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Myomodulin Increases I_h and Inhibits the Na/K Pump to Modulate Bursting in Leech Heart Interneurons

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Abstract

In the medicinal leech, a rhythmically active 14-interneuron network composes the central pattern generator for heartbeat. In two segmental ganglia, bilateral pairs of reciprocally inhibitory heart interneurons (oscillator interneurons) produce a rhythm of alternating bursts of action potentials that paces activity in the pattern-generating network. The neuropeptide myomodulin decreases the period of this bursting and increases the intraburst spike frequency when applied to isolated ganglia containing these oscillator interneurons. Myomodulin also decreases period, increases spike frequency, and increases the robustness of endogenous bursting in synaptically isolated (with bicuculline) oscillator interneurons. In voltage-clamp experiments using hyperpolarizing ramps, we identify an increase in membrane conductance elicited by myomodulin with the properties of a hyperpolarization-activated current. Voltage steps confirm that myomodulin indeed increases the maximum conductance of the hyperpolarization-activated current I_h . In similar experiments using Cs^+ to block I_h , we demonstrate that myomodulin also causes a steady offset in the ramp current that is not associated with an increase in conductance. This current offset is blocked by ouabain, indicating that myomodulin inhibits the Na/K pump. In current-clamp experiments, when I_h is blocked with Cs^+ , myomodulin decreases period and increases spike frequency of alternating bursting in synaptically connected oscillator interneurons, suggesting that inhibiting the Na/K pump modulates these burst characteristics. These observations indicate that myomodulin decreases period and increases spike frequency of endogenous bursting in synaptically isolated oscillator heart interneurons and alternating bursting of reciprocally inhibitory pairs of interneurons, at least in part, by increasing I_h and by decreasing the Na/K pump.

INTRODUCTION

Rhythmic motor patterns such as swimming, walking, chewing, and breathing are driven in part by rhythmically active neural networks called *central pattern generators* (CPGs) (Marder and Calabrese 1996). The ability of these networks to produce rhythmic output is determined by the membrane currents and the synaptic interconnections of their neurons (for reviews, see Calabrese 1998; Dale and Kuenzi 1997). Studies of vertebrate and invertebrate CPG networks have shown that peptide modulators alter their rhythmic activity by modifying membrane currents and/or synaptic connections of network neurons (Harris-Warrick et al. 1995; Kiehn et al. 1996; Marder and Richards 1999; Nadim and Calabrese 1997; Swensen and Marder 2000). Characterizing which currents and synapses are targeted by modulators aids in assessing the mechanisms of rhythm generation and the function of these currents in the network and,

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ultimately, to understand how neuromodulators change rhythmic network function to adapt to a changing environment.

Because of its small number of component neurons (14 interneurons) and well-defined synaptic connectivity, the central pattern-generating network controlling leech heartbeat is useful for exploring how rhythmic networks function (Calabrese et al. 1995; Thompson and Stent 1976b,c). Within this network, two bilateral pairs of oscillator interneurons in the third and fourth ganglia pace the heartbeat rhythm. Each pair is configured as a half-center oscillator where each neuron bursts in alternation with its contralateral homolog through reciprocally inhibitory synapses (Peterson 1983a). In isolated ganglia, pairs of oscillator interneurons maintain alternating bursting and are considered elemental half-center oscillators (Masino and Calabrese 2002a; Peterson 1983a). When synaptically isolated, the oscillator interneuron can display endogenous bursting (Cymbalyuk et al. 2002). Because the rhythmic activity and membrane currents of these oscillator interneurons are well characterized (for review, see Hill et al. 2001), they are an excellent system for exploring how modulation of specific membrane currents shapes rhythmic activity.

The leech heartbeat CPG has been shown to be modulated by a variety of sensory, neurosecretory, and motor pathways (Arbas 1984). Modulation by the endogenous leech peptide FMRFamide has been characterized (for a review, see Calabrese et al. 1995). Bath application of FMRFamide to the third or fourth ganglion elicits an acceleration of rhythmic bursting in the oscillator heart interneurons (Simon et al. 1992). Voltage-clamp studies found that FMRFamide causes a negative shift in the activation and inactivation of a K current (I_{K1}) in the oscillator interneurons (Simon et al. 1992), activation of a persistent Na current (Schmidt et al. 1995), and a decrease in spike-mediated synaptic transmission (Simon et al. 1994). Additionally, FMRFamide activates a slowly activating voltage-gated K current, I_{KF} (Nadim and Calabrese 1997). Modeling studies identified this slowly activating K current as the most important mechanism for FMRFamide-mediated acceleration of the leech heartbeat rhythm.

Another endogenous leech peptide, leech myomodulin (Wang et al. 1998), has also been shown to accelerate the period of oscillator interneurons. When it [or its molluscan analog myomodulin A (Cropper et al. 1987b), subsequently referred to as myomodulin] is bath applied (1 μ M) to isolated ganglia, it causes a decrease in period (Masino and Calabrese 2002b). In this study, we demonstrate that, in addition to decreasing period, myomodulin also increases intraburst spike frequency. In synaptically isolated interneurons, it produces similar changes in burst characteristics and increases the robustness of endogenous bursting. We used voltage-clamp and current-clamp techniques to explore which membrane currents are affected by myomodulin to modulate the bursting of oscillator interneurons. We found that myomodulin enhances the hyperpolarization-activated cation current (I_h) and inhibits the electrogenic Na/K pump. By separately blocking I_h and the Na/K pump and determining the effects on burst characteristics, we demonstrate that the changes in membrane properties elicited by myomodulin may contribute to the decrease in period and increase in intraburst spike frequencies.

METHODS

Dissection and superfusion

Leeches (*Hirudo medicinalis*) were supplied by Leeches USA (Westbury, NY) and Biopharm (distributed by Carolina Biological Supply, Burlington, NC) and maintained in artificial pond water at 15°C. Before dissection, animals were cold anesthetized (1–4°C). Ganglia from midbody segments 3 and 4 were removed, individually pinned in petri dishes lined with silicone elastomer (Sylgard, Dow Corning, Midland, MI), and covered with saline. Immediately before

the experiment, the glial sheath was removed with microscissors or scalpel, then the preparation was superfused continuously with saline (bath volume about 0.5 ml; flow rate about 2 ml/min). All experiments were performed at room temperature.

Unless otherwise indicated, experiments were performed in normal saline, containing (in mM): 115 NaCl, 4 KCl, 1.8 CaCl₂, 10 glucose, 10 HEPES buffer; pH was adjusted to 7.4 with NaOH. For 0 Ca²⁺ salines, an equimolar amount of MnCl₂ replaced CaCl₂ (Angstadt and Calabrese 1991, 1989). For 0 Na⁺ salines, an equimolar amount of *N*-methyl-D-glucamine replaced NaCl, and the pH was adjusted to 7.4 with HCl. Where indicated, 2 mM CsCl was added to the saline. When used, ouabain (Sigma, St. Louis, MO) was dissolved, 10 mg/ml, in 95% ethanol, and this solution was added to saline for a concentration of 100 μ M ouabain, resulting in an ethanol concentration of 0.6%. For ethanol control conditions, 95% ethanol was added to saline for an ethanol concentration of 0.6%. Where indicated, 0.5 mM bicuculline methiodide (Sigma) was added to the saline. Molluscan myomodulin (SynPep, Dublin, CA) was dissolved to a concentration of 1 mM in distilled water and frozen in 50 μ l aliquots. Immediately before use, each aliquot was thawed and diluted in saline to produce a final concentration of 1 μ M.

