Activity-Independent Coregulation of $I_A$ and $I_h$ in Rhythmically Active Neurons

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MacLean, Jason N., Ying Zhang, Marie L. Goeritz, Richard Casey, Ricardo Oliva, John Guckenheimer, and Ronald M. Harris-Warrick. Activity-independent coregulation of $I_A$ and $I_h$ in rhythmically active neurons. J Neurophysiol 94: 3601–3617, 2005. First published July 27, 2005; doi:10.1152/jn.00281.2005. The fast transient potassium or $A$ current ($I_A$) plays an important role in determining the activity of central pattern generator neurons. We have previously shown that the shal $K^+$ channel gene encodes $I_A$ in neurons of the pyloric network in the spiny lobster. To further study how $I_A$ shapes pyloric neuron and network activity, we microinjected RNA for a shal-GFP fusion protein into four identified pyloric neuron types. Neurons expressing shal-GFP had a constant increase in $I_A$ amplitude, regardless of cell type. This increase in $I_A$ was paralleled by a concomitant increase in the hyperpolarization-activated cation current $I_h$ in all pyloric neurons. Despite significant increases in these currents, only modest changes in cell firing properties were observed. We used models to test two hypotheses to explain this failure to change firing properties. First, this may reflect the mislocalization of the expressed shal protein solely to the somata and initial neurites of injected neurons, rendering it electrically remote from the integrating region in the neuropil. To test this hypothesis, we generated a multicompartment model where increases in $I_A$ could be localized to the soma, initial neurite, or neuropil/axon compartments. Although spike activity was somewhat more sensitive to increases in neuropil/axon versus somatic/primary neurite $I_A$, increases in $I_A$ limited to the soma and primary neurite still evoked much more dramatic changes than were seen in the shal-GFP–injected neurons. Second, the effect of the increased $I_A$ could be compensated by the endogenous increase in $I_h$. To test this, we modeled the compensatory increases of $I_A$ and $I_h$ with a cycling two-cell model. We found that the increase in $I_h$ was sufficient to compensate the effects of increased $I_A$, provided that they increase in a constant ratio, as we observed experimentally in both shal-injected and noninjected neurons. Thus an activity-independent homeostatic mechanism maintains constant neuronal activity in the face of dramatic increases in $I_A$.

INTRODUCTION

The interaction of neuronal intrinsic firing properties with the synaptic connectivity between component neurons determines the output patterns of rhythm generating networks called central pattern generators (CPGs) (Getting 1989; Selverston and Moulins 1985). The electrophysiological properties of neurons are in turn determined by the palette of ion channels they express. Changes in the balance of a neuron’s ionic conductances can dramatically change the output of the neuron and the CPG (Harris-Warrick et al. 1998; Llinas 1988).

The pyloric CPG, in the stomatogastric ganglion (STG) of crustaceans, is among the best understood neural networks in any species (Harris-Warrick et al. 1992). In the spiny lobster, Panulirus interruptus, the pyloric CPG is composed of 14 neurons in six major neuronal classes, each of which has distinct electrophysiological properties and exhibits a unique firing pattern. The intrinsic properties of pyloric neurons are determined, in part, by differences in the fast transient potassium ($K^+$) or $A$ current ($I_A$). $I_A$ operates in the subthreshold range of membrane potentials and is activated by depolarization after a period of hyperpolarization that removes resting inactivation (Graubard and Hartline 1991). $I_h$ differs between the six pyloric neuronal classes in its amplitude, voltage dependency, and kinetics (Baro et al. 1997; Tierney and Harris-Warrick 1992). This variability of $I_h$ helps to set the phase relationships and firing frequency of each neuron within the pyloric motor pattern (Tierney and Harris-Warrick 1992).

In arthropods, the shaker and shal genes both encode $\alpha$-subunits of $A$-type $K^+$ channels (Wei et al. 1990). We have cloned and expressed these genes from P. interruptus. Several arguments strongly suggest that shal encodes $I_h$ in the somatodendritic compartment of pyloric neurons. First, when expressed in Xenopus oocytes, lobster shal encodes a fast transient $K^+$ current similar to the endogenous pyloric $I_h$ (Baro et al. 1996), whereas shaker generates an $A$-current with quite different properties (Kim et al. 1997, 1998). Second, there is a strong linear relationship between the level of shal mRNA expression and the amount of $I_h$ in the six cell types in the pyloric network (Baro et al. 1997). Third, immunocytochemical studies, using antibodies raised against lobster shal, reveal extensive labeling of the somata and the neuropil, but not peripheral axons, of the pyloric neurons, whereas shaker proteins are selectively localized to axons of STG neurons (Baro et al. 2000).

