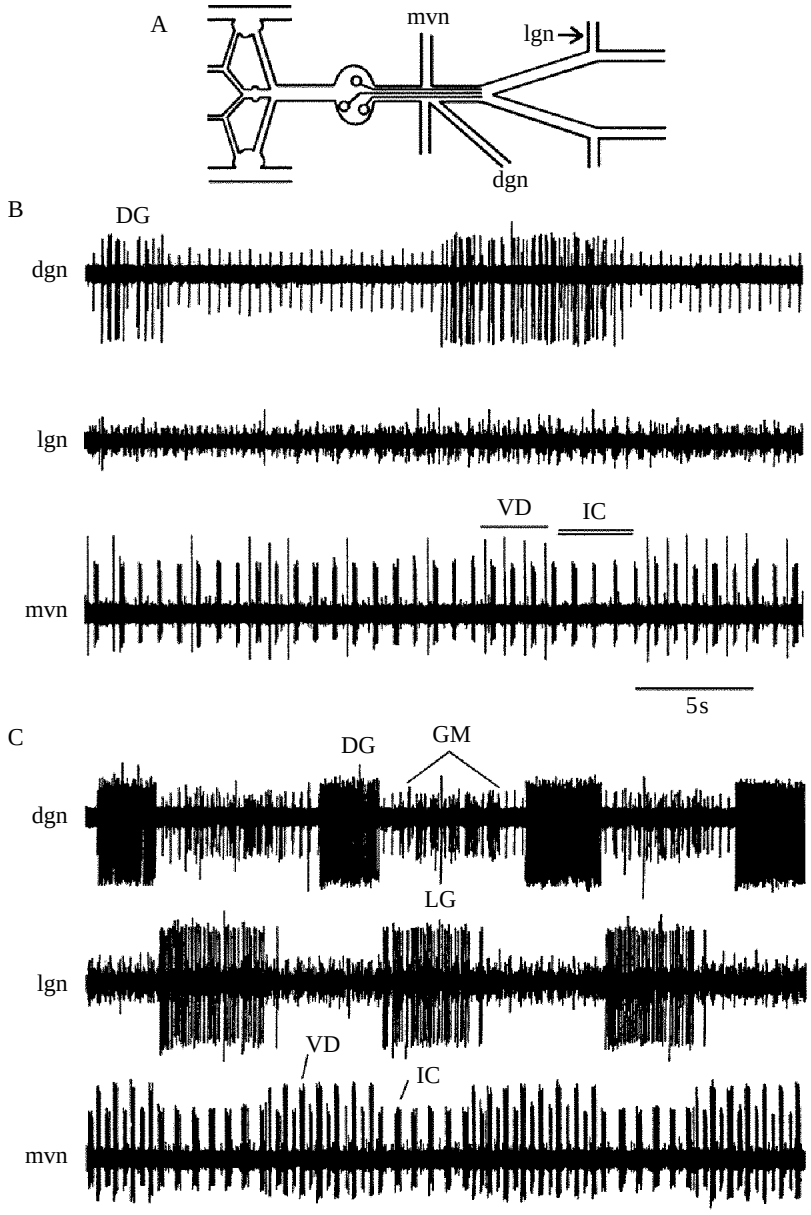

Fig. 7. Activation of the gastric network by SDRNFLRFamide. (A) Schematic drawing of the experimental preparation showing the unblocked stn. (B) In control saline, DG fired periodically (large unit in dgn trace) and a pyloric rhythm was evident (seen in the mvn trace). (C) After application of 10^{-7} mol l^{-1} SDRNFLRFamide, a gastric rhythm emerged. The gastric rhythm is seen as the rhythmic alternation of the DG, GM and LG neurones. lgn, lateral gastric nerve.

pyloric rhythm, as monitored by PD, was not altered significantly. In this experiment, MG was firing pyloric bursts that increased in spike intensity from the beginning of the DG burst into the initial phase of the LG burst. It is clear, in this case, that the individual bursts were in pyloric time, but the pattern shows an overall gastric envelope. The IC neurone also showed this pyloric activity with increased firing rate within a gastric envelope. In this example, the IC neurone produced a double burst in the gastric cycle (see figure legend).