Electrophysiology

For all recordings, borosilicate microelectrodes (1 mm OD, 0.75 mm ID) were used. For extracellular recordings, cell somata were guided by gentle suction into saline-filled electrodes whose tips were pulled to about 20 μ m; methods are described in Masino and Calabrese (2002a). For intracellular recordings, electrodes were pulled to sharp tips, and filled with 4 M K-acetate, 20 mM KCl solution, resulting in electrode resistances of 15 to 30 M Ω . Where indicated, TEA electrodes were filled with 1.5 M tetraethyl ammonium acetate tetrahydrate, 1.5 M Cs-acetate, 1 M K-acetate, 20 mM KCl solution. To decrease capacitance, some electrodes were coated with silicone elastomer to within nearly 500 μ m of the tip, whereas all intracellular electrode tips were dipped in dimethyl polysiloxane (Sigma).

Heart interneurons were identified by position in the ganglion and by their characteristic bursting pattern (Fig. 1A1). For all intracellular recordings, neurons were impaled in normal saline, and only neurons with input resistances >60 M Ω , as measured with a -0.1-nA pulse, were accepted for experiments. At the end of each experiment, the bath potential was recorded, and only those experiments where it was within $\pm$ 5 mV were accepted.

Data acquisition and analysis

Current-clamp and voltage-clamp experiments were performed using an Axoclamp-2A or Axoclamp-2B amplifier (Axon Instruments, Union City, CA) operating in discontinuous current-clamp or discontinuous single-electrode voltage-clamp mode with a sample rate of 2.5–2.8 kHz. The electrode potential was monitored to ensure that it settled during each sample cycle. Output bandwidth was 0.3 kHz. Voltage-clamp gain was 0.8 to 2.0 nA/mV. Extracellular signals were amplified with a differential AC amplifier (model 1700, A-M Systems, Carlsburg, WA). Clampex software (pClamp 9.0, Axon Instruments) generated current or voltage waveforms during current-clamp and voltage-clamp recordings, respectively, and digitized and stored all electrophysiology recordings. Time-constant fitting was performed with either Clampfit (pClamp 9.0, Axon Instruments) or scripts written for MATLAB (The MathWorks, Natick, MA); all other data analysis was performed using MATLAB.

Spike detection and burst discrimination were performed as described in Masino and Calabrese (2002a). Briefly, spikes were detected when the voltage crossed a threshold. To prevent double counts of one spike, a 20-ms refractory period was used. Bursts were defined as groups of five or more spikes with $\geq$ 800 ms between each group of spikes. Burst period was calculated as the time between the median spikes of consecutive bursts (see Fig. 1A). Burst duration was defined

as the time from the first to last spike of each burst, and duty cycle was defined as the ratio of burst duration and period for each cycle.

Voltage-clamp protocol and analysis

For all voltage-clamp experiments, except where indicated, protocols were first run in the control saline, indicated for each experiment below, then myomodulin or control saline was applied for 3–4 min and the protocol was run again. The pretreatment recordings were made to ensure the preparations in the two treatment groups were not significantly different before treatment. In all voltage-clamp protocols, at least three times the stimulus duration elapsed between stimulus repetitions, during which time the holding potential was maintained.

SYNAPTIC CURRENT—One neuron of a pair of oscillator interneurons was recorded extracellularly to detect spikes for off-line spike-triggered averaging of postsynaptic currents. The other neuron was recorded intracellularly and held at -50 mV in single-electrode voltage-clamp mode. Synaptic currents were recorded for about 1 min (seven to ten burst cycles). Synaptic currents were averaged according to their sequence within a burst by triggering from spikes in the presynaptic neuron. For those experiments where myomodulin was applied, to ensure myomodulin had taken effect, only experiments where the period decreased were analyzed for changes in synaptic potential (two experiments out of 14 in the myomodulin group were excluded). Experiments in which the clamped neuron was spiking too much to discriminate synaptic potentials were also excluded from analysis (two experiments out of 12 in the myomodulin group were excluded).

RAMP CURRENT—For all ramp protocols, 1.8 mM Mn^{2+} replaced Ca^{2+} in the saline, to synaptically isolate the cells. Where indicated, 2 mM Cs^+ and 100 μM ouabain were added to the 0 Ca^{2+} saline. The membrane potential was held at -40 mV and then linearly ramped to -85 mV in one second, then linearly ramped back to the -40 mV holding potential in one second. Ramp conductances were calculated as the slope of the linear fit to the I - V curve between -55 and -80 mV. Average holding current was calculated as the mean of 25 ms of holding current (uncontaminated by unclamped spiking) preceding the ramp. One experiment (out of nine) was eliminated from the control group of the 0 Ca^{2+} , 1.8 mM Mn^{2+} ramps because the slope was >9 SDs from the mean during the control treatment indicating an unacceptable level of damage to the neuron.

H-CURRENT—Cells were bathed for 3–4 min in 0 Ca^{2+} , 1.8 mM Mn^{2+} saline. Membrane potential was held at -40 mV, stepped to -60 mV for 3,040 ms, and stepped back to the -40 mV holding potential. This was performed three times, with steps to -60 , -80 , and -100 mV. I_h amplitude was measured as the difference between the average current between 75 and 85 ms after pulse onset and the average current between 2,990 and 2,995 ms after pulse onset. This section of the current trace was fit to a single exponential to extract the time constant of activation of I_h . The conductance of I_h was calculated by dividing the amplitude of I_h by the driving force, calculated as the voltage of the corresponding voltage step minus the I_h reversal potential, $E_h = -21$ mV (Angstadt and Calabrese 1989). In a separate set of experiments measuring the effect of ZD7288 (Tocris Cookson, Ellisville, MO) on I_h , the protocol was as above except membrane potential was stepped to between -50 and -100 mV in 10-mV increments. ZD7288 (0.1 mM) was applied, and the voltage-clamp protocol was run at 5 and 8 min of application.

