Cellular/Molecular

Overexpression of a Hyperpolarization-Activated Cation Current (I_h) Channel Gene Modifies the Firing Activity of Identified Motor Neurons in a Small Neural Network

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The hyperpolarization-activated cation current (I_h) is widely distributed in excitable cells. I_h plays important roles in regulation of cellular excitability, rhythmic activity, and synaptic function. We previously showed that, in pyloric dilator (PD) neurons of the stomatogastric ganglion (STG) of spiny lobsters, I_h can be endogenously upregulated to compensate for artificial overexpression of the Shal transient potassium channel; this maintains normal firing properties of the neuron despite large increases in potassium current. To further explore the function of I_h in the pyloric network, we injected cRNA of *PAIH*, a lobster gene that encodes I_h, into rhythmically active PD neurons. Overexpression of PAIH produced a fourfold increase in I_h, although with somewhat different biophysical properties than the endogenous current. Compared with the endogenous I_h, the voltage for half-maximal activation of the PAIH-evoked current was depolarized by 10 mV, and its activation kinetics were significantly faster. This increase in I_h did not affect the expression of I_A or other outward currents. Instead, it significantly altered the firing properties of the PD neurons. Increased I_h depolarized the minimum membrane potential of the cell, reduced the oscillation amplitude, decreased the time to the first spike, and increased the duty cycle and number of action potentials per burst. We used both dynamic-clamp experiments, injecting the modeled PAIH currents into PD cells in a functioning STG, and a theoretical model of a two-cell network to demonstrate that the increased I_h was sufficient to cause the observed changes in the PD activity.

Key words: **I_h; I_A; STG; computational modeling; gene expression; neuronal network**

Introduction

The hyperpolarization-activated cation current (I_h) was first identified in cardiac sinoatrial node cells as a pacemaker current (Noma and Irisawa, 1976; Brown and Difrancesco, 1980; Yanagihara and Irisawa, 1980). I_h has now been found in many cell types in the CNS (DiFrancesco, 1993; Pape, 1996). Besides playing important roles in controlling rhythmic activity in the heart (Noma and Irisawa, 1976; Brown and Difrancesco, 1980; Yanagihara and Irisawa, 1980) and the brain (Pape and McCormick, 1989; Bal and McCormick, 1997; Luthi and McCormick, 1998), I_h also regulates cellular excitability (Pape and McCormick, 1989; Thoby-Brisson et al., 2000), synaptic transmission and plasticity (Beaumont and Zucker, 2000; Mellor et al., 2002), and dendritic integration (Poolos et al., 2002) in different preparations. Four I_h channel genes, HCN1 to HCN4, have been cloned in mammals (Santoro et al., 1997, 1998; Ludwig et al., 1998). When expressed in *Xenopus* oocytes or heterologous cell lines, HCN1- to HCN4-expressing currents show very different biophysical properties. HCN channels can also interact with auxiliary proteins such as minK-related protein (MinK-R or KCNE2) (Yu et al., 2001), which can further contribute to the diversity of H-currents *in vivo*.

Despite this molecular knowledge, our understanding of the functional roles of I_h within the context of intact neural networks is still uncertain (Vassalle, 1995; Chevaleyre and Castillo, 2002; Miake et al., 2002). The complexity of vertebrate systems makes these system-level studies difficult. In our study, we took advantage of a small and clearly defined network, the 14-neuron pyloric network in the stomatogastric ganglion (STG) of the spiny lobster, *Panulirus interruptus*. In this system, we can manipulate the expression of single genes in a single identified neuron. This allows us to study the function of the channel both in individual neurons and in the intact, functioning network. I_h is present in STG neurons (Golowash and Marder, 1992; Kiehn and Harris-Warrick, 1992), and can be modulated by dopamine (Harris-Warrick et al., 1995), red pigment-concentrating hormone (Dickinson et al., 2001), and serotonin (Kiehn and Harris-Warrick, 1992).

One possible physiological role of I_h is to regulate postinhibitory rebound that drives resumption of bursting after synaptic inhibition in the pyloric rhythm. We previously showed that postinhibitory rebound after hyperpolarizing current injections is shaped by a complex opposition between I_h and the transient

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potassium current, I_A (Kiehn and Harris-Warrick, 1992). Recent work from our lab suggests that I_h is involved at the molecular level in balancing the activity of the I_A gene, *shal*, in pyloric neurons (MacLean et al., 2003). Overexpression of *shal* by RNA injection into pyloric dilator (PD) neurons produced a very large I_A, but did not significantly change the firing properties of the neurons. This is attributable to an activity-independent compensatory increase in I_h that accompanied the *shal*-induced overexpression of I_A, thus maintaining the normal activity of the network. We asked whether this compensatory response is bidirectional: does overexpression of the I_h protein upregulate I_A as well?

PAIH, a gene encoding an I_h channel, has been cloned from the closely related spiny lobster *Panulirus argus* (Gisselmann et al., 2003). In our present study, we overexpressed *PAIH* in PD neurons by direct microinjection of its RNA. Overexpression of *PAIH* produced very large increases in I_h in PD neurons. However, no compensatory enhancement of I_A or other outward currents was detected. Instead, it significantly changed the firing properties of the injected PD neurons. These experimental results were confirmed by dynamic-clamp experiments, injecting an artificial *PAIH* current into PD cells, and a theoretical model of a PD neuron in a simplified oscillating network.

Materials and Methods

STG dissection and PD cell identification

Pacific spiny lobsters (*P. interruptus*) were purchased from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in artificial seawater at 16°C until use. Animals were anesthetized by cooling on ice for 30 min before dissection. The STG was dissected along with its motor nerves and associated commissural and esophageal ganglia (Mulloney and Selverston, 1974), and pinned in a Sylgard-lined dish. The preparation was superfused continuously (3 ml/min) with saline (16°C) containing (in mM): 479 NaCl, 12.8 KCl, 13.7 $CaCl_2$, 3.9 Na_2SO_4, 10.0 $MgSO_4$, 2 glucose, and 11.1 Tris base, pH 7.35 (Mulloney and Selverston, 1974). Extracellular recordings were made from identified motor nerves using glass suction electrodes. Individual somata were impaled with glass microelectrodes (10–25 MΩ; 3 M KCl). The PD neurons were identified by the 1:1 correspondence of their intracellular action potentials with those recorded extracellularly on the PD motor nerve and by their typical shape of membrane potential oscillations and synaptic inputs (Kloppenburg et al., 1999).