Peptide activation of plateau and oscillatory properties of the DG neurone

The DG neurone plays a critical role in the gastric rhythm. The activation of bursting and plateau properties in the DG neurone by TNRNFLRFamide and SDRNFLRFamide appears to play a significant role in the activation of the gastric rhythm. SDRNFLRFamide and TNRNFLRFamide enhance the ability of the DG neurone to generate plateau potentials (Fig. 10). In this preparation, the STG was isolated from

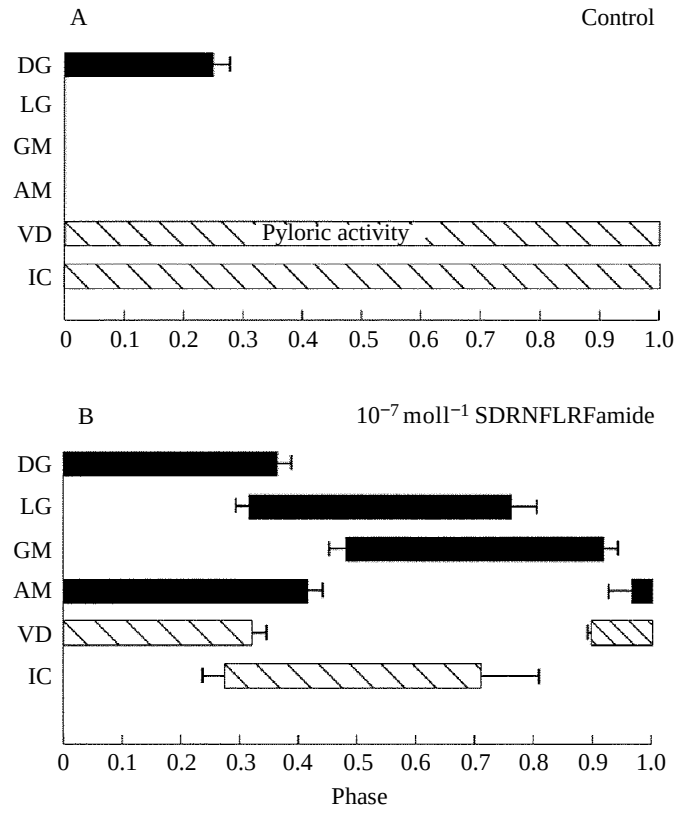

Fig. 8. Phase plots of peptide-activated gastric activity. (A) In control saline with the stn intact, most of the gastric neurones are silent and VD and IC burst in pyloric time (hatched). In 6/19 preparations, the DG was spontaneously active (solid bar). (B) After bath application of SDRNFLRFamide (10^{-7} mol l^{-1}), gastric activity was initiated. The VD and IC neurones fire within a gastric envelope but also maintain their pyloric activity within the gastric envelope. Error bars are standard deviations about the mean.

inputs by blocking impulse traffic in the stn with sucrose. In control saline, when the DG neurone was depolarized it fired action potentials only during the time of the injected current (Fig. 10A). After TNRNFLRFamide (10^{-7} mol l^{-1}) had been applied to the STG,