CA CURRENTS—Cells were penetrated with TEA electrodes (containing 1 M K-acetate, 1.5 M Cs-acetate, 1.5 M tetraethylammonium acetate tetrahydrate, 20 mM KCl) to block potassium currents. Cells were bathed for 3–4 min in 0 Na^+ , 5 mM Ca^{2+} saline. The membrane potential was held at -70 mV, stepped to -50 mV for 2 s, and back to -70 mV. This procedure

was performed four times, with steps to -50 , -45 , -40 , and -35 mV. Leak current was subtracted on-line by summing four negative prepulses, each of equal duration and one fourth the amplitude of the following protocol pulse. Linearity of leak currents was visually monitored with Axoscope (pClamp 9.0, Axon Instruments). Peak I_{CaF} amplitude was visually ascertained as the peak current within the first 200 ms of the pulse. Peak I_{CaS} amplitude was visually ascertained as the peak current, after the I_{CaF} peak, within the first 500 ms of the pulse. To measure I_{CaS} inactivation time constant for the steps to -40 and -35 mV, the voltage-clamp current was fit by four exponentials using the Levenberg–Marquart fitting procedure in Clampfit. To aid fit convergence to biologically relevant values, a standard seed value for each of the exponentials was used for all fits, obtained from an oscillator heart interneuron model (based on prior published data: Hill et al. 2001). The seed was as follows: $A_1 = 30$ nA, $\tau_1 = 24$ nA, $A_2 = -30$ nA, $\tau_2 = 26$ nA, $A_3 = 0.6$ nA, $\tau_3 = 28$ nA, $A_4 = -0.6$ nA, $\tau_4 = 1200$ ms; offset = -0.2 nA, where the A values represent the coefficients of each exponential term and the τ values represent the time constants of each exponential term; offset is a constant offset added to the fitting equation. Each trace was fit twice, varying the start and end time points of the fits by 5–10 ms. The fit yielding the lowest SE for the slowest time constant was chosen. In one experiment (myomodulin application), the I_{CaS} inactivation time constant for the current elicited by a step to -40 mV could not be fit successfully with our methods. This data point was excluded from the data, but the peak I_{CaF} and peak I_{CaS} values for this trace, as well as the inactivation time constant for the subsequent step to -35 mV, were included. To avoid erroneous measurements caused by Ca-current escape arising from imperfect space clamp, peak I_{CaF} measurements were excluded when conductance measured at -35 mV was less than that measured at -40 mV.

K CURRENTS—No pretreatment protocols were run. Cells were bathed for 3–4 min in 0 Ca^{2+} , 0 Na^+ saline with myomodulin or without (control). Membrane potential was held at -35 mV, stepped to -40 mV for 3 s, and stepped back to the -35 -mV holding potential. This procedure was performed six times, with steps to between -40 and -100 mV in 10-mV increments. K-current amplitude was measured as the difference between the average current between 10 and 20 ms before pulse onset and the average current between 2,040 and 2,060 ms after pulse onset.

STATISTICS—Paired and unpaired t -test were performed with either Microsoft Excel or SigmaStat (Systat Software). All other statistical tests, Wilcoxon signed-rank test, Pearson's correlation, and one-way and two-way repeated-measures (RM) ANOVAs, were performed with SigmaStat. Tukey's test was used for post hoc analysis of ANOVAs. Significance of $P < 0.05$ is indicated on graphs by asterisks (*). All error bars indicate SE of the mean [$\text{SE} = \text{SD} / \sqrt{(n - 1)}$]; all values are quoted as means $\pm$ SE.

RESULTS

The heartbeat central pattern generator of the leech is paced by two pairs of oscillator interneurons. Each pair is connected by reciprocally inhibitory synapses to form a half-center oscillator, producing alternating bursts of action potentials. The intrinsic currents in these cells have been identified by voltage-clamp studies. In addition to the fast sodium current (I_{Na}) involved in generating action potentials, and the leak current (I_{leak}), the following currents have been identified: two low-threshold Ca currents (Angstadt and Calabrese 1991), one rapidly inactivating (I_{CaF}) and one slowly inactivating (I_{CaS}); three outward currents (Simon et al. 1992), a fast transient K current (I_{A}) and two delayed rectifier-like K currents, one inactivating (I_{K1}) and one persistent (I_{K2}); a hyperpolarization-activated inward cation current (I_{h}) (Angstadt and Calabrese 1989); and a low-threshold persistent Na current (I_{p}) (Opdyke and Calabrese 1994). The inhibitory synaptic transmission between the cells has both spike-mediated (Simon et al. 1994) and graded components (Angstadt and Calabrese 1991). The

endogenous leech myomodulin-related neuropeptide (Wang et al. 1998) and molluscan myomodulin A (Cropper et al. 1987b), subsequently referred to as myomodulin, have been shown to decrease the period of bursting (Masino and Calabrese 2002b) when applied to isolated ganglia. We examined the effects of myomodulin on membrane currents: the spike-mediated synaptic current, I_h , I_{CaF} , I_{CaS} , K currents between -100 and -40 mV, and on the Na/K pump.

Myomodulin decreases period and increases spike frequency in oscillator heart interneurons

In accordance with previous results (Masino and Calabrese 2002b), we found that bath-applying $1 \mu\text{M}$ myomodulin to oscillator heart interneurons in isolated ganglia significantly decreased the period by $17.3 \pm 0.5\%$ and significantly increased the mean, maximum, and minimum intraburst spike frequencies (by 13.2 ± 0.7 , 14.2 ± 0.6 , and $22.8 \pm 2.4\%$, respectively, $n = 20$; period compared using a Wilcoxon signed-rank test attributed to nonnormality, $P \leq 0.001$. Mean, maximum, and minimum spike frequencies compared with paired t -test, $P \leq 0.001$, $P \leq 0.001$, $P < 0.05$, respectively) (Fig. 1). Myomodulin did not change the duty cycle, (paired t -test, $P \leq 0.30$). These changes in burst characteristics were the same in the oscillator interneurons of ganglia 3 and 4, so we do not distinguish between those interneurons for the subsequent experiments in this paper. During washout of myomodulin, the period returned to premyomodulin values; mean period in wash was 3.6% greater than premyomodulin values ($P = 0.7$, paired t -test). The spike frequency remained elevated above premyomodulin values (mean, 10.5% ; maximum, 10.4% ; minimum, 7.1% of premyomodulin values; $P = 0.03$, $P = 0.01$, $P = 0.50$, respectively, paired t -test). Although bursting can persist for many minutes with less-invasive extracellular recordings, cells deteriorate during intracellular recordings and voltage clamp, so wash was not examined for voltage-clamp studies.

Although on average myomodulin decreased the period, its effect on period varied from a 2.5% increase to a 42.3% decrease. Similarly, myomodulin's effect on mean spike frequency ranged widely from a 12.0% decrease to a 40.2% increase. We found that there was a significant negative correlation between period change and initial period, (Pearson's $R = -0.629$; $P < 0.01$), and a significant positive correlation between spike frequency change and initial spike frequency (Pearson's $R = 0.493$; $P < 0.05$) (Fig. 2, A and B). The slower the initial period, the more the rhythm sped in the presence of myomodulin; and the slower the initial intraburst spike frequency, the more it increased in the presence of myomodulin. Therefore the large range of initial burst characteristics (period = $[6.1 \text{ to } 13.2 \text{ s}]$, mean spike frequency = $[6.8 \text{ to } 15.3 \text{ Hz}]$) may explain the large range of myomodulin-induced changes in the burst properties.