To further study the role of shal in shaping pyloric neuron properties, we previously overexpressed the shal potassium channel in the pyloric dilator (PD) neurons by microinjection of shal RNA (MacLean et al. 2003; Zhang et al. 2003). This leads to a greatly enhanced expression of $I_h$. The detailed biophysical properties of the enhanced current, although similar, are not identical to those of the endogenous $I_A$ in PD neurons, apparently because of the lack of an auxiliary KCaP-like protein (Zhang et al. 2003). Surprisingly, despite very
large increases in $I_A$ after $shal$ RNA injection, only very subtle changes in pyloric cycle activity are observed (MacLean et al. 2003). We proposed that this may arise from a compensatory activity-independent increase in a completely different current, the hyperpolarization-activated inward current $I_h$, which counteracts the action of the increased $I_A$ (MacLean et al. 2003).

Our previous work examined only a single neuron type in the pyloric network, the Pyloric Dilator (PD). Herein we have established the generality of the compensatory effect by showing that microinjection of $shal$ RNA into other pyloric neurons evokes both an increase in $I_A$ and a homeostatic increase in $I_h$. In addition, we performed a more detailed study of the biophysical properties of the $shal$-evoked $I_A$ and $I_h$. Finally, we report two modeling studies to test hypotheses on why the neurons retain their normal firing properties despite these large changes in ionic currents. These studies support the hypothesis that homeostatic upregulation of $I_h$ can compensate the upregulation of $I_A$ over a wide range in all pyloric neurons.

**METHODS**

**Construction of $shal$-GFP fusion protein**

The cDNA fragment coding for the full length of Green Fluorescent Protein (GFP, GIBCO) was amplified by polymerase chain reaction using the primers 5'-ACT ACT CCT GCT CTA GGG AGC AAG GCC GAG GAA CTG TT-3' and 5'-ACT ACT CCT AGA GCA GCC TTT TCG ACG TC-3', digested by EcoRI and subcloned at the EcoRI cutting site of the C-terminal of $shal$-e15 (between amino acids 649 and 650), a splice form of lobster $shal$ (Baro et al. 2001), in Bluescript vector.

**Preparation**

California spiny lobsters (*Panulirus interruptus*: Don Tomlinson Commercial Fishing, San Diego, CA) were anesthetized by cooling on ice for 30 min. The stomatogastric nervous system (STNS), consisting of the stomatogastric ganglion (STG), with its motor nerves and associated commissural and esophageal ganglia, was dissected and pinned in a UV-sterilized Sylgard-coated dish. Individual somata were impaled with glass microelectrodes (10–25 MΩ; 3 M KCl) and identified using three criteria: 1) a 1:1 correspondence of action potentials recorded intracellularly in the soma and extracellularly from an identified motor nerve; 2) characteristic phasing and synaptic input during the pyloric motor pattern; and 3) characteristic shape of the membrane potential oscillations and action potentials in the pyloric rhythm.

**Microinjection of neurons**

After physiological identification, pyloric neurons were injected with RNA using pressure pulses (40 psi, 10- to 90-ms duration, 0.2 Hz, for 2–10 min) with a home-built pressure injector. Microelectrodes were broken to a tip diameter of 1–2 μm and had resistances of about 1 MΩ when filled with 3 M KCl. The tips were filled with 0.2–1.0 μg/μl $shal$ or $shal$-GFP RNA with 0.04–0.07% Fast Green in sterile distilled water. Control neurons were injected with the Fast Green solution alone or with RNA encoding GFP; both control injections had few detectable effects on the control neurons (described below).

**Organotypic culture of the STG**

After RNA injection, the STNS was placed into filter-sterilized, oxygenated L15 media and incubated at 16°C. The L15 media contained Leibovitz’s L15 media with t-glutamine (GIBCO), penicillin (2500 unit/l)-streptomycin (2.5 mg/l) (Sigma), 250 μg/l Fungizone (GIBCO), and additional salts to raise the osmolarity to that of normal lobster saline. The medium was changed every 24 h.

**Voltage-clamp recordings**

$I_A$ and $I_h$ were isolated by a combination of pharmacological block, voltage inactivation, and digital current subtraction protocols as previously described (Kloppenburg et al. 1999). Briefly, neurons were voltage clamped, using an Axoclamp 2B amplifier and pClamp software (Axon Instruments), in lobster saline containing tetrodotoxin (TTX, 0.1 μM) and picrotoxin (PTX, 5 μM) to block most synaptic inputs; CdCl2 (600 μM) to eliminate calcium and calcium-dependent currents; and tetraethyl ammonium (TEA chloride, 20 mM), to block rectifying voltage-gated $K^+$ currents. $I_h$ was measured by a digital subtraction method where the leak-subtracted currents evoked by depolarizing steps from −50 mV (where $I_A$ is nearly completely inactivated) were subtracted from the leak-subtracted currents evoked by similar steps with a 500- to 700-ms prestep to −120 mV to remove inactivation. For $I_h$ the neurons were held at −40 mV (where $I_A$ is inactive) then hyperpolarized with 7- to 10-s steps at 5- or 10-mV increments from −60 to −100 mV. These currents were not leak subtracted.