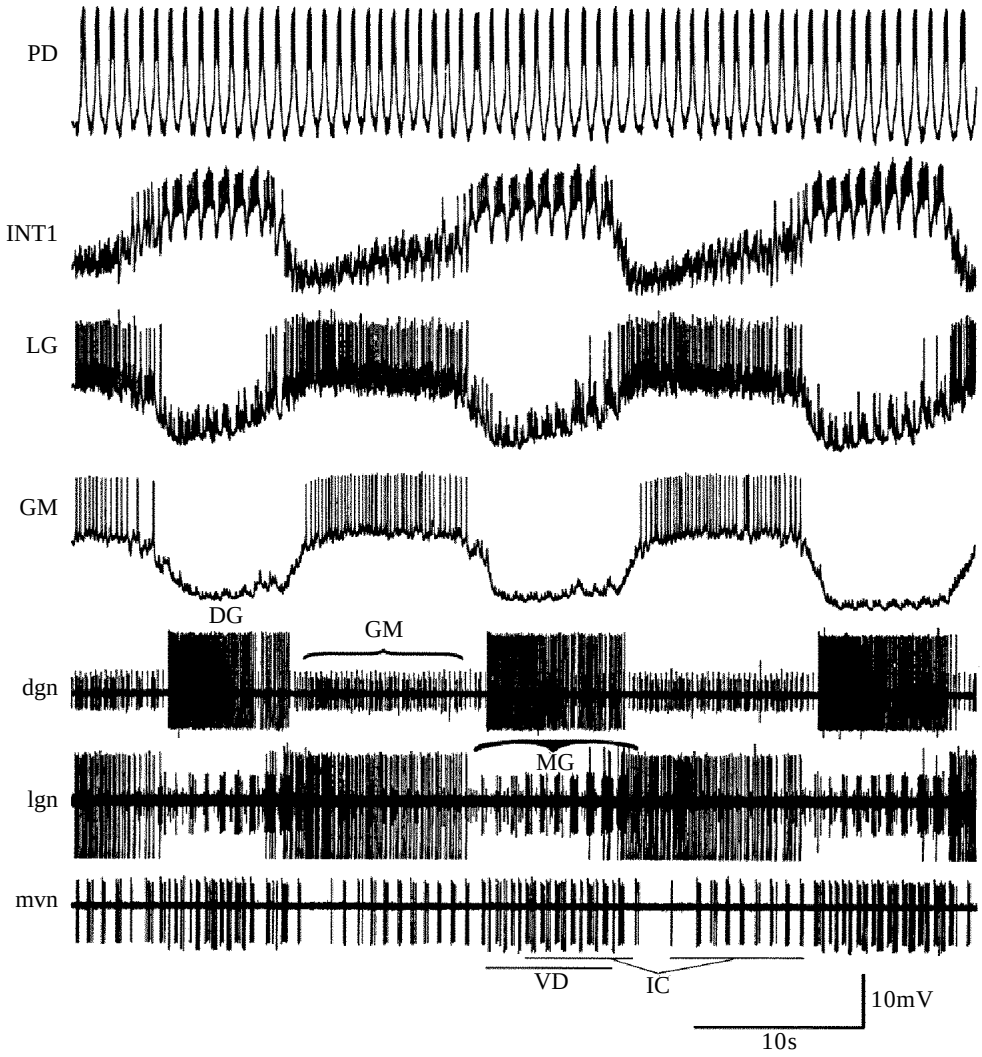

Fig. 9. Simultaneous intracellular recordings from the PD, INT1, LG and GM neurones and extracellular recordings from the dgn, lgn and mvn show activity patterns of the gastric rhythm. The preparation was bathed in 10^{-7} mol l^{-1} SDRNFLRFamide and 10^{-6} mol l^{-1} pilocarpine. In this example, LG, GMs and DG fired in a gastric-like pattern only while INT1, MG, VD and IC fired pyloric bursts within a gastric envelope. The MG produces the smaller spikes under the bracket. VD is inhibited during the LG burst, whereas IC produces double bursts, first at the end of the VD burst and again at the end of the LG burst. These recordings show patterns of activity representative of those seen in 35 preparations in which SDRNFLRFamide and pilocarpine were co-applied. The most hyperpolarized point of the membrane potentials were: PD, −62mV; INT1, −51mV; LG, −58mV; GM, −68mV.

the DG neurone started to generate irregular bursts of action potentials (Fig. 10C). The depolarized phase of these plateaux could be prematurely terminated by short pulses of hyperpolarizing current (Fig. 10B), as is typical for plateau potentials. Short pulses of depolarizing current triggered sustained depolarized plateaux that outlasted the depolarizing pulse (Fig. 10B). Several minutes after the peptide has been removed from the bath, these plateau potentials could no longer be elicited (Fig. 10D).

In some experiments, TNRNFLRFamide or SDRNFLRFamide appeared to cause the DG neurone to develop properties characteristic of a true oscillator. Fig. 11 illustrates the behaviour of a DG neurone in 10^{-7} mol l^{-1} TNRNFLRFamide as a function of the amount of injected current. The inset in Fig. 11 is a plot of the period of the stable bursts that were seen 1–2min after the current had been applied as a function of the injected current. Between imposed depolarizations, the neurone recovered to its baseline activity pattern. Without injected current (0nA), the period of the DG burst was 43s (see inset). Injection of 0.2nA of current caused the DG neurone to produce a burst of action potentials followed by irregularly timed bursts for the next 3min. The oscillations then stabilized into a regular burst pattern with a period of 26s. Injection of 0.3 nA of current caused the DG neurone to fire and burst irregularly for approximately 45 s before regular bursting, with a period of 20s, began. Depolarizing the DG neurone with 0.4nA of current brought the membrane potential to −39 mV, which is only a few millivolts more depolarized than in the previous cases (as measured in the soma). This depolarization caused sustained high-frequency firing with only two slight oscillations