Myomodulin does not affect spike-mediated synaptic currents

A previous study using a model of the oscillator interneurons and their synaptic interactions with coordinating neurons (simulating the condition of an isolated ganglion) demonstrated that decreasing spike-mediated inhibitory synaptic conductance increased spike frequency and decreased the period of model neurons (Hill et al. 2001). To investigate whether myomodulin affected spike-mediated inhibitory postsynaptic currents (IP-SCs), we voltage-clamped one neuron of an oscillator inter-neuron pair at -50 mV while recording extracellularly from its contralateral homolog (Fig. 3A) to obtain spike-triggered average IPSCs (Fig. 3B). Under these conditions, the peak amplitude of IPSCs increased during the first few spikes of each burst, stabilized between the 11th and the 30th spikes, and waned slightly at the end of the burst (Fig. 3C). To assess the effect of myomodulin on IPSC amplitude, we averaged the postsynaptic current amplitudes from the 11th to the 30th spikes, when the IPSC amplitude was most stable. Myomodulin had no significant effect on this average postsynaptic current amplitude (Fig. 3D, unpaired t -test, $P = 0.12$). Moreover, the average change in IPSC amplitude for the control and myomodulin applications was the same, further demonstrating no effect of myomodulin

(control: -52.2 ± 20.6 pA, myomodulin: -51.9 ± 6.1 pA, unpaired t -test, $P = 0.99$). To assess any changes in IPSC dynamics, we fit each IPSC, from the 11th to the 30th, to a double-exponential function and extracted the rise and fall time constants (τ_1 and τ_2 , respectively). Myomodulin did not change either of the averaged time constants (myomodulin: $\tau_1 = 3.1 \pm 0.5$ ms, $\tau_2 = 7.5 \pm 0.3$ ms, $n = 10$; control: $\tau_1 = 2.1 \pm 0.2$ ms, $\tau_2 = 7.0 \pm 0.6$ ms, $n = 9$; unpaired t -test, τ_1 : $P = 0.07$; τ_2 : $P = 0.98$).

Myomodulin decreases period, increases spike frequency, and enhances burst robustness in synaptically isolated oscillator heart interneurons

In isolated ganglia 3 and 4, when one oscillator heart interneuron is voltage-clamped, the unclamped contralateral interneuron continues to receive phasic inhibition from and provide inhibition to ipsilateral axons arising from coordinating heart interneurons in ganglia 1 and 2 (Peterson 1983a). This inhibition is functionally weaker than that from the contralateral oscillator interneuron. Under these conditions, the unclamped neuron exhibits irregular bursting that is thought to arise as a result of endogenous burst properties in the oscillator interneuron and network interactions with the coordinating axons (Cymbalyuk et al. 2002) (Fig. 4A1). During myomodulin application, however, the unclamped neuron always exhibited regular bursting (Fig. 4A2). To quantify this effect, we calculated the coefficient of variation of period and of burst duration. Both values were smaller in the presence of myomodulin (Fig. 4, B and C; control: $n = 9$, myomodulin, $n = 10$; unpaired t -test, $P < 0.05$). In addition to increasing burst robustness, myomodulin significantly decreased the period (control: 12.0 ± 1.2 s; myomodulin: 6.77 ± 1.0 s; unpaired t -test, $P = 0.05$). Although within every preparation myomodulin increased the mean spike frequency with respect to the pretreatment condition (mean increase of $21.7 \pm 4.0\%$); there was no significant difference between the control and myomodulin mean spike frequencies (control: 9.6 ± 0.2 Hz; myomodulin: 11.5 ± 1.3 Hz; unpaired t -test, $P = 0.18$). The lack of a significant myomodulin-elicited increase in spike frequencies may be a result of the initial spike frequency of the control group (10.2 ± 0.4 Hz), which is higher than that of the myomodulin group (9.4 ± 1.0 Hz). Nonetheless, these experiments demonstrated that myomodulin can enhance the robustness of bursting in an oscillator interneuron whose contralateral homolog is silenced. Furthermore, myomodulin decreased period while the contralateral homolog was voltage-clamped, supporting the idea that myomodulin has effects on burst characteristics that are independent of spike-mediated synaptic transmission between the oscillator heart interneurons.

To test further whether myomodulin's effects are independent of synaptic transmission, we studied the effects of myomodulin on interneurons during complete synaptic isolation. Bilateral pairs of oscillator interneurons were recorded extracellularly in 0.5 mM bicuculline methiodide, which blocks all inhibitory synaptic input in heart interneurons (Schmidt and Calabrese 1992). Although oscillator interneurons are capable of regular endogenous bursting in bicuculline (1 mM), they also can exhibit bouts of irregular or tonic firing interspersed with regular bursting (Cymbalyuk et al. 2002). However, our experiments show irregular bursting in bicuculline (0.5 mM) (Fig. 5A1), and when myomodulin was applied, bursting became regular (Fig. 5A2). The coefficient of variation of period significantly decreased (Fig. 5B, $n = 7$, one-way RM ANOVA, $P < 0.01$), and the burst duration significantly decreased (Fig. 5C, one-way RM ANOVA, $P < 0.05$). After washout, the coefficient of variation returned to pre-myomodulin values ($P = 1.0$), but the burst duration did not fully return to the pre-myomodulin value ($P = 0.11$). To determine whether myomodulin affected the mean period and mean intraburst spike frequency, only those regions where the neuron exhibited discrete bursting (assessed by a silent phase of ≥ 700 ms between groups of at least five spikes) were analyzed. Myomodulin application significantly decreased the period by $26.6 \pm 6.1\%$ (pre-myomodulin: 6.6 ± 0.6 s; myomodulin: 5.4 ± 0.4 s; wash 6.2 ± 0.6 s, $n = 5$, one-way RM ANOVA, $P < 0.05$) and significantly increased mean spike frequency by $36.1 \pm 11.3\%$ (pre-myomodulin: $7.2 \pm$

1.2 Hz; myomodulin: 9.5 ± 0.9 Hz; wash: 7.2 ± 0.8 Hz, one-way RM ANOVA, $P < 0.05$). After washout, the period did not fully return to pre-myomodulin values, but the spike frequency did recover ($P < 0.05$).

Myomodulin enhances the hyperpolarization-activated cation current I_h in oscillator heart interneurons

The preceding experiments indicate that myomodulin affects membrane currents other than synaptic current. To identify which currents, we synaptically isolated interneurons with 0 Ca^{2+} (substituted with 1.8 mM Mn^{2+}) saline and applied hyperpolarizing voltage-clamp ramps (-40 to -85 mV) (Fig. 6A). In the presence of myomodulin, the conductance, measured as the slope of the linear current (see METHODS), during the ramps was significantly larger than that in the control group (control: 25.2 ± 1.3 nS, $n = 8$; myomodulin: 30.7 ± 2.3 nS, $n = 9$, unpaired t -test, $P < 0.05$), whereas the holding current was the same (control: 100.2 ± 31.7 pA; myomodulin: 229.3 ± 67.3 pA, unpaired t -test, $P = 0.10$). For further analysis of the myomodulin-elicited conductance, the averaged current waveforms from the control group were subtracted from those of the myomodulin group to obtain the “average difference current” (Fig. 6, B and C). Linear fits of the difference current revealed that the descending portion of the difference current had a slope of 5.5 nS with a reversal potential of -69.5 mV; the ascending portion corresponded to a slope of 3.6 nS with a reversal potential of -53.2 mV. The hysteresis in the current, indicated by the inward rectification during the down ramp, but the linearity during the up ramp, suggested a hyperpolarization activated conductance with slow activation and deactivation. Thus an increase in leak conductance seemed unlikely. It seems most likely that the myomodulin-elicited conductance was an inward-rectifier K current (I_{KIR}) and/or a hyperpolarization-activated cation current (I_h).