RNA microinjection into neurons

The *PAIH* clone in a pTracer2 vector was obtained from H.H.; the accession number is GenBank AY280847. *PAIH* DNA was linearized with *Xho*I and transcribed *in vitro* using a T7 mMessage mMachine kit (Ambion, Austin, TX). The transcripts were cleaned using the RNeasy mini kit (Qiagen, Valencia, CA). After neuronal identification, PD neurons were injected with an RNA solution using pressure pulses (40 psi; 0.2 Hz) driven by a homemade pressure injector and a pulse generator (Master-8; AMPI, Jerusalem, Israel). The RNA solution contained 0.4–0.7 μg/μl *PAIH* and 0.04% Fast Green to monitor the injection. PD cells were injected with approximately equivalent amounts of RNA based on the color of the coinjected Fast Green. Fast Green alone was injected into control neurons, which were otherwise treated identically to the RNA-injected neurons. After injection, the ganglion, with intact connections to the commissural and esophageal ganglia, was incubated in sterilized recording saline without Tris base but containing 5 mM HEPES, pH 7.4, 2 gm/l glucose, 50,000 U/l penicillin, and 50 mg/l streptomycin at 16°C for 5 d to allow the expression of the proteins.

Electrophysiological analysis of currents

PD neurons. After 5 d in organ culture, PD neurons were recorded intracellularly and voltage clamped using an Axoclamp 2B amplifier driven by pClamp8 software (Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had a tip resistance of 14–20 MΩ for voltage recording and ≤8 MΩ for current injection. To isolate PD neurons from most synaptic input, we superfused the ganglion with saline containing 10^{-7} M tetrodotoxin and 5×10^{-6} M picrotoxin. The currents of interest were additionally isolated from most other currents with the following channel blockers: for I_h, 2×10^{-4} M $CdCl_2$ (to block calcium and calcium-activated currents), 2×10^{-2} M tetraethylammonium chloride (TEA) (to block most potassium currents), and 4×10^{-3} M 4-aminopyridine (to block I_A); for I_A, 2×10^{-4} M $CdCl_2$, 5×10^{-3} M CsCl (to block I_h), and 2×10^{-2} M TEA; for noninactivating outward currents [Ca^{2+}-activated potassium current ($I_{K(Ca)}$) and delayed rectifiers ($I_{K(V)}$)], 5×10^{-3} M CsCl; the neuron was held at −40 mV, where I_A is almost completely inactivated in PD cells.

To measure I_h, the cells were held at −40 mV, and the voltage dependence of activation was measured with a series of 8 sec hyperpolarizing voltage steps at 20 sec intervals from −50 to −130 mV in 10 mV increments. These steps were not leak subtracted. The reversal potential of I_h was measured from the tail currents after a preactivating pulse to −100 mV for 8 sec with a series of 4 sec pulses from −60 to −20 mV in 5 mV increments.

To measure the peak currents of I_A, the cells were held at −50 mV and depolarized to +20 mV for 400 msec after a deinactivating prepulse to −120 mV for 400 msec. Traces were leak subtracted using a P/6 protocol with steps opposite to the sign of activation.

To measure the non-I_A potassium currents (a mixture of $I_{K(Ca)}$ and $I_{K(V)}$), the cell was held at −40 mV. A series of 400 msec voltage steps were delivered from −40 mV to +30 mV with 10 mV increments. The traces were leak subtracted as described above.

Current analysis. I_h amplitudes were measured from single exponential fits of the data performed in Clampfit, version 8.1 (Axon Instruments), extrapolated back to the beginning of the hyperpolarizing step and forward to approximate the steady state at 12 sec. Currents were converted to conductances using a reversal potential for I_h (V_{Rev}) of −30 mV, which we determined from analysis of the tail currents. The conductance–voltage data were fit to the Boltzmann equation:

$$g/g_{max} = 1/(1 + e^{-(V_m - V_{1/2})/s}) \quad (1)$$

where g is the conductance, g_{max} is the maximal conductance, $V_{½}$ is the voltage of half-activation, and s is the slope factor.

Analysis of rhythmic activity

We analyzed rhythmic activity in PD neurons using Spike2 (Cambridge Electronic Design, Cambridge, UK). The period was the time between the first spike of two adjacent oscillations, and the frequency was the inverse of the period. The minimal membrane potential (V_{min}) was measured at the most hyperpolarized potential in the trough of the oscillation. The oscillation amplitude was the difference between V_{min} and the most depolarized potential of the slow wave oscillation (at the base of the action potentials). The time to the first spike was the time from V_{min} to the top of the first spike.

Dynamic-clamp studies

We used the dynamic clamp (Sharp et al., 1993a,b) to inject an artificial I_h into PD neurons during their normal oscillating firing pattern. The membrane potential V_m recorded with a microelectrode in the PD cell body was fed into a Digidata 1200A board (Axon Instruments) and digitized at 2 kHz with in-house software that was kindly modified by Dr. A. A. Prinz (Brandeis University, Waltham, MA) from a C++ program written by Dr. R. D. Pinto (Physics Institute, University of Säo Paulo, Säo Paulo, Brazil) (Pinto et al., 2001). The dynamic-clamp software calculated the I_h that would be active given a set of model parameters and V_m, and the injected current was calculated as follows:

$$I_{inj} = g_{max} m (V - E_{rev}) \quad (2)$$

where m changed according to $dm/dt = (m_\infty - m)/\tau_m$ and m_∞ was given by $m_\infty = 1/(1 + \exp((V_m - V_{½})/V_{slope}))$. The parameters g_{max}, E_{rev}, τ_m, $V_{½}$, and V_{slope} were derived from electrophysiological recordings of I_h in *PAIH*-injected PD neurons. The activities of the neurons during dynamic clamp were recorded on a separate computer using Axoscope (Axon Instruments).

Table 1. Model equations

$$C_1 \frac{dV_1}{dt} = I_{ext,1} - I_{Na,1} - I_{Ca,1} - I_{K(V),1} - I_{K(Ca),1} - I_{A,1} - I_{leak,1} - \kappa_1(V_1 - V_2)$$

$$C_2 \frac{dV_2}{dt} = I_{ext,2} - I_{Na,2} - I_{Ca,1} - I_{K(V),2} - I_{K(Ca),2} - I_{A,2} - I_{leak,2} - I_h - \kappa_2(V_2 - V_1)$$

$$I_{Na,i} = \bar{g}_{Na} \cdot (m_{\infty,i})^3 \cdot h_i \cdot (V_i - E_{Na})$$