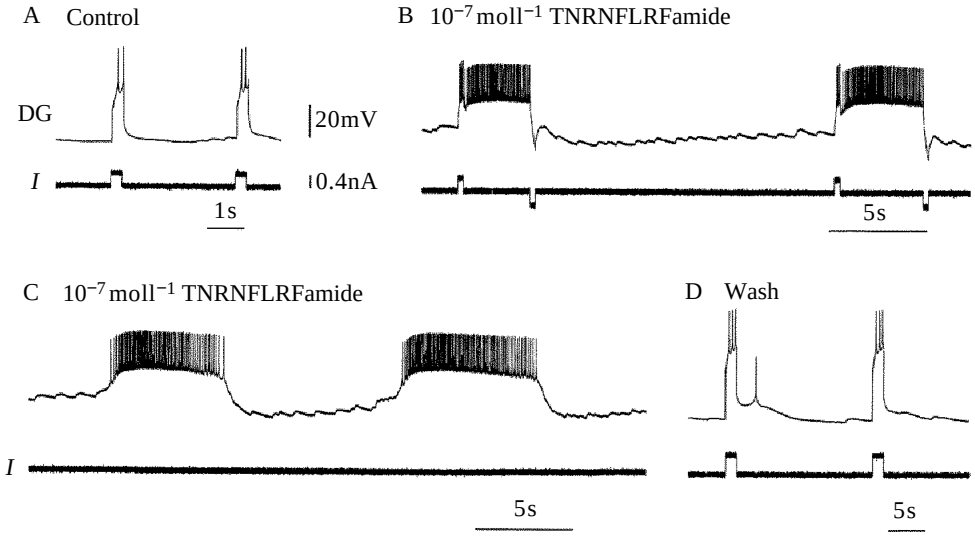

Fig. 10. TNRNFLRFamide (10^{-7} mol l^{-1}) activates plateau potentials in the DG neurone. The stn was blocked with sucrose. (A) In control saline, depolarizing current pulses (*I*) do not evoke plateau potentials. (B) Depolarizing current pulses of the same amplitude as in A can initiate plateaux that outlast the pulse and are terminated by short hyperpolarizing pulses. (C) Spontaneous plateaux recorded in the presence of TNRNFLRFamide. (D) After a wash, depolarizing current pulses no longer elicit plateaux. Resting potential, −62mV.

(barely visible at this sweep speed). After 2min, a regular burst pattern with a period of 18 s emerged.