To identify the myomodulin-elicited conductance, we measured steady-state K currents during 3-s hyperpolarizing voltage-clamp steps (held at -35 mV, stepped to between -40 and -100 mV in 10-mV increments) in 0 Na^+ , 0 Ca^{2+} saline (Mn^{2+} replaced Ca^{2+} , N -methyl-D-glucamine replaced NaCl), which eliminates inward currents, including I_h (Angstadt and Calabrese 1989) (Fig. 7A). We used a step protocol, instead of a ramp, to observe the full dynamics of the current at each potential. There was no difference in the amplitude of steady-state K currents between control and myomodulin groups (Fig. 7B) (control: $n = 5$, myomodulin: $n = 5$; two-way RM ANOVA, $P > 0.05$). We concluded that a change in K currents in the region of -40 to -100 mV did not account for the myomodulin-elicited conductance revealed by the ramp protocol, and thus I_h was likely to be the myomodulin-elicited conductance.

The conductance elicited by myomodulin during the hyperpolarizing voltage ramps had characteristics that suggested an increase in the hyperpolarization-activated inward cation current I_h . To test whether myomodulin altered I_h , we synaptically isolated interneurons with 0 Ca^{2+} , 1.8 mM Mn^{2+} saline and applied 3-s hyperpolarizing voltage-clamp steps (held at -40 mV, stepped to between -60 and -100 mV in 20-mV increments) (Fig. 8A). The conductance of I_h was significantly greater in myomodulin than in control saline for steps to -80 and -100 mV (Fig. 8B) (control: $n = 9$; myomodulin: $n = 10$; two-way RM ANOVA, $P < 0.05$). Furthermore, for all voltages, the change in I_h conductance was significantly greater when myomodulin was applied than in the control experiments (Fig. 8C) (two-way RM ANOVA, $P < 0.001$).

Myomodulin causes an inward offset of the current during hyperpolarizing voltage ramps in Cs^+ that is blocked by ouabain

To test whether the myomodulin enhancement of I_h could account fully for the myomodulin-elicited conductance revealed during voltage ramps, we repeated the ramp protocol, blocking I_h with 2 mM CsCl (Fig. 9A). There was no difference between the conductance of the

myomodulin and control groups (control: 14.7 ± 1.9 nS, $n = 9$; myomodulin: 16.6 ± 1.7 nS, $n = 10$; unpaired t -test, $P = 0.44$), indicating that the myomodulin-elicited conductance revealed by the ramp protocol was indeed blocked by Cs^+ and could indeed be attributed to an increase in I_h . However, there was a near constant offset between the averaged ramp current of the control and myomodulin groups, although this offset was not indicated as a statistically significant difference in holding currents (control: 100.6 ± 8.3 pA; myomodulin: 42.3 ± 7.0 pA), nor in the current at the trough of the ramp (control: -512.1 ± 19.9 pA; myomodulin: -639.8 ± 27.1 pA; two-way RM ANOVA, $P = 0.09$). Nonetheless, this offset indicated a further potential effect of myomodulin on interneurons. A constant offset in voltage-clamp current (not accompanied by a commensurate difference in conductance) that is present throughout all voltages could be explained by a myomodulin-induced inhibition of the Na/K pump. The absence of this offset during the ramps in 0 Ca^{2+} saline without Cs^+ may be attributable to potential enhancement of the Na/K pump by Cs^+ ; such enhancement by Cs^+ has been observed in other leech neurons (Catarsi and Brunelli 1991; Skou 1960, 1965). The effect of myomodulin on the Na/K pump may be more prominent when the pump is enhanced by Cs^+ .

If the myomodulin-elicited current offset in Cs^+ was caused by inhibiting the Na/K pump, we expected the offset to disappear when the Na/K pump was blocked with ouabain. We repeated the ramp protocol with $100 \mu\text{M}$ ouabain added to the saline (with 0.6% ethanol as vehicle) and found no myomodulin-induced offset in the ramp current (Fig. 9B). There was no difference in the conductance between myomodulin and control groups (control: 16.8 ± 1.2 nS, $n = 9$; myomodulin: 16.9 ± 1.0 nS, $n = 11$; unpaired t -test, $P < 0.93$), nor in holding current (control: -7.3 ± 8.6 pA; myomodulin: 47 ± 6.9 pA), or trough current (control: -680.3 ± 21.9 pA; myomodulin: -672.3 ± 13.8 pA; two-way RM ANOVA, $P = 0.47$). Thus ouabain blocked the apparent myomodulin-elicited ramp offset.

Myomodulin affects the period and spike frequency in Cs^+

Our results so far show that myomodulin increases I_h conductance and, in saline with 2 mM Cs^+ saline (Cs^+ saline), likely inhibits the Na/K pump. To determine whether myomodulin might change burst characteristics when I_h is blocked by Cs^+ , we recorded from oscillator interneurons extracellularly in saline, in Cs^+ saline, and in Cs^+ saline with myomodulin (Fig. 10A). When the superfusion was switched from saline to Cs^+ saline, the period significantly increased by $23.9 \pm 2.5\%$ ($n = 6$; paired t -test, $P < 0.001$), in accordance with previous studies (Masino and Calabrese 2002b), and the spike frequency significantly decreased by $8.7 \pm 2.8\%$ (paired t -test, $P < 0.05$). If increasing I_h is the only mechanism by which myomodulin changes burst properties, no further change should occur when myomodulin is added to Cs^+ saline. However, application of myomodulin significantly decreased the period and increased the spike frequency (Fig. 10B, paired t -test, period: $P < 0.01$, spike frequency: $P < 0.001$). These changes demonstrate that myomodulin also affects membrane properties other than I_h . Moreover, the modulation of these other membrane properties decreases the period and increases the spike frequency. The current offset revealed by the ramp protocol in 0 Ca^{2+} , 2 mM Cs^+ saline and the subsequent disappearance of this offset in 0 Ca^{2+} , 2 mM Cs^+ saline with $100 \mu\text{M}$ ouabain suggests that, in the presence of Cs^+ , myomodulin inhibits the Na/K pump and thus changes the period and spike frequency.

To evaluate the effects of myomodulin using another I_h blocker, we measured in a pilot study whether ZD7288, shown to block I_h in several vertebrate preparations (Gasparini and DiFrancesco 1997; Luthi and McCormick 1998; Thoby-Brisson et al. 2000), would block I_h in heart interneurons. ZD7288 (0.1 mM) did not block I_h in leech heart interneurons; after 5 min of ZD7288 application, the maximum conductance of I_h decreased by $29.4 \pm 3.7\%$ and at 8 min, by $37.0 \pm 23.0\%$, compared with pretreatment values (for steps to -50 , -60 , -70 , -80 , -90 , and -100 mV, respectively; pretreatment: 1.0 ± 1.0 , 1.3 ± 1.3 , 2.4 ± 2.3 , 3.4 ± 3.3 , $3.1 \pm$

3.1, 3.1 ± 3.1 nS; ZD7288: 0.6 ± 0.5 , 1.1 ± 0.9 , 2.1 ± 2.1 , 1.9 ± 1.7 , 2.0 ± 2.9 , 2.1 ± 2.1 nS, $n = 3$). Because ZD7288 was ineffective at blocking I_h , Cs^+ remains the only known blocker of I_h in leech heart interneurons.