$$m_{\infty,i} = 1/(1 + e^{-(V_i+10)/10})$$

$$a_{h,i} = c_h \cdot (1 + e^{-(V_i+39)/7})$$

$$b_{h,i} = 1/(1 + e^{-(V_i+4)/5})$$

$$\frac{dh_i}{dt} = k_h \cdot (a_{h,i} - h_i(a_{h,i} + b_{h,i}))$$

$$I_{K,i} = \bar{g}_K \cdot n_i^4 \cdot (V_i - E_K)$$

$$a_{n,i} = 1/(1 + e^{-(V_i+23)/20})$$

$$\frac{dn_i}{dt} = c_{n,i}/(1 + e^{-(V_i-10)/45}) \cdot (a_{n,i} - n_i)$$

$$I_{A,i} = \bar{g}_A \cdot (a_{\infty,i})^3 \cdot a_i \cdot (V_i - E_K)$$

$$a_{\infty,i} = 1/(1 + e^{-(V_i+43)16})$$

$$b_{\infty,i} = 1/(1 + e^{(V_i+62)/6})$$

$$\frac{da_i}{dt} = k_a \cdot (b_{\infty,i} - a_i)$$

$$I_{Ca,i} = \bar{g}_{Ca} \cdot s_i \cdot \frac{0.5}{0.5 + [Ca]_i}(V_i - E_{Ca})$$

$$s_{\infty,i} = 1/(1 + e^{-(V_i+50)/6.7})$$

$$\frac{ds_i}{dt} = k_s \cdot e^{-(V_i+22)^2/10^4} \cdot (S_{\infty,i} - s_i)$$

$$\frac{d[Ca]_i}{dt} = -c_{Ca} I_{Ca} + k_{Ca}(0.05 - [Ca]_i)$$

$$I_{K(Ca),i} = g_{K(Ca)} \cdot \frac{[Ca]_i}{0.3 + [Ca]_i}(V_i - E_K)$$

$$I_{leak,i} = \bar{g}_{leak} \cdot (V_i - E_{leak})$$

$$I_h = \bar{g}_h \cdot (V_2 - E_h)$$

$$i = 1, 2$$

Student's *t* tests were used to assess statistical significance. Throughout this paper, all of the calculated values are reported as means ± SD.

Modeling

We investigated how an increase in I_h affects the firing properties of PD neurons using a mathematical model of these cells. The model is based on a previous model of the lateral pyloric (LP) neuron of the crab (Buchholtz et al., 1992; Golowash and Marder, 1992). The model incorporates the known relevant currents for the PD neurons in the STG: Hodgkin–Huxley-type currents for sodium (I_{Na}), calcium (I_{Ca}), a delayed-rectifier potassium current ($I_{K(V)}$), a calcium-activated potassium current ($I_{K(Ca)}$), a transient potassium current (I_A), and a leak current (I_{leak}). Parameters for I_{Ca}, $I_{K(V)}$, $I_{K(Ca)}$, I_A, and I_{leak} were obtained from our previous work (Kloppenburg et al., 1999; Willms et al., 1999; Johnson et al., 2003). The experimentally induced H-current (I_h) was modeled as a constant conductance parameter g_h. We use this simple representation of I_h, because the activation time constant at physiological voltages is much slower than the time scales of oscillations examined in our model simulations. During any simulation with a model that included a voltage-dependent H-current with its measured time constants, changes in its conductance would be negligible.

Our modeled PD neuron is electrically coupled to a second cell that embodies the rest of the pyloric network that normally drives the PD cell into bursting. The parameters in our model PD cell have been tuned to reflect values experimentally measured, whereas the parameter values of the driver cell were chosen to produce a bursting mode characteristic of the network oscillations that normally drive the PD neuron. The coupling coefficients were chosen so that, in the absence of I_h, the PD cell follows the bursting pattern of the driver cell. In addition, the coupling was set to be asymmetric. This asymmetrically coupled two-cell model is a surrogate for a more detailed model that would represent each of the individual neurons in the pyloric circuit and include chemical as well as electrical synapses. Although in the pyloric network the strongest electrical coupling of each PD cell is its electrical coupling to the other PD cell, we hypothesize that modification of the driven PD cell will have a smaller feedback to the remainder of the network than the input it receives from the network. In the absence of more detailed models, this hypothesis has not been tested.

The equations of the model are shown in Table 1. Quantities with subscript 1 correspond to the driver cell; and quantities with subscript 2 correspond to the modified PD cell. The constant parameters in the model are listed in Table 2. Under control conditions, I_h in the PD cell is very small, and we include it in our calculations of the leak current. Therefore, we set g_h equal to 0. Our model assumes that the effects of any I_h in the remainder of the network are reflected in the leak current of the driver cell. We then model the experimentally induced I_h in the PD cell by setting g_h to a value similar to the increase in I_h seen in the *PAIH* injection experiments.

Table 2. Parameter for the modeled cells

Symbol	Meaning	Value	Units
C_1, C_2	Membrane capacitance	0.002	nF
I_{ext}	External current	1	μA
$\bar{g}_h$	Maximal I_h conductance	0.07	μS
$\bar{g}_A$	Maximal I_A conductance	2.60	μS
$\bar{g}_K$	Maximal $I_{K(V)}$ conductance	3.00	μS
$\bar{g}_{K(Ca),1}$	Maximal $I_{K(Ca)}$ conductance	0.75	μS
$\bar{g}_{K(Ca),2}$	Maximal $I_{K(Ca)}$ conductance	0.85	μS
$\bar{g}_{leak}$	Maximal I_{leak} conductance	0.10	μS
$\bar{g}_{Ca}$	Maximal I_{Ca} conductance	0.07	μS
$\bar{g}_{Na}$	Maximal I_{Na} conductance	2000	μS
k_a	Rate constant	10	1/sec
k_s	Rate constant	43	1/sec
k_{Ca}	Rate constant	3	1/sec
k_h	Rate constant	200	1/sec
c_h	Rate constant	0.07	1/sec
c_{Ca}	Rate constant	0.01	1/sec
$c_{n,1}$	Rate constant	5	1/sec
$c_{n,2}$	Rate constant	3	1/sec
E_{Na}	Reversal potential of I_{Na}	50	mV
E_{Ca}	Reversal potential of I_{Ca}	140	mV
E_K	Reversal potential of I_K	−86	mV
E_{leak}	Reversal potential of I_{leak}	−50	mV
E_h	Reversal potential of I_h	−35	mV
κ_1	V_1 coupling coefficient	0.002	MΩ
κ_2	V_2 coupling coefficient	0.050	MΩ

The differential equations for the model were numerically integrated using the Radau integrator (Hairer and Wanner, 1991) for a time span of 18 sec. This time span was sufficient for transients to die out, and the time interval between the minimum voltage points on the $V(t)$ curve during the last two cycles of the PD cell oscillations was selected for analysis. The oscillation frequency, duty cycle, oscillation amplitude, minimum volt-

age, and number of spikes were obtained using the same criteria applied to the experimental data.

Results

PAIH overexpression produces large I_h in PD neurons

The crustacean gene for I_h channels, *PAIH*, was cloned from *P. argus* and characterized by Gisselmann et al. (2003). In the pyloric network of the lobster STG, there are two PD neurons with identical physiological properties. Therefore, we injected *PAIH* RNA with 4% Fast Green as an injection indicator into one PD neuron and injected Fast Green alone into the other PD neuron as an internal control. After these injections, the STG, with attached commissural and esophageal ganglia, was cultured for 5 d to allow *PAIH* expression before recordings were made. Our previous work has shown that the STG with the attached anterior ganglia can maintain normal oscillatory pyloric motor pattern for at least 1 week in organ culture (MacLean et al., 2003).