These experiments demonstrate that SDRNFLRFamide and TNRNFLRFamide not

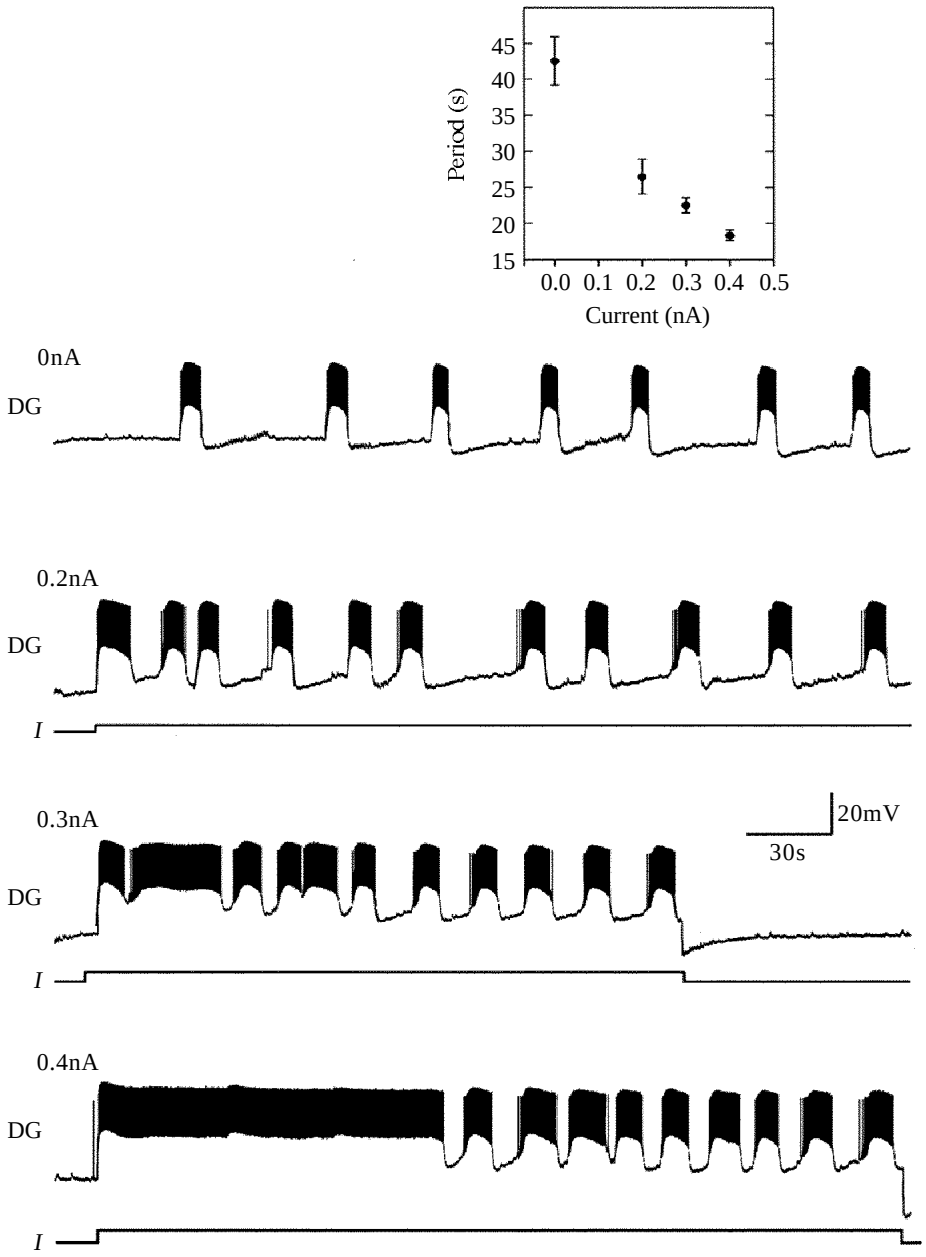

Fig. 11. The effect of membrane potential on the period of spontaneous DG bursts of a preparation bathed in 10^{-7} mol l^{-1} TNRNFLRFamide. The inset shows the resultant period (mean ± S.D.) of the DG with respect to the amount of current injected (*I*) into the soma. The stn was blocked with sucrose. Interburst membrane potential, −62mV.

only induce plateau potentials in the DG neurone but also activate membrane currents that are sensitive to potential.

Control of neuronal switching in the STG

Many neurones in the STG spontaneously switch from a gastric activity pattern to a pyloric pattern and *vice versa*. Bath applications of SDRNFLRFamide and TNRNFLRFamide allowed experimental control of pyloric/gastric switching (Fig. 12). In control saline (Fig. 12A) (anterior inputs intact), two 'gastric' neurones, the lateral posterior gastric (LPG) and INT1, were firing in time with the pyloric rhythm, while LG was inhibited in pyloric time. The DG neurone (bursts in dgn trace) was the only neurone firing in gastric time. Five minutes after the application of 10^{-7} mol l^{-1} SDRNFLRFamide and 10^{-6} mol l^{-1} pilocarpine (Fig. 12B), the intensity and duration of the gastric bursts were increased. Note that the LG now fired in a 'hybrid pattern' and that INT1 activity was inhibited by each gastric-timed LG burst. Ten minutes later (Fig. 12C), a strong gastric rhythm was evident. Note that LG had lost its pyloric-timed activity. INT1 retained its pyloric-timed modulation, but fired in alternation with LG. The LPG neurone retained its pyloric-timed pattern. In summary, INT1 and LG have switched from pyloric-timed to gastric-timed activity in this modulatory environment.