Ouabain stops bursting in oscillator heart interneurons

To characterize how blocking the Na/K pump affects burst characteristics in oscillator heart interneurons, we recorded extracellularly from interneurons first in saline, then in saline plus 0.6% ethanol (the solvent vehicle for ouabain) for 4 min, followed by application of 100 μM ouabain (in 0.6% ethanol). No change in burst characteristics was seen with ethanol vehicle alone, indicating it is an innocuous vehicle for ouabain application (period change: $1.8 \pm 3.1\%$, mean spike frequency change: $10.9 \pm 5.9\%$, $n = 7$, one-way RM ANOVAs, period: $P = 0.95$, spike frequency: $P = 0.16$). Application of 100 μM ouabain resulted in the cessation of bursting within 3 min of application ($n = 7$). We compared burst characteristics in saline with vehicle alone to those during ouabain application (up to burst cessation). Blocking the Na/K pump with ouabain decreased period ($-15.9 \pm 5.6\%$) and increased mean intra-burst spike frequency ($39.9 \pm 6.7\%$) (one-way RM ANOVAs, period: $P < 0.05$, spike frequency: $P < 0.001$). These effects support our hypothesis that by inhibiting the Na/K pump, myomodulin decreases period and increases spike frequency.

Myomodulin does not alter low-threshold Ca currents

Although the results reported here are all consistent with the idea that myomodulin modulates burst characteristics by increasing I_h conductance and decreasing the Na/K pump activity, we cannot exclude other possible targets of modulation. For example, modeling studies indicate that decreasing the inactivation time constant of the slow, low-threshold Ca current (I_{CaS}) in oscillator interneurons would decrease the period and increase spike frequency (Hill et al. 2001). We measured the effect of myomodulin on Ca currents in 0 Na^+ , 5 mM Ca saline and found no change in the peak amplitudes of the slow (I_{CaS}) or fast (I_{CaF}) low-threshold Ca currents or the inactivation time constant of I_{CaS} (τ_{hCaS}). Figure 11, two-way RM ANOVAs, peak I_{CaF} : $P = 0.064$, peak I_{CaS} : $P = 0.52$, τ_{hCaS} : $P = 3.047$.

DISCUSSION

Motor patterns need to adjust to changing environmental conditions, and this adjustment is often brought about by modulating the central pattern generator (Harris-Warrick and Marder 1991; Katz 1995, 1996). We have explored how the rhythmic activity of oscillator interneurons that pace the leech heartbeat are modulated by myomodulin, which is similar to an endogenous leech neuropeptide (Wang et al. 1998). We showed that myomodulin decreases the period and increases spike frequency of oscillator interneurons, both in a half-center oscillator (Figs. 1 and 2) and in synaptically isolated interneurons. In addition, we showed that myomodulin enhances burst robustness of endogenously bursting interneurons (Figs. 4 and 5). During hyperpolarizing voltage ramps, myomodulin enhanced a hyperpolarization-activated current (Fig. 6), which was blocked by Cs^+ (Fig. 9A). Using voltage steps, we confirmed that myomodulin enhances the maximal conductance of I_h (Fig. 8). Blocking I_h during voltage ramps revealed a second effect of myomodulin: a voltage-independent offset of the voltage-clamp current that was not associated with a change in conductance (Fig. 9A). Moreover, additional evidence supports the idea that myomodulin changed membrane properties in addition to I_h . When I_h was blocked, myomodulin still decreased the period and increased spike frequency of the alternating bursting of oscillator interneurons (Fig. 10), indicating that myomodulin modulates burst properties by a second mechanism. This I_h -independent effect of myomodulin is likely associated with the current offset seen in hyperpolarizing voltage ramps. Because this current offset was not coupled with a change in conductance, and because it was not observed in the presence of ouabain (Fig. 9B), a Na/K pump blocker, it is consistent

with an inhibition of the Na/K pump, which is electrogenic in other leech neurons (Baylor and Nicholls 1969). We conclude that myomodulin decreases the period and increases spike frequency by enhancing I_h and inhibiting the Na/K pump.

Comparison of the modulation of I_h to other systems

I_h has been established as a regulator of many rhythmically active neurons (Bal and McCormick 1997; Luthi and McCormick 1998) and, as such, it is a common target for neuropeptide modulation (Harris-Warrick et al. 1995; Kiehn and Harris-Warrick 1992; Lee and Cox 2003; Luthi and McCormick 1999; Marder and Thirumalai 2002). For example, in the lateral pyloric (LP) neuron of the rhythmic pyloric network of the crab stomatogastric nervous system, 5-HT was found to increase the conductance of I_h by shifting the voltage dependency of activation in a more positive direction. The resulting increase in I_h enhanced postinhibitory rebound, which phase-advanced the bursts of the LP neuron with respect to the other bursting neurons in the circuit and increased the LP burst spike frequency (Kiehn and Harris-Warrick 1992). Whereas in many rhythmic systems the depolarizing effect of I_h causes an acceleration of rhythm, in the pre-Bötzinger complex in the mammalian brain, where respiratory rhythm is thought to be generated, blocking I_h with Cs^+ or ZD 7288 increases respiratory frequency; the underlying mechanisms are as of yet unknown (Thoby-Brisson et al. 2000). Thoby-Brisson and colleagues (2000) found that the degree to which the frequency of the respiratory network changed by blocking I_h depended on the initial frequency, similar to our findings that the degree to which myomodulin changed period and spike frequency were dependent on their initial values (Fig. 2). Such state-dependent neuromodulation has been observed in a number of neuronal networks (Marder and Thirumalai 2002; Nusbaum et al. 2001).

The hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels that mediate I_h have cyclic-nucleotide-binding domains (Robinson and Siegelbaum 2003), and I_h is known to be sensitive to intracellular cyclic AMP (cAMP) (DiFrancesco 1993; Luthi and McCormick 1998, 1999; Robinson and Siegelbaum 2003). Moreover, the effects of myomodulin A in the *Aplysia* ARC neuromuscular system (where the peptide was first characterized; Cropper et al. 1987a,b) are mediated by cAMP (Brezina et al. 1994; Hooper et al. 1994). Thus the modulation we have observed here suggests that cAMP may mediate myomodulin's effects.