In control, Fast Green-injected PD neurons, the hyperpolarization-activated inward current recorded from the soma was normally very small. At −100 mV, the peak current was less than −10 nA, and the time constant for activation was ~4.5 sec (Fig. 1*A*, Table 3). Current activation was voltage dependent and could be fitted by a single exponential. We extended the exponential fitting curve of the raw data to estimate the steady state of the peak current at each voltage. The activation *g*/*V* curve was fitted by a Boltzmann equation (Eq. 1). The average maximal conductance was ~0.15 μS; the voltage for half-maximal activation ($V_{½}$) was −86 mV, whereas the slope was 10.5 mV (Fig. 1*B*, Table 3) ($n = 12$).

Compared with the control, PD cells expressing *PAIH* had much larger I_h with significantly different biophysical properties (Fig. 1, Table 3). The average maximal conductance was increased nearly fivefold, showing successful expression of the RNA. The current was activated at significantly more depolarized potentials than the control: $V_{½}$ was shifted by >10 mV in the positive direction (−75 mV), whereas the slope factor decreased by 3 mV. In addition, the current was activated more rapidly at each voltage (Fig. 1*A*, Table 3); at −100 mV, for example, τ_m was reduced by approximately one third (Table 3). However, there were similarities in other parameters between the endogenous and the new currents: both currents had similar reversal potentials, close to −30 mV (Fig. 1*C*), and I_h in both control PD and *PAIH*-expressing cells were blocked by >85% by 5 mM Cs^+ or 300 μM ZD7288 (*D*, *E*). These data indicate that *PAIH* encodes a standard H-current. When compared with the PAIH currents expressed in HEK293 cells (Gisselmann et al., 2003), I_h in PD neurons was activated at a significantly more positive voltage ($V_{½}$ of −75 vs −119 mV). Thus, as seen with other H-current genes, the biophysical properties of the *PAIH*-evoked current depend markedly on the cells in which it is expressed.

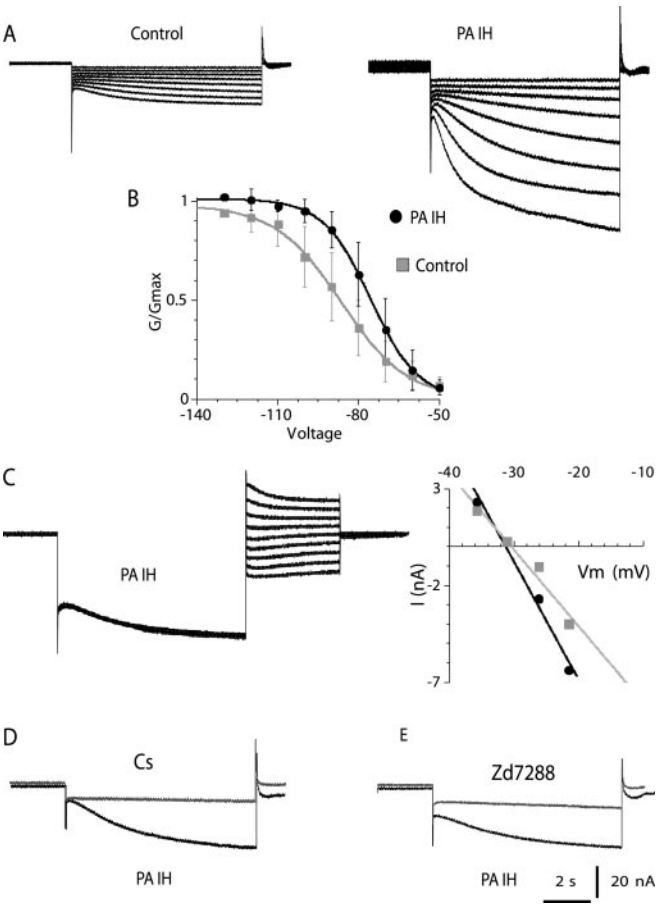

Figure 1. Injection of *PAIH* cRNA produces a large I_h in PD neurons. *A*, I_h was recorded in control and *PAIH*-injected PDs. The cells were held at −40 mV, and a series of 8 sec pulses was applied from −50 to −120 mV in 10 mV increments. Recordings were performed as described in Materials and Methods. *B*, Plots of g/g_{max} versus voltage for activation of I_h in control (■) and PAIH-expressing (●) PD neurons ($n = 12$ control and 19 *PAIH*-injected neurons). Error bars indicate SDs. *C*, Left, Measurement of the reversal potential from tail currents of I_h in a PAIH-expressing PD neuron. Currents were measured after a preactivating pulse to −100 mV for 8 sec with a series of 4 sec pulses from −60 to −20 mV in 5 mV increments. The tail currents were plotted against the tail voltage to determine the reverse potential. Right, Determination of V_{Rev} for a control cell (■) and a PAIH-expressing cell (●). *D*, The PAIH currents could be blocked by 5 mM Cs^+ and 300 μM ZD7288.

PAIH overexpression does not affect I_A or other outward currents

We previously showed that overexpression of the transient potassium current gene, *shal*, can induce a compensatory increase of I_h in PD neurons, nullifying the physiological effects of *shal* injection (MacLean et al., 2003). To determine whether overexpression of *PAIH* would evoke a parallel homeostatic upregulation of I_A, we measured the effect of *PAIH* overexpression on I_A, as well as additional outward currents including the calcium-activated potassium current ($I_{K(Ca)}$) and the delayed rectified outward current ($I_{K(V)}$). The average peak current of I_A at +20 mV in control PD neurons was 379 ± 69 nA ($n = 6$), whereas that in *PAIH*-expressing PDs was 344 ± 64 nA ($n = 11$) (Fig. 2*A*). There were no significant differences in I_A amplitude or inactivation kinetics between the control and *PAIH*-injected PD neurons ($p > 0.5$). In several neurons, we also checked the complete voltage dependence of activation and inactivation of I_A: again, there was no significant difference in these parameters between control and *PAIH*-expressing PD neurons (data not shown). By holding the membrane at −40 mV, I_A is almost completely inactivated, and the remaining currents activated by depolarizing pulses represent the sum of $I_{K(V)}$ and $I_{K(Ca)}$. These combined outward currents also showed no significant difference between the control and *PAIH*-expressing PD neurons (Fig. 2*B*). The average peak current at +30 mV was 566 ± 64 nA ($n = 9$) in control and 570 ± 66 nA in PAIH-expressing cells ($n = 9$). Thus, unlike our previous experiments, in which the effect of an artificial increase in I_A was compensated by an endogenous upregulation of I_h (MacLean et al., 2003), our experiments show that overexpression of *PAIH* produces a large I_h, but does not induce an endogenous compensatory change in outward currents.