Discussion

TNRNFLRFamide and SDRNFLRFamide

Since the first discovery of FMRFamide-like peptides in molluscs (Price and Greenberg, 1977), a large number of related peptides have been purified from many different phyla (Evans *et al.* 1991; Cottrell, 1989; Cowden *et al.* 1989; Dockray *et al.* 1983; Grimmelikhuijzen *et al.* 1988; Kingan *et al.* 1990; Krajniak and Price, 1990; Price *et al.* 1987; Robb *et al.* 1989; Trimmer *et al.* 1987), and the genes encoding some of these peptides have been sequenced (Nambu *et al.* 1988; Rosoff *et al.* 1992; Linacre *et al.* 1990; Schaefer *et al.* 1985; Schneider and Taghert, 1988; Taghert and Schneider, 1990; Taussig and Scheller, 1986). Trimmer *et al.* (1987) purified and sequenced two of the FMRFamide-like peptides present in extracts of the nervous system of the lobster *Homarus americanus*. These peptides are extended forms of FLRFamide: TNRNFLRFamide and SDRNFLRFamide. In this paper we show that TNRNFLRFamide and an SDRNFLRFamide-like peptide are present in the stomatogastric nervous system of the crab *Cancer borealis*. Although Trimmer *et al.* (1987) focused their attention on the two fractions that they eventually established were TNRNFLRFamide and SDRNFLRFamide, in their initial work they saw additional FMRFamide-like immunoreactive fractions that they did not fully purify or sequence. Likewise, our chromatograms show several additional peaks that were immunoreactive, leading us to conclude that there are almost certainly other FMRFamide-like peptides in the crab nervous system that may play important biological roles. Presumably, the identity of the full FMRFamide-like family of these peptides in these crustacean species will come with the cloning and sequencing of their genes.

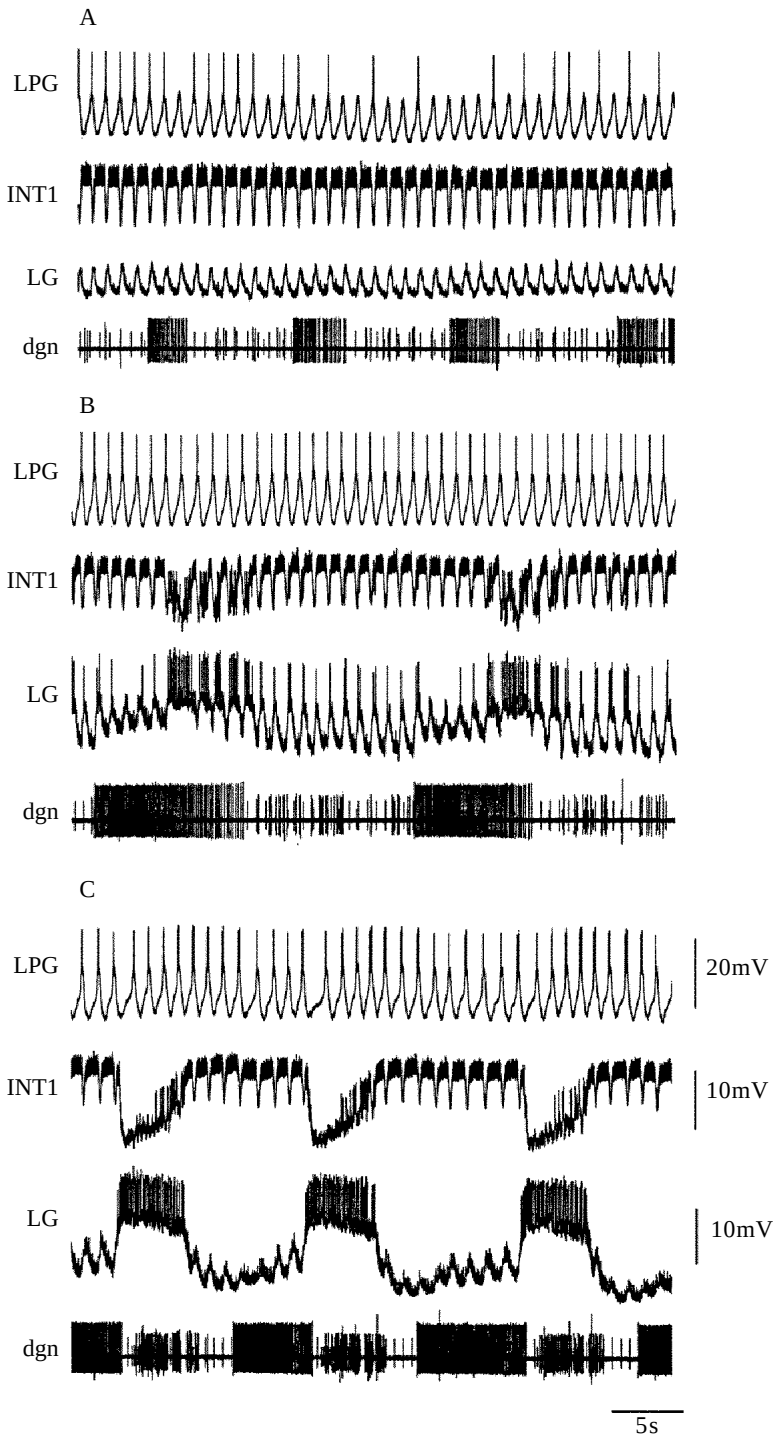
A
LPG
INT1
LG
dgn
B
LPG
INT1
LG
dgn
C
LPG
20mV
INT1
10mV
LG
10mV
dgn
5s

