Modulation of a central pattern generator by two neuropeptides, proctolin and FMRFamide

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The neuropeptides, proctolin and FMRFamide, increase the frequency of, and modify the motor pattern produced by, the stomatogastric ganglion (STG) of the crab, Cancer irroratus. Both proctolin-like and FMRFamide-like immunoreactivities are present in fibers in the stomatogastric nerve which terminate in the neuropile of the STG. The neural output of the STG thus appears to be modulated by at least two different groups of peptidergic input fibers.

The stomatogastric ganglion (STG) of decapod crustaceans consists of about 30 neurons which control the movements of the animal's stomach. The approximately half of the STG neurons that constitute the pyloric system are responsible for producing rhythmic dilations and constrictions of the pyloric region of the stomach, and can do so even when the STG is completely isolated from the rest of the animal. However, the frequency and the phase relations of the pyloric motor pattern are influenced by sensory stimuli and by inputs which travel in the stomatogastric nerve (STN) to the STG from the paired commissural ganglia (CG) and the single oesophageal ganglion (OG). Previous work has shown or suggested that serotonin, dopamine, octopamine, histamine, and acetylcholine are present in input fibers to the STGs of various decapod species, and that each of these substances induces reproducible and specific changes in the motor pattern. We now report that two neuropeptides originally isolated from invertebrate tissues, proctolin and FMRFamide, modulate the pattern of neural activity generated by the pyloric neurons in characteristic and different ways. Furthermore, proctolin-like and FMRFamide-like immunoreactivities are present in the neuropile of the STG and in fibers in the STN. We suggest that proctolin (or a proctolin-like substance) and FMRFamide (or a FMRFamide-like substance) are present in inputs to the STG that serve the role of modulating the neural output of the STG.

Experiments were done on 42 specimens of 200–400 g Cancer irroratus obtained from Boston fishermen and kept in sea-water aquaria until used. The stomatogastric nervous systems of crabs were dissected, desheathed, and prepared for simultaneous intracellular and extracellular recording using the techniques routinely applied to this preparation. An example of the pyloric motor pattern produced by the isolated STG of the crab in control saline is shown in Fig. 1A. The top trace is an extracellular recording from the lateral ventricular nerve (lvn) which carries the output of the lateral pyloric (lp) motor neuron, the pyloric (py) motor neurons, and the pyloric dilator (pd) motor neurons. The second trace is an extracellular recording from the pyloric nerve (pyln) and shows py neuron activity. The third and fourth traces are intracellular recordings from a PD neuron and the LP neuron. This recording illustrates the typical pattern of pyloric activity, with the LP, PY, and PD neurons firing in a sequence which is then repeated. This cyclical pattern is determined by the membrane properties of, and synaptic connections among, the pyloric neurons.
Fig. 1. Effects of FMRFamide and proctolin on the pyloric motor output. A: pyloric motor output from an isolated STG in control saline (mM: NaCl, 440; KCl, 11; MgCl₂, 26; CaCl₂, 13; Tris base 11, malic acid, 5.2; pH 7.6). The preparation was continuously superfused with chilled saline (10–12 °C) at 6–10 ml/min. The top two traces are extracellular recordings of motor output made with stainless steel pin electrodes insulated from the bath solution with vaseline. The bottom two traces are intracellular recordings from the somata of the PD neuron and the LP neuron made with glass microelectrodes (20–40 MQ) filled with 2.5 M KCl. LP neuron activity is seen extracellularly as the largest unit on the lvn as well as intracellularly. PY neuron activity is shown extracellularly on the pyn and as the medium size units on the lvn following LP neuron activity. PD neuron activity is seen intracellularly as well as extracellularly as the unit on the lvn following PY neuron activity. B: same preparation as in A, 5 min after switching the saline to one containing 3 × 10⁻⁵ M FMRFamide. C: same preparation, after 15 min of washing in control saline. D: same preparation, 5 min after switching the saline to one containing 10⁻⁶ M proctolin. In A, the most hyperpolarized point of the membrane potential excursion of the LP neuron was −65 mV and of the PD neuron was −74 mV. These remained virtually unchanged in B, C, and D.

Bath application of 10⁻⁵ M FMRFamide (Peninsula Laboratories, Inc.) caused an increase in the frequency of the pyloric cycle. In the experiment shown here (Fig. 1B), the PY neurons fired for a greater percentage of the cycle length and the LP neuron no longer fired action potentials, presumably due in part to the increase in activity of the PY neurons, which inhibit the LP neuron. In all experiments (n = 5) PY
neuron activity increased, and in all but one experiment LP neuron activity decreased. After washing the preparation with saline (Fig. 1C) the activity returned to control levels. Bath application of $10^{-6}$ M proctolin (Bachem, Inc.) was also excitatory ($n = 11$), but had very different effects than FMRFamide on the LP and PY neurons (Fig. 1D). Proctolin produced an increase in pyloric cycle frequency, an increase in the length of the LP neuron’s burst, and an increase in the number of LP neuron action potentials/burst. The intracellular recording from the LP neuron (Fig. 1D) shows an extremely rapid depolarizing phase, and an increased peak-to-trough membrane potential excursion, or ‘plateau’$^{23,24}$. The effects of both proctolin and FMRFamide were rapid in onset, and entirely reversed after 30 min of washing.