Table 3. H-currents in control and PAIH-expressing PD cells

	g_{max} (μS)	$V_{1/2}$ (mV)	Slope (mV)	τ_{act} (at −100 mV)
Control ($n = 12$)	0.15 ± 0.06	−86 ± 8.2	10.5 ± 2.1	4.48 ± 0.89
PAIH ($n = 19$)	0.72 ± 0.20**	−75.2 ± 6.0*	7.5 ± 1.4*	2.87 ± 0.96*

*Significantly different from control cells, $p < 0.05$.

**Very significantly different from control cells, $p < 0.01$.

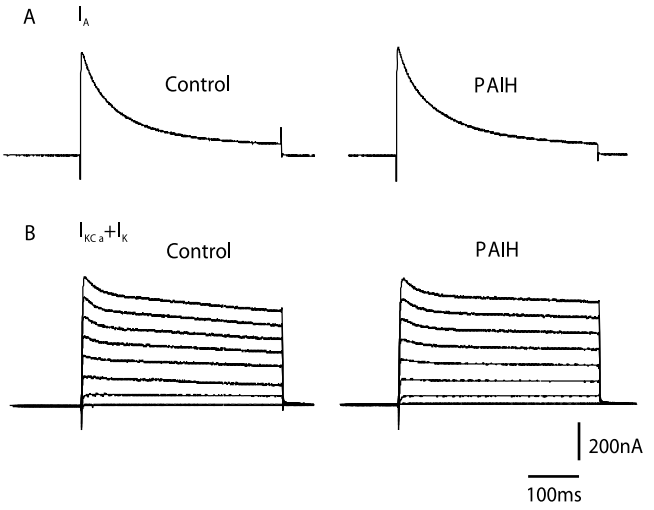

Figure 2. Overexpression of PAIH does not affect outward potassium currents. *A*, I_A measured at +20 mV was not significantly different in control and *PAIH*-expressing PD neurons. The cells were held at −50 mV, and after a 400 msec deinactivating prepulse to −120 mV, a 400 msec step to +20 mV was given. *B*, The control and PAIH-expressing cells had similar noninactivating outward potassium currents. The cell was held at −40 mV, where I_A is inactivated. A series of 400 msec voltage steps were delivered from −40 to +30 mV in 10 mV increments. Drugs added to isolate the currents are described in Materials and Methods.

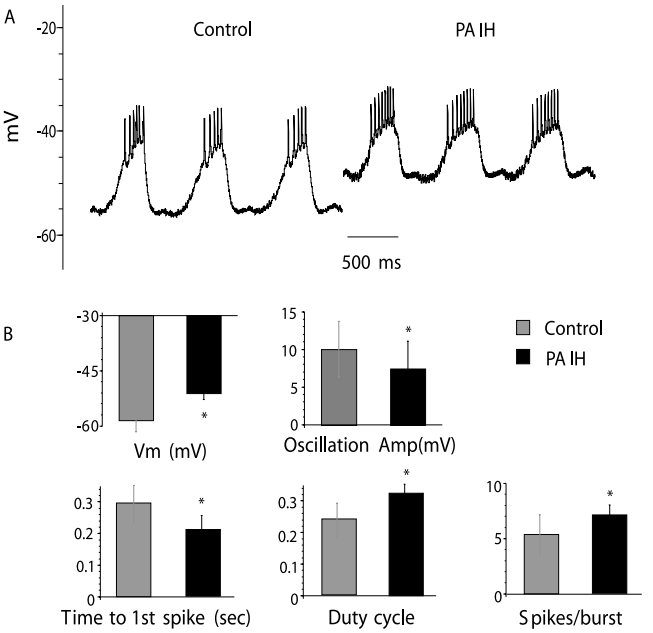

Figure 3. Overexpression of PAIH changed PD activity. *A*, Rhythmic activity recorded in paired control and PAIH-expressing PD neurons from the same ganglion. *B*, Comparison of the different firing properties in paired control (gray) and PAIH-expressing (black) PD neurons ($n =$ 6 pairs; *$p < 0.05$, significantly different from control PDs). Error bars indicate SDs. The measurements of the parameters are described in Materials and Methods. Amp, Amplitude.

PAIH overexpression alters the firing properties of the PD neurons

Because upregulation of I_h is not compensated by outward currents in PD neurons, we expected the increased I_h to change the firing properties of the injected PD neuron, relative to the control PD neuron in the same ganglion, and this turned out to be correct (Fig. 3). Compared with the control PD cell, the *PAIH*-expressing cell had a more depolarized minimal membrane potential, V_{min}, at the trough of the membrane oscillation (−51 ± 1.7 vs −58 ± 2.9 mV; $n = 6$ for all of the measurements), and the oscillation amplitude was somewhat smaller than the control value (7.4 ± 3.2 vs 10.0 ± 3.7 mV). The *PAIH*-injected PD neuron rebounded from inhibition more quickly than the control PD neuron: it had a shorter time from the V_{min} to the first spike (208 ± 50 vs 294 ± 58 msec), more spikes per burst (7 ± 1 vs 5 ± 2), and consequently a larger duty cycle (fraction of the cycle during which the cell was spiking, 0.32 ± 0.054 vs 0.22 ± 0.064) (Fig. 3*B*). All of these differences were significantly different between the injected and control PD neurons ($p < 0.05$; $n = 6$ pairs).

Cesium ions block I_h (Fig. 1*D*). When we applied 5 mM Cs^+ to a ganglion with one *PAIH*-expressing PD cell, the oscillatory frequency of the entire network significantly slowed down (from 1.15 ± 0.38 Hz down to 0.98 ± 0.31 Hz; $p < 0.01$; $n = 5$), and the oscillatory properties of *PAIH*-expressing cells moved back toward those seen in control, noninjected PDs (Fig. 4). The minimal membrane potential in the *PAIH*-expressing PD cell was hyperpolarized by 3.7 ± 1.2 mV (Fig. 4*A*), and the oscillation amplitude was increased by 2.9 ± 1.3 mV. As expected, the postinhibitory rebound of the cell was delayed: the time from the minimal voltage of the oscillation to the first spike was doubled (258 ± 98 vs 556 ± 172 msec), and the duty cycle was decreased by 17% (0.32 ± 0.01 vs 0.27 ± 0.03). These values were all significantly different from the values of the *PAIH*-expressing neuron before Cs^+ application (Fig. 4*B*) ($p < 0.05$; $n = 5$) and more similar to those seen in the control cells.