Fig. 12

In many cases, the first indication of the presence of a peptide in a region of the nervous system comes from the use of antibody raised against a peptide isolated from a different species, or even a different phylum. In the case of the stomatogastric nervous system, immunocytochemical results indicated the presence of one or more FMRFamide-like peptides (Hooper and Marder, 1984; Marder and Hooper, 1985), although the peptide against which the antibody had been raised was only a poor agonist in physiological studies. Indeed, it was only after Trimmer *et al.* (1987) had provided the sequence in a closely related species that reliable physiological effects at reasonably low concentrations (this paper) were seen. Not surprisingly, when we used antibodies raised against arthropod peptides, such as proctolin, RPCH and crustacean cardioactive peptide (CCAP), to demonstrate the presence of a peptide in the stomatogastric nervous system, these peptides were also good agonists (Hooper and Marder, 1984; Marder *et al.* 1986; Nusbaum and Marder, 1988; Weimann *et al.* 1992). In contrast, antibodies raised against peptides from phylogenetically distant species have sometimes shown the presence of a peptide in the stomatogastric nervous system (Goldberg *et al.* 1988) that is probably quite different from the peptide that was used to raise the antibody, because that peptide does not elicit physiological responses.

In the experiments reported in this paper, we bath-applied SDRNFLRFamide and TNRNFLRFamide. These peptides are found in numerous neurosecretory structures in crustaceans (Kobierski *et al.* 1987) as well as in neural inputs that ramify throughout the neuropile of the STG, the CGs and in the stn. Therefore, these peptides could play hormonal roles in these animals. Additionally, there are sites of release in several places in which we expect that modulation of the motor patterns produced by the STG might occur. For example, the peptides are almost certainly released into the STG neuropile where they could influence not only the STG neurones but also the terminals of stomatogastric nerve axons (SNAXes, Nusbaum *et al.* 1992). Additionally, the peptides could influence SNAX activity in the stn, or in the CGs, where some of the SNAXes originate.

Peptide activation of the pyloric rhythm

TNRNFLRFamide and SDRNFLRFamide join a list of a large number of substances that can activate the pyloric rhythm in a relatively quiescent preparation. These include amines, other peptides and muscarinic agonists (Hooper and Marder, 1984; Flamm and Harris-Warrick, 1986*a*; Marder and Weimann, 1992). The pyloric rhythms expressed in the presence of SDRNFLRFamide and TNRNFLRFamide are relatively conventional;

Fig. 12. Modulatory control of pyloric/gastric switching. All panels show simultaneous intracellular recordings from the LGP, INT1 and LG neurones and an extracellular recording of the dgn showing DG activity. (A) Control saline; note that LPG and INT1 are firing in pyloric time, LG is being rhythmically inhibited in pyloric time and DG is firing weakly in gastric time. (B) 5min after application of 10^{-7} mol l^{-1} SDRNFLRFamide and 10^{-6} mol l^{-1} pilocarpine. Note the hybrid pattern of activity in which LG is firing in pyloric time with gastric modulation. (C) 15min after modulator application. The most hyperpolarized point of the membrane potentials were: LPG, −80 mV; LG, −80 mV; INT1, −40 mV in A and −48 mV in C.

that is, they do not show the major changes in relative phasing among the elements that are produced by many of the other modulatory substances, such as proctolin (Hooper and Marder, 1987), the amines (Flamm and Harris-Warrick, 1986*a*) or CCAP (Weimann *et al.* 1992).