I_h affects period and spike frequency in oscillator heart interneurons

Since its first characterization (Angstadt and Calabrese 1989), I_h has been implicated as an important mechanism for burst generation and, potentially, regulation in leech oscillator heart interneurons (Calabrese 1998; Calabrese and De Schutter 1992; Hill et al. 2001). Multiple generations of the single-compartment model of an oscillator interneuron (Hill et al. 2001; Nadim et al. 1995; Olsen et al. 1995) have all demonstrated that changing the maximum conductance of I_h is an effective mechanism for modulating both the period and the spike frequency. Increasing the maximum conductance of I_h , $\bar{g}_h$, allows the inhibited neuron to escape earlier from synaptic inhibition and begin spiking, terminating the decelerating burst of the opposite neuron. Thus the cycle period is decreased and mean spike frequency is increased (Hill et al. 2001). The same mechanism may contribute to the increase in both mean and minimum spike frequencies measured in the presence of myomodulin in the living neurons (Fig. 1B). Second, increasing $\bar{g}_h$ augments depolarization of the initial part of the burst before I_h slowly deactivates. This increased depolarization increases the mean spike frequency in the model (Hill et al. 2001) and may contribute to the increase in mean and maximum spike frequencies elicited by myomodulin in the living neurons (Fig. 1B). Studies of a hybrid half-center oscillator, consisting of a hardware model oscillator interneuron connected to a living oscillator interneuron, found that increasing I_h in either the hardware model or the living interneuron (through dynamic clamp; Prinz et al. 2004; Sharp et al. 1993) decreased the period

(Sorensen et al. 2004). These results support the conclusions reached here, by showing that our measured changes in I_h could cause the observed myomodulin-elicited changes of oscillator interneuron burst characteristics.

In an oscillator interneuron model tuned to exhibit endogenous bursting, Cymbalyuk et al. (2002) found that increasing the maximal conductance of I_h expanded the parameter space in which endogenous bursting was possible, supporting our hypothesis that myomodulin's increase of I_h may enhance the robustness of endogenous bursting in interneurons (Figs. 4 and 5).

Modulation of the Na/K pump in neurons

There is little evidence of neuromodulation of the Na/K pump in rhythmically active neurons. During pharmacologically induced rhythmic bursting, the Na/K pump has been implicated in generating rhythmic activity in neonatal rat spinal networks (Rozzo et al. 2002), and rat midbrain dopaminergic neurons (Johnson et al. 1992). In all examined neurons of the leech CNS, replacing extracellular Ca^{2+} with Co^{2+} , Ni^{2+} , or Mn^{2+} induced rhythmic oscillations that were disrupted by application of Na/K pump blockers (Angstadt and Friesen 1991). Their computer simulations indicate that Na^+ influx during a burst may activate the Na/K pump, thus causing hyperpolarization to end each burst. This mechanism is consistent with the finding that partial inhibition of the Na/K pump by low concentrations of ouabain (10–25 μM) increased the cycle period of Co^{2+} -induced oscillations (Angstadt and Friesen 1991). However, in the normal cycling of oscillator heart interneurons, the period decreased as ouabain (100 μM) began to take effect, indicating the pump has a different role in influencing this bursting.

Modulation of the Na/K pump has been implicated in several nonrhythmic neurons, such as inhibition by DA_1 receptors in cultured rat striatal neurons (Aizman et al. 2000), and by serotonin in leech T sensory neurons (responsive to light touch) (Catarsi and Brunelli 1991) mediated by an increase in cAMP (Catarsi et al. 1993). Modulation of the Na/K pump by cyclic nucleotides has been demonstrated in many vertebrate tissues, including the PNS and CNS (for a review, see Therien and Blostein 2000). Thus the modulation of the Na/K pump we have observed here again suggests that cAMP may mediate the effects of myomodulin.

Modulation of the Na/K pump in heart interneurons had not been previously observed before this study, and its functional effects have not yet been fully explored. If the myomodulin-induced inhibition of the pump is constant during myomodulin application, it would provide a voltage-independent depolarization of the neuron without an associated change in membrane conductance, similar to injecting positive current. Modeling this effect would be useful to determine whether the enhancement of the Na/K pump that we measured when blocking I_h with Cs^+ might contribute to the myomodulin-induced change in burst properties measured in both Cs^+ saline and normal saline. In leech T sensory neurons, Cs^+ (4 mM, replacing extracellular K^+) enhances the Na/K pump (Catarsi and Brunelli 1991; Skou 1960, 1965), which may affect myomodulin's inhibition of the Na/K pump. However, to measure the effect of myomodulin's inhibition of the pump in normal saline requires an I_h blocker other than Cs^+ . The I_h blocker ZD7288 is ineffective in leech heart interneurons (Tobin, unpublished observations), and thus we have no way to separate, in normal saline, the effects of increasing I_h from those of decreasing the Na/K pump on heart interneuron burst characteristics.

Comparison of the modulatory effects of myomodulin to those of FMRFamide

Another well-characterized neuropeptide modulator of heart interneurons is FMRFamide (Evans et al. 1991), which, like myomodulin, accelerates leech heartbeat (Simon et al. 1992). FMRFamide has been shown to 1) cause a negative shift of activation and inactivation of I_{K1} (Simon et al. 1992), 2) activate a persistent Na current (Schmidt et al. 1995), 3) reduce

spike-mediated synaptic transmission (Simon et al. 1994), and 4) activate a slowly activating and deactivating K current, I_{KF} (Nadim and Calabrese 1997). Here we demonstrate that, although FMRFamide and myomodulin accelerate heart inter-neuron oscillations, they appear to act on different cellular targets. For example, we show that myomodulin increases I_h (Fig. 8), whereas FMRFamide has no effect (Angstadt and Calabrese 1989), and myomodulin does not change spike-mediated transmission (Fig. 3E), whereas FMRFamide reduces it (Simon et al. 1994). The changes that we have observed and that have been reported are small, however, and thus failure to observe an effect may not be definitive. We have yet to identify whether myomodulin affects K currents as FMRFamide does (Nadim and Calabrese 1997; Simon et al. 1992). Because these modulators both accelerate the heart rate, but apparently by different mechanisms, it has yet to be determined whether combined application of FMRFamide and myomodulin would have an effect different from that of applying either modulator alone, as has been described for muscles of the feeding apparatus in *Aplysia* (Brezina et al. 1996; Nusbaum et al. 2001) and the stomatogastric nervous system of crustaceans (Nusbaum et al. 2001).

The neuropeptide myomodulin modulates burst properties in leech oscillator heart interneurons by modulating at least two membrane targets: increasing I_h and decreasing the Na/K pump. Although I_h has long been implicated as an important membrane current in producing the oscillatory activity of heart interneurons, this is the first demonstration of neuropeptide modulation of I_h in leech heart interneurons. Furthermore, our studies reveal a role for the Na/K pump as a modulating influence of rhythmic neuronal activity.

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Fig. 1.

Myomodulin changes the burst characteristics of oscillator heart interneurons. *A*: example extracellular recordings from heart interneurons in ganglion 3: HN(L,3) and HN(R,3). Period is measured as the time between median spikes (diamonds) of consecutive bursts. Burst duration is measured as the time from first to last spike in a burst. Before myomodulin application (1), HN(R,3) burst period was 10.2 s and mean intraburst spike frequency was 10.6 Hz. When 1 μ M myomodulin was added (2), HN(R,3) period decreased to 7.4 s and mean spike frequency increased to 12.7 Hz. *B*: myomodulin decreased burst period while increasing mean, maximum, and minimum intraburst spike frequencies. * indicates significant difference from 0.