Dynamic-clamp simulation of I_h alters the firing properties of PD neurons in the same way as PAIH overexpression

Dynamic-clamp experiments use a computer to generate and apply a modeled ionic current to a neuron *in situ* (Sharp et al., 1993a,b). The computer monitors the membrane potential of the neuron, calculates the conductance of the modeled current that would arise as a consequence of the changing membrane potential, and injects the appropriate current into the soma of the cell. The parameters of the modeled conductance can be altered to explore what effect the modeled conductance has on the intrinsic properties of the biological neuron under investigation. We established a dynamic-clamp system that could inject a modeled I_h into PD neurons. This modeled I_h had the same properties as the PAIH-expressed current in PD cells. The basic parameters we used were derived from the experiments described above: $g_{max} =$ 0.75 μS; $V_{½} = -72$ mV; $V_{slope} = 7.5$ mV; $E_{rev} = -30$ mV; and $\tau_m = 8$ sec. The maximal currents injected were ~1–2 nA, and the output current reached steady state after 7 sec (Fig. 5*A*).

This artificial I_h produced a similar effect on noninjected PD cells in the acutely isolated STG as overexpression of *PAIH* in the

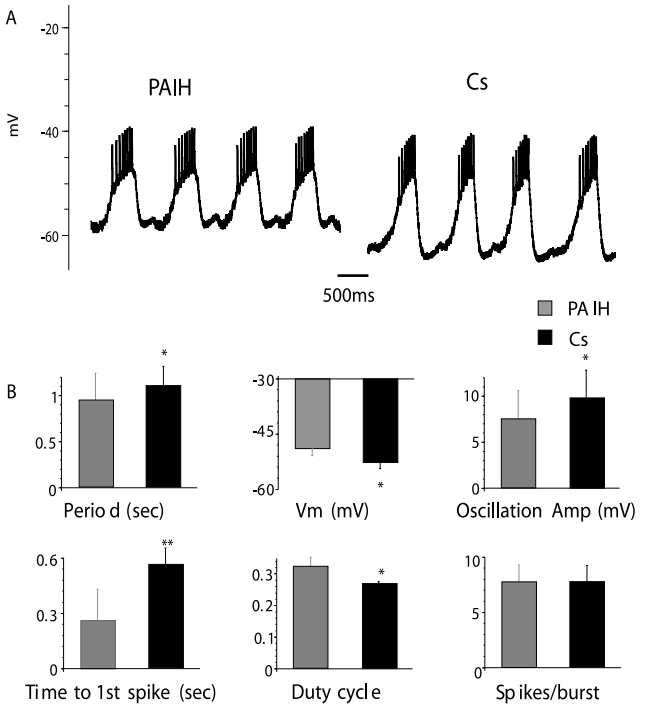

Figure 4. Cs^+ restores the activity of PAIH-expressing PD neurons toward control values. *A*, Rhythmic activity in a PAIH-expressing PD neuron before and after application of 5 mM Cs^+. *B*, Comparison of the firing properties in a PAIH-expressing PD before (gray) and after (black) application of 5 mM Cs ($n = 5$; $*p < 0.05$, significantly diferent from PAIH-expressing neurons; $**p < 0.01$, very significantly different from PAIH-expressing neurons). Error bars indicate SDs. Amp, Amplitude.

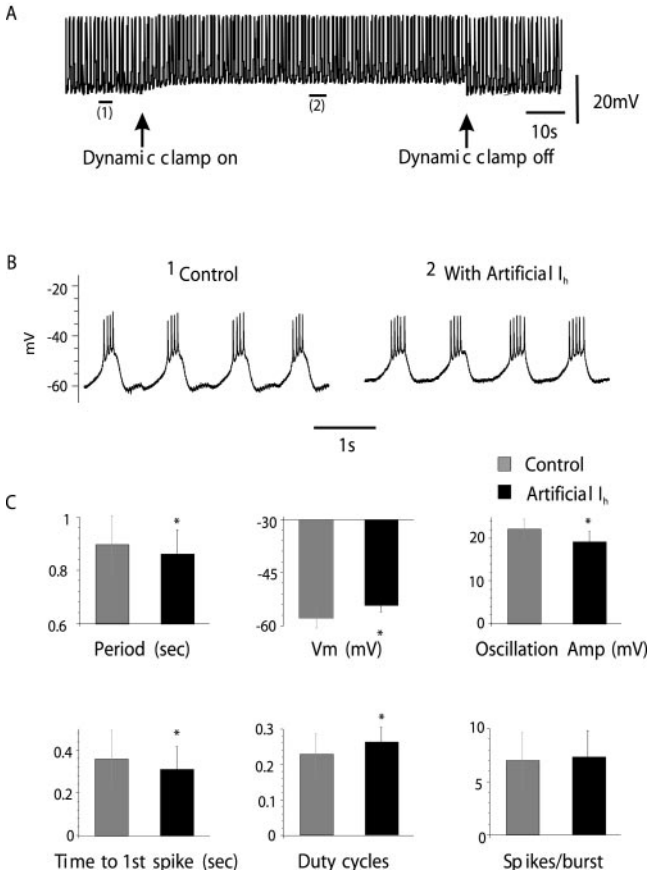

Figure 5. Dynamic clamp simulation of PAIH-evoked I_h alters the electrophysiological activity of noninjected PD neurons. *A*, A 2 min continuous recording of the activity of a PD cell, with the dynamic clamp of the PAIH-evoked I_h turned on and off at the arrows. (1) and (2) are the regions that are expanded in *B*. *B*, Examples of rhythmic activity in a PD neuron before (1) and after (2) activating the artificial I_h. *C*, Comparison of the average firing properties in a PD neuron before (gray) and after (black) dynamic-clamp addition of the artificial I_h ($n = 5$; $*p < 0.05$, significantly different from control status). Error bars indicate SDs. Amp, Amplitude.

cultured preparations (Fig. 5*B*,*C*). Compared with the oscillation before the dynamic clamp was turned on, the cycle frequency was slightly, but significantly, increased from 1.11 to 1.16 Hz; the minimal membrane potential was depolarized by 3.5 ± 0.7 mV, and the oscillation amplitude was decreased from 22.1 ± 2.3 to 19.1 ± 2.4 mV; the time from the trough of the oscillation to the first spike was decreased from 358 ± 135 to 309 ± 105 msec, and the duty cycle was increased from 0.22 ± 0.06 to 0.26 ± 0.05 ($p < 0.05$; $n = 5$). All of these alterations in parameters were in the same direction as we found in the *PAIH*-expressing PDs, but were somewhat less pronounced.