The most dramatic action of proctolin was seen on 3 isolated STG which were quiescent after removal from the animal. In each of these experiments proctolin application induced robust pyloric cycling, as can be seen in the example shown in Fig. 2. Before proctolin application to this preparation the LP neuron fired irregularly, the PD neuron showed small membrane potential oscillations, and no pyloric cycling was evident. Shortly after the application of proctolin (triangle), the LP neuron began to fire in long bursts, the PD neuron membrane potential excursions increased in amplitude, the PD neuron started to fire action potentials, and the full pyloric cycle of activity was evident. In these experiments a single proctolin application had long-lasting excitatory effects which persisted after extensive washing (the ganglia continued to display cyclical pyloric activity after washing out the proctolin, although both cycle frequency and the number of LP neuron action potentials/burst and LP burst length decreased).

To determine whether these peptides or close
structural analogs are present in the STG, we carried out immunohistochemical experiments with antisera raised against proctolin and FMRFamide conjugates. Fig. 3, a line drawing of the STG of the crab, shows that the 30 STG somata encircle a central neuropile area. Extensive FMRFamide-like (Fig. 4A) and proctolin-like (Fig. 4B) immunoreactive material was found within the neuropile of the STG, but STG somata were unstained. Both antisera stained fibers in the STN and somata in the OG and CG. The pattern of immunoreactivities suggests that different OG somata show proctolin-like or FMRFamide-like immunoreactivity (data not shown).

Preincubation of the antisera with the appropriate peptide ($2 \times 10^{-5} \text{ M}$) resulted in a complete block of the staining, but preincubation of the antisera raised against proctolin with FMRFamide (10^{-5} \text{ M}) or the antisera raised against FMRFamide with proctolin (10^{-5} \text{ M}) had no effect. Therefore it appears that the antisera define two independent sets of peptide-containing fibers which have terminals within the neuropile of the STG.

The chemical identity of the peptides responsible for the immunoreactivity still remains open. Proctolin was originally identified in and purified from Arthropod tissues, and has been isolated from a closely related decapod crustacean, *Homarus americanus*. It is thus likely, but not proven, that the

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**Fig. 4.** Distribution of FMRFamide-like and proctolin-like immunoreactivity in the neuropile of the STG. Whole mount preparations were processed by the method of Belz and Kravitz. Preparations were fixed in 4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.2, at 4°C for 10–20 h. Fixed ganglia were extensively washed in 0.1 M sodium phosphate, pH 7.2 containing 0.3% Triton-X100 and 0.1% sodium azide. Ganglia were then reacted with primary antibody (1:500 dilution of rabbit anti-FMRFamide serum (Cambridge Research Biochemicals) or 1:500 dilution of rabbit anti-proctolin serum (gift of K. K. Sewick and E. A. Kravitz)) for 10–20 h at 4°C, and then extensively washed again. Ganglia were then reacted with secondary antibody (1:20 dilution of goat anti-rabbit FITC; Boehringer-Mannheim), washed in sodium phosphate, rinsed briefly with 4 mM sodium carbonate, pH 9.5, and then mounted on glass coverslips in 80% glycerine, 20% 20 mM sodium carbonate. A: extensive neuropil processes showing FMRFamide-like immunoreactivity and fibers in the STN. B: extensive neuropil processes showing proctolin-like immunoreactivity and fibers in the STN. In both cases the 30 neurons of the STG were unstained. Calibration bars: 100 μm.
proctolin-like immunoreactivity reported here is due to proctolin. With regard to the FMRFamide-like immunoreactivity, however, the issue is less certain. FMRFamide was originally isolated from extracts of molluscan tissue, and has not to date been biochemically isolated from a crustacean species. Furthermore, although FMRFamide-like immunoreactivity has been observed in a wide range of both vertebrate and invertebrate species, at least some of this FMRFamide-like immunoreactivity is due to other, related peptides. Thus, it will be necessary to characterize biochemically the peptide(s) responsible for the FMRFamide-like and proctolin-like staining in the crustacean.

Most of the previous physiological studies on the action of proctolin and FMRFamide have focused on their role as modulators of neuromuscular preparations or muscle contractions. Several reports have previously suggested that proctolin and FMRFamide may also act on central neurons. However, this report is the first to correlate the presence of peptide-containing fibers with physiological actions of the peptides on neurons of a central pattern generator, the pyloric system of the STG. The ability of proctolin to initiate rhythmic activity in a quiescent preparation suggests that it may act as a modulator of voltage-dependent conductances, as has been previously suggested to explain the effects of acetylcholine and dopamine on this system. It will be interesting to compare directly the physiological actions of these peptides and amines on the same neurons.

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