Fig. 2.

Myomodulin-induced changes in burst properties are correlated with their premyomodulin values. Black diamonds indicate heart interneurons from ganglion 3 [HN(3)], gray squares indicate heart interneurons from ganglion 4 [HN(4)], and white triangles indicate data where ganglion number was not recorded [HN(?)]. *A*: myomodulin decreased period more for oscillator heart interneurons with larger initial periods. *B*: myomodulin increased spike frequency more for oscillator heart interneurons with smaller initial mean spike frequencies.

Fig. 3.

Myomodulin does not change the amplitude of spike-mediated synaptic currents. *A, 1*: extracellular (*top*) and intracellular (*bottom*) recordings from a pair of heart interneurons in ganglion 4; *2*: left interneuron [HN(L,4); *bottom*] was voltage-clamped at -50 mV and its voltage-clamp current is shown. *B*: inhibitory postsynaptic currents (IPSCs) were averaged according to their sequence in each burst. Dashed line indicates zero current. *C*: average individual IPSC amplitude increased then slightly waned during each burst. Amplitudes of IPSCs 11–30 were averaged to obtain the total average IPSC amplitude for each experiment. *D*: myomodulin (MM) did not change the total average IPSC amplitude.

Fig. 4.

Myomodulin increases burst robustness in an oscillator heart inter-neuron whose partner is voltage clamped. *A*: heart interneurons from ganglion 3 burst in alternation. *1*: when the right neuron [HN(R,3)] was prevented from bursting by voltage clamp, the left neuron [HN(L,3)], recorded extracellularly, exhibited prolonged, irregular bursts; *2*: when 1 μ M myomodulin was added, the unclamped neuron maintained regular bursting while the partner neuron was voltage clamped. Robust bursting in the presence of myomodulin (MM) was indicated by a decrease in the coefficient of variation of period (*B*) and a decrease in burst duration (*C*).

Fig. 5.

Myomodulin increases burst robustness in synaptically isolated heart interneurons. *A, 1*: heart interneurons from ganglion 3, HN(R,3) and HN(L,3), burst endogenously when synapses were blocked by 0.5 mM bicuculline. Irregular bursting sometimes changed to tonic spiking for many seconds; *2*: when 1 μ M myomodulin was added, bursting became regular and bursts became more discrete. Robust bursting in myomodulin (MM) was indicated by a decrease in the coefficient of variation of period (*B*) and decrease in burst duration (*C*), compared with the pre-myomodulin (pre-MM) condition.

Fig. 6. Myomodulin increases membrane conductance during hyperpolarizing voltage ramps. *A*: average voltage-clamp current for myomodulin application (black) and control (gray), from 9 and 8 experiments each. Myomodulin increased conductance, as indicated by the steeper slope of the clamp current. *Inset*: command voltage. *B* and *C*: subtracting the average voltage-clamp current with myomodulin application from that with control yielded the difference current. Difference current corresponding to hyperpolarization is indicated by downward arrows; current corresponding to repolarization is indicated by upward arrows. *C*: myomodulin elicited a current that was slowly activated by hyperpolarization, as shown by plotting the difference current vs. voltage.

Fig. 7.

Myomodulin does not change K currents elicited during hyperpolarizing voltage-clamp steps. *A*: example voltage (*top*) and current traces (*1, 2*) during a voltage-clamp protocol to elicit K currents. Amplitude of K currents was measured as the difference between the current 10–20 ms before pulse onset (*left arrow*) and the steady-state current (*right arrow*). *1, 2*: example voltage-clamp current before (*1*) and after (*2*) application of 1 μ M myomodulin. *B*: myomodulin (black triangles) does not change the amplitude of steady-state K currents, compared with control (gray squares).

Fig. 8. Myomodulin increases maximal conductance of I_h elicited by voltage-clamp steps in 0 Ca^{2+} , 1.8 mM Mn^{2+} saline. **A:** example voltage (top) and current traces (1, 2) during a voltage-clamp protocol to elicit I_h . Amplitude of I_h was measured as the difference between the current 75–85 ms after pulse onset (left arrow) and the steady-state current (right arrow). 1, 2: example voltage-clamp currents before (1) and after (2) application of $1 \mu\text{M}$ myomodulin. **B:** myomodulin (black triangles) increased I_h conductance for steps to -80 and -100 mV, compared with control (gray squares). **C:** change in I_h conductance from pretreatment measurements was significantly larger in myomodulin than in control experiments for all voltage steps.

Fig. 9.

In 0 Ca^{2+} , 1.8 mM Mn^{2+} saline with 2 mM Cs^+ , myomodulin causes an offset in voltage-clamp current during hyperpolarizing voltage ramps that is blocked by ouabain. *A*: average voltage-clamp current for myomodulin application (black) and control (gray), from 10 and 9 experiments, respectively. Slopes of the current during the hyperpolarizing and depolarizing phases of the ramp were the same for the myomodulin application and control condition, indicating similar conductance. Holding and ramp current during the myomodulin application were offset from the control condition. *Inset*: command voltage. *B*: in experiments where ouabain was present in 0 Ca^{2+} saline with 2 mM Cs^+ , there was no myomodulin-induced current offset. There was no offset in holding or ramp current between myomodulin and control conditions; averages from 11 and 9 experiments, respectively.

Fig. 10.

Myomodulin-induced changes of burst characteristics in Cs⁺. *A, 1*: a heart interneuron from ganglion 3, HN(R,3), burst when I_h was blocked by 2 mM Cs⁺; *2*: when 1 μM myomodulin was added, period decreased and mean spike frequency increased. *B*: average change in period and mean spike frequency. * indicates significant difference from 0.

Fig. 11.

Myomodulin does not change low-threshold Ca currents. *A*: example voltage (*top*) and current traces (*1*, *2*) from a voltage-clamp experiment to measure slow and fast Ca currents. Ca^{2+} concentration was elevated to 5 mM to enhance Ca currents. Amplitude of the fast Ca current (I_{CaF}) was measured as the peak Ca current within the first 200 ms of the step (*left arrow*). Amplitude of the slow Ca current (I_{CaS}) was measured as the subsequent peak Ca current within the first 500 ms of the step (*right arrow*). I_{CaS} inactivation time constant (τ_{hCaS}) was measured as the longest time constant in a 4 exponential fit of the Ca current waveform. *1*, *2*: example voltage-clamp currents before (*1*) and after (*2*) application of 1 μ M myomodulin. *B*: myomodulin does not change peak I_{CaF} amplitude (for steps to -50, -45, -40, and -35 mV,

respectively: control, $n = 9, 9, 9, 5$; myomodulin, $n = 9, 9, 8, 6$). *C*: myomodulin does not change peak I_{CaS} amplitude (control, $n = 10$; myomodulin, $n = 9$). *D*: myomodulin does not change τ_{hCaS} (for steps to -40 and -35 mV, respectively: control, $n = 9, 9$; myomodulin, $n = 9, 10$).