We also varied the parameters of the simulated current to study their effects on the cells ($n = 5$). In most of our experiments, we set the activation time constant of I_h at 8 sec, which was close to τ_m for PAIH at −60 mV. When we increased τ_m to 2 sec with the other parameters unchanged, it produced the same effects on oscillatory properties as above but sped up the time course to reach the steady state (data not shown). When we varied the maximum conductance, as we expected, all of the measured oscillatory parameters changed in parallel. For example, when the g_{max} was reduced to 0.3 μS, the minimal membrane potential was depolarized by only 2.03 ± 0.67 mV, and the oscillation amplitude was decreased by 1.6 ± 0.5 mV. The time to the first spike was decreased by 32 ± 31 msec, the duty cycle was increased by 0.014 ± 0.012, and the firing frequency showed no change. In contrast, when g_{max} increased to 1.2 μS, the minimal membrane potential was now depolarized by 4.9 ± 1.3 mV, and the oscillation amplitude was decreased by 3.9 ± 0.7 mV. The time to the first spike was decreased by 82 ± 76 msec, the duty cycle was increased by 0.036 ± 0.017, and the firing frequency increased by 0.05 ± 0.03 Hz. Shifting the value of $V_{½}$ also had strong effects on the firing properties of the PD neuron. When we changed the $V_{½}$ to −90 mV (near the value for endogenous I_h in the control cells), there was no effect at all of the injected I_h on the oscillatory properties of the cell, with the maximal conductance at 0.75 μS. In contrast, when the $V_{½}$ was depolarized beyond its normal value, the firing and oscillatory parameters increased accordingly (data not shown).

Theoretically modeled simple network confirms our experimental results

To further test the effects of I_h on the firing properties of PD cells, we examined the response of the two-cell model described in Materials and Methods to an I_h added to the PD cell. In this model, we had a driver cell, which mimicked the function of the pacemaker cell AB with one PD cell, and a follower cell, which represented the *PAIH*-injected PD cell. In the control state of the model, the endogenous I_h is included in the leak conductance, because its activation time constant is very long relative to an oscillation. With added g_h set to 0, the modeled PD cell showed a firing pattern characteristic of normal PD cells in the pyloric network (Fig. 6*A*). Analogous to our dynamic-clamp experiments, we then added an H-current to the modeled PD neuron with constant conductance $g_h = 0.07$ μS, representing the I_h observed in this voltage range in the *PAIH*-injected cells or the I_h

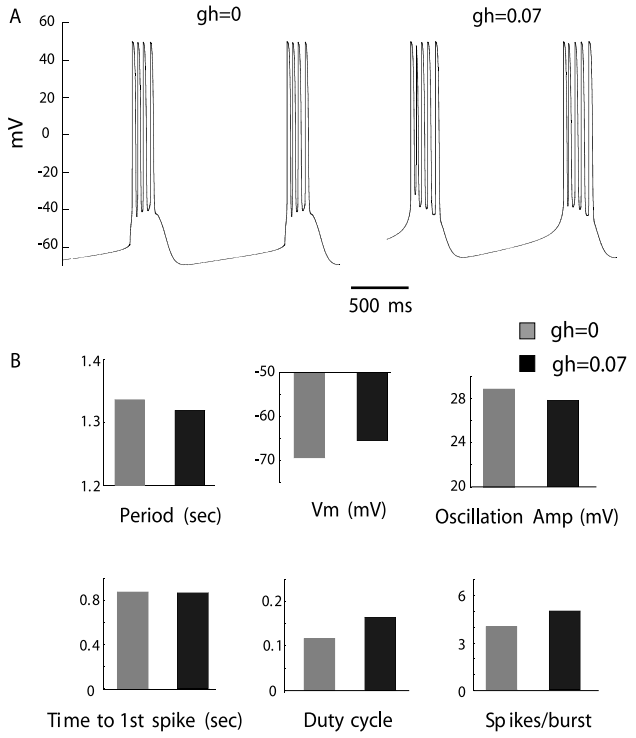

Figure 6. A mathematical model of a two-cell network is altered by adding a PAIH-like I_h conductance. *A*, Oscillatory traces from the modeled PD cell with and without an added *PAIH*-like g_h. *B*, Comparison of the electrophysiological parameters in the modeled PD without g_h (gray) andwith g_h (black). Amp, Amplitude.

added in the dynamic-clamp experiments. With this added I_h, the oscillatory properties of the modeled PD cell were altered in the same direction as changes observed in the *PAIH* injection and dynamic-clamp experiments (Fig. 6): the cycle frequency of the two-cell network was slightly increased from 0.748 to 0.758 Hz. The minimal membrane potential was depolarized from −69 to −65 mV, and the oscillation amplitude was decreased from 28.9 to 27.9 mV. The time to the first spike was shortened from 877 to 867 msec, the duty cycle was increased from 0.118 to 0.165, and the spike number was increased by 1 (4 vs 5). All of these changes are qualitatively in the same direction as seen in the other experiments, although, again, they are quantitatively smaller than seen with *PAIH* injection.

Discussion

Our work shows that injection of cRNA for *PAIH*, a lobster gene encoding I_h channels, increases I_h in the PD neuron of the pyloric network. This increase in I_h channel protein did not provoke a compensatory increase in I_A. Combining experimental with theoretical modeling studies, we were able to see how an increase in I_h can regulate the excitability and firing property of the cell in a functioning network.

PAIH currents in PD neurons

Gisselmann et al. (2003) have studied PAIH channel properties when expressed in HEK293 cells. When we injected *PAIH* RNA into PD neurons, the enhanced I_h had somewhat different properties than were seen in HEK293 cells. In particular, the $V_{½}$ for activation was >40 mV depolarized (−119 mV in HEK293 and −75 mV in PD). This variable expression of I_h genes in different cell types has been noted previously: Qu et al. (2001, 2002) found that HCN2- and HCN4-evoked currents have very different properties when expressed in neonate versus adult cardiac ventricular cells, or in HEK293 cells. In addition, Chen et al. (2001) showed that the $V_{½}$ for HCN1 shifted from −69 mV in an intact oocyte to −116 mV in an inside–out patch from the oocyte, and this was not merely caused by the loss of cAMP. All of these indicate that the characteristics of H-channels are profoundly modified by the cellular environment. This could arise from differences in the concentration of cAMP, the auxiliary subunits expressed by the cells, the state of phosphorylation of the channels, or cell-specific posttranslational modifications (Chen et al., 2001; Yu et al., 2001; Vargas and Lucero, 2002).

The *PAIH*-evoked current was also somewhat different from the endogenous I_h in PD neurons: the PAIH-encoding current had more positive $V_{½}$ and more rapid activation kinetics (Kiehn and Harris-Warrick, 1992; Peck et al., 2000). There are several possible explanations for this discrepancy. First, although they are very closely related species, the gene(s) encoding I_h in *P. interruptus* may have somewhat different properties from its homolog in *P. argus*. Second, there may be more than one gene encoding I_h in lobsters, so *PAIH* alone could not produce the normal current. Third, *PAIH* may undergo alternative splicing, yielding splice variants with different properties. Preliminary data have shown that, in *Drosophila*, there are several splice variants in the I_h gene that generate H-currents with somewhat different biophysical properties; splice variants at similar positions of the gene have been also detected in *Panulirus* lobsters (G. Gisselmann, T. Marx, and H. Hatt, unpublished data). Finally, overexpression of PAIH may exhaust available stores of critical modulation factors or auxiliary subunits, which may normally shape the properties of the endogenous I_h. To address these possibilities, we are performing additional gene cloning from *P. interruptus*.