The work we have done thus far does not provide many indications of which neurones within the pyloric network are likely to be direct targets of TNRNFLRFamide and SDRNFLRFamide. Pyloric network activation is often accomplished by direct activation of bursting in the anterior burster (AB) neurone (Flamm and Harris-Warrick, 1986*b*; Hooper and Marder, 1987; Marder and Meyrand, 1989), and the AB neurone is likely to be a direct target of peptide action. However, general excitation of many of the elements of the pyloric network or of SNAXes (Nusbaum *et al.* 1992) may also account for activation of the pyloric rhythm.

We were unable to detect any obvious difference between TNRNFLRFamide and SDRNFLRFamide in the physiological experiments reported here. This may indicate that the receptors involved require an extended RNFLRFamide peptide, and that, as long as that sequence is present, other changes are relatively unimportant. However, further structure–activity work would be required to determine this.

TNRNFLRFamide and SDRNFLRFamide had state-dependent actions on the pyloric rhythm, reminiscent of the actions of proctolin (Hooper and Marder, 1987; Nusbaum and Marder, 1989*a*,*b*). Proctolin also increases the pyloric rhythm when applied to inactive or slowly active preparations, and has little or no effect on pyloric rhythm frequency when applied to preparations with starting frequencies in the 1Hz range. In the case of proctolin, the state-dependence of the activation depends on both circuit (Hooper and Marder, 1987) and cellular mechanisms (Golowasch and Marder, 1992). To determine why TNRNFLRFamide and SDRNFLRFamide produce state-dependent activation of the pyloric rhythm it will be necessary to establish which neurones are directly activated by the peptides and the biophysical mechanisms underlying these effects.

Peptide activation of the gastric rhythm

Although this paper is not intended as a comprehensive characterization of the crab gastric mill rhythm, it contains the first analysis of fictive gastric mill rhythms in *C. borealis*. TNRNFLRFamide and SDRNFLRFamide can activate gastric mill rhythms in many preparations of *C. borealis*. In those preparations in which the peptides alone fail to activate full gastric rhythms, the co-application of the peptides with low concentrations of muscarinic agonists is effective. Recent intracellular recordings from stomatogastric nerve input axons (Nusbaum *et al.* 1992) have demonstrated that some SNAXes are part of the pattern-generating circuitry of the gastric rhythm. Therefore, it is possible that both cholinergic and SDRNFLRFamide- or TNRNFLRFamide-containing inputs may be needed for full gastric activity.

TNRNFLRFamide and SDRNFLRFamide significantly modify the intrinsic membrane properties of the DG neurone. In the presence of the peptides, the DG expresses plateau properties and/or becomes a true oscillator. Other work (Katz and Harris-Warrick, 1990; Kiehn and Harris-Warrick, 1992*a*,*b*) showed that serotonin can also evoke these

properties in the *C. borealis* DG neurone, and Elson and Selverston (1992) found that muscarinic agonists activate plateau and bursting properties in the DG neurone of the lobster *Panulirus interruptus*. Kiehn and Harris-Warrick (1992*b*) suggest that the serotonin induction is produced by changes in the hyperpolarization-activated inward current I_h and in the Ca^{2+}-activated K^+ current in the DG neurone. It will be interesting to determine whether the peptide actions on the DG neurone involve modulation of the same or different conductances.

The induction of plateau and oscillatory properties in the DG neurone is an important step in the activation of the full gastric rhythm. The activation of the DG neurone is almost always the first effect of the peptides; moreover, even in preparations in which the peptides do not fully activate a gastric rhythm, they almost always evoke gastric-timed bursts in the DG neurone.

Peptide-induced pyloric/gastric switching

Previous work on the crab STG (Weimann *et al.* 1990, 1991; Marder and Weimann, 1992) demonstrated that many of the STG neurones could switch their patterns from pyloric-timed to gastric-timed activity. In this paper, we demonstrate that SDRNFLRFamide and TNRNFLRFamide can be used to produce these switches and, therefore, it is now possible to study experimentally the changes in the neurones and the circuit that are responsible for these switches. One of the aims of our future work will be to understand the cellular mechanisms that allow INT1, LG, MG, etc. to fire in pyloric time in control saline and then to fire in gastric time in the presence of peptides. The results presented in this paper indicate clearly that peptides can reorganize the motor patterns produced by a network. Indeed, one might argue that the gastric rhythm in the crab is generated by a network that is 'constructed' by the peptides, since they modify the membrane properties and synaptic connectivity of the component neurones so that the appropriate motor patterns may be produced.

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