Overexpression of *PAIH* does not evoke detectable homeostatic compensation by outward currents

In a previous study, we overexpressed the transient potassium channel gene, *shal*, in PD neurons of the *P. interruptus* STG, and found that this did not significantly alter the firing properties of the injected neurons (MacLean et al., 2003). This unusual result was attributable to an activity-independent homeostatic response: the artificially evoked increase in I_A was compensated by an endogenous cellular response to upregulate I_h, which balanced and negated the physiological effects of the enhanced I_A. In this study, we wanted to determine whether overexpression of *PAIH* also leads to a cellular upregulation of I_A. Our current results suggest that *PAIH* overexpression does not significantly affect I_A or the other outward currents that we measured.

There are several possible explanations for this unexpected result. First, as we mentioned above, there may be some difference between the I_h gene in *P. argus* and that in *P. interruptus*, or we may be using the incorrect splice variant of I_h that could evoke the homeostatic upregulation of I_A. Second, there may be more than one H-current gene in the lobster, and *PAIH* may not be the one that coregulates with I_A. In fact, the I_h evoked by *shal* overexpression had very different biophysical properties from both the endogenous I_h and the *PAIH*-evoked currents (MacLean et al., 2003; J. N. McLean, Y. Zhang, and R. M. Harris-Warrick, unpublished observations). Additional cloning efforts will help to address this possibility. Finally, it is possible that the homeostatic compensation we observed with *shal* RNA injections is unidirectional: that is, I_A proteins might cause an upregulation of I_h, but not vice versa. When Qu et al. (2001, 2002) overexpressed HCN2 or -4 in cardiac ventricular cells, they did not detect any

compensatory change in potassium currents. From the point of view of cellular economy, these cells may not need to regulate the consequences of enhanced I_h expression as closely as I_A. We showed that subtle changes in I_A markedly alter the firing properties of pyloric neurons (Kiehn and Harris-Warrick, 1992; Harris-Warrick et al., 1995a,b; Kloppenburg et al., 1999). In our theoretical model, when we increase the maximal conductance of I_A to the value seen in *shal*-injected neurons, the cell is unable to oscillate normally. However, the model was much less sensitive to increases in I_h: although the oscillatory properties of the cell changed as described above, it still maintained a relatively stable oscillation. Thus, it may be relatively more important to maintain homeostatic control for overexpression of I_A than of I_h. This might be a reason that I_h can be a good target for neuromodulation and a significant factor shaping the firing properties of neurons.

Overexpression of PAIH modulates the firing properties of PD neurons

The function of I_h under normal physiological conditions is still unclear. Preliminary data suggest that I_h is small and does not play a major role in the pyloric rhythm under control conditions. Many endogenous neuromodulators of the pyloric motor pattern, such as dopamine, can shift the $V_{1/2}$ of I_h in the depolarizing direction, so that I_h can become active in the physiologically relevant voltage range (Harris-Warrick et al., 1995b; Dickinson et al., 2001). Neuromodulators exert a combination of changes in synaptic function and a variety of changes in the intrinsic properties of the cells. It is always difficult to single out the function of each individual conductance. In this study, we introduced an I_h into PD neurons with a $V_{1/2}$ ~10 mV more depolarized than the control I_h; this is similar to the I_h in the presence of dopamine, which depolarizes its $V_{1/2}$ in LP neurons by 20 mV (Harris-Warrick et al., 1995b). This enhanced I_h significantly altered the firing properties of the PD neurons and the pyloric network.

In the pyloric network, there are two PD cells with identical biophysical and oscillatory properties. Normally, these cells fire synchronous bursts of action potentials. However, overexpression of *PAIH* in one PD cell separated it from its partner cell. The PAIH-expressing cell was more depolarized and had a smaller oscillation amplitude. Significantly, it showed a more rapid onset of spiking, with a larger duty cycle and more spikes per burst, such that the injected cell became the leading cell of the two PD neurons. We tested whether the major cause of these changes was in fact an increase in I_h by reversing them with the I_h blocker, Cs^+. Five millimolar Cs^+ eliminated I_h in the *PAIH*-expressing PD neuron and restored most of its oscillatory properties toward the control values in an uninjected PD neuron.

To further test whether the *PAIH*-expressed current is sufficient to cause these changes in cellular activity, we conducted dynamic-clamp experiments, modeling the additional *PAIH* current and using a computer to inject the calculated current into a PD neuron. In addition, we used a mathematical model of a two-cell network, comparing the response of the modeled PD with or without an additional *PAIH*-like current. In both experiments, application of the extra I_h evoked changes in firing properties of the PD neuron that were all in the same direction as seen after *PAIH* RNA injection: the modeled current depolarized the cell, reduced the oscillation amplitude and the onset time of the spikes, and increased the duty cycle. The changes in both dynamic clamp and modeled network were quantitatively less dramatic than those seen in *PAIH*-overexpressing cells. However, neither of our theoretical models completely represents the situation in real cells. In the dynamic-clamp experiments, the modeled current is injected only into the soma rather than distributed throughout the neuropil and in the vicinity of the spike initiation zone, in which we expect the PAIH channels to be expressed. Our previous studies have shown that the effects of ionic currents on oscillatory properties are very sensitive to the location of the currents relative to the spike initiation zone (MacLean, Zhang, R. A. Ricardo, R. Casey, J. Guckenheim, and Harris-Warrick, unpublished data). The mathematical model of the two-cell network was not intended as an accurate representation of the pyloric circuit. Instead, we used it as a computational test bed for examining the effects of adding an I_h to a PD cell driven by rhythmic oscillations. In this model, the PD cell was represented as a single compartment that lacks the structural complexity of the real neurons. Because of these limitations, we can only show that the responses of the PD cell in the dynamic-clamp and theoretical simulations are qualitatively consistent over a number of different parameters with our data from *PAIH*-injected PD cells. Overall, these simulations support the hypothesis that alteration of I_h by itself is sufficient to explain the effects of *PAIH* RNA injection; there is no need to consider additional compensatory current changes. This is consistent with the lack of compensation by I_A, $I_{K(V)}$, and $I_{K(Ca)}$.

In conclusion, our study has demonstrated that overexpression of an I_h gene, *PAIH*, causes significant changes in the membrane potential and firing properties of the PD neurons within the pyloric network. Overexpression of PAIH does not alter the expression of I_A or other outward currents, so the homeostatic compensation we previously observed after upregulation of I_A is not bidirectional.

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