**Current analysis**

The voltage dependency activation of $I_A$ was determined by converting the peak current to peak conductance $g_A$ (assuming $E_{K}$ of −94 mV for the oocytes and −86 mV for neurons; Hartline and Graubard 1992). The resulting $g/V$ curve was fitted to a Boltzmann equation of the form

$$g_A = g_{max} \left[1/(1 + e^{-V_{act} - V_s})\right]$$  (1)

where $g_{max}$ is the maximal conductance, $V_{act}$ is the voltage of half-maximal activation of each gating subunit, $s$ is the slope factor, $n = 3$ for activation, and $n = 1$ for inactivation. The inactivation kinetics were fit with two exponentials, using the least-squares minimization procedure of pClamp (Axon Instruments).

The voltage dependency of activation of $I_h$ was determined by converting the peak current to peak conductance $g$ (using a reversal potential of −30 mV for neurons; Zhang et al. 2003). The resulting $g/V$ curve was fitted to a Boltzmann equation (see above). The activation and deactivation kinetics were both fit with single exponentials, using the least-squares minimization procedure of pClamp (Axon Instruments).

**Analysis of rhythmic activity**

The minimal membrane potential was measured as the most hyperpolarized potential in the trough of the rhythmic activity. The amplitude of the oscillation was the difference between the most depolarized potential of the slow wave oscillation (under the spikes) and the minimal membrane potential. The slope of the rise phase was calculated using a straight line from the minimal membrane potential to the threshold point of the first spike. The frequency was the inverse of the time between the most hyperpolarized potential of two adjacent oscillations. All measures were based on average measures of ∼20 cycles.

**Xenopus oocyte expression**

Xenopus oocyte expression studies were performed as previously described (Baro et al. 1996). Briefly, capped RNA was transcribed from linearized $shal$ or $shal$-GFP DNA clones with a T3 mMessage mMachine kit (Ambion) using T3 RNA polymerase. A Sutter Instrument microinjector (model NA-1; San Rafael, CA) was used to inject about 100 nl of cRNA (concentration about 50 ng/μl) into Xenopus...
oocytes, which were isolated and maintained according to Quick et al. (1992). Recordings were made by two electrode voltage clamp 3 days later.

Immunocytochemistry

After voltage clamp, the STG was fixed in 3.2% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. The fix was washed out with eight changes of PBST (PBS + 0.3% Triton X-100) over 2–8 h. The tissue was incubated for 48 h in a rabbit anti-shal primary antibody [Baro et al. 2000; 1:2,000 PBST + 5% normal goat serum (NGS)]. The primary antibody was washed out with PBST for 2 h. The secondary antibody was preabsorbed with lobster brain ganglia to reduce nonspecific binding. The STG was then incubated overnight with the preabsorbed donkey anti-rabbit-Cy3 secondary antibody (Jackson ImmunoResearch Laboratories; 1:400 dilution in PBST + 5% NGS). The secondary antibody was washed out with PBS for 2–8 h. All incubations were performed at 4°C with constant shaking. The STG was mounted on a polylysine-coated coverslip, run through an ethanol dehydration series (30, 50, 70, 2 × 95%, 5 min each), and cleared in xylene (2 × 5 min each). The STG was mounted on a slide with DPX mounting media (Fluka) for 24 h. The slides were visualized with a Biorad 600 confocal microscope.

Statistics

Student’s t-test and ANOVA were performed using Excel software (Microsoft, Redmond, WA). Pearson correlation was performed using SPSS. Values are given as means ± SD.

Mathematical model of a pyloric neuron

We based our three-compartment model of a pyloric neuron on modifications of a previous single-compartment model for the LP cell in P. interruptus (Harris-Warrick et al. 1995a). This model was itself based on a model of Buchholz et al. (1992) for the LP neuron in Cancer borealis. The compartments represent the soma, the primary neurite, and the neuropile/axon. We chose to model the neurons with three compartments after fitting passive voltage responses to hyperpolarizing current steps with a sum of exponentials, each representing a compartment; the residual was very small with three terms. The three compartments are coupled by axial resistances with a coupling coefficient of 0.1. This value, along with the leak parameters for each compartment, was obtained from the multieponential fits. Each compartment has differential equations representing the major currents present in that region. The equations and parameter values used for the model are shown in Tables 4 and 5. We represent the location of the spike initiation zone (SIZ) and Ca²⁺ channels by including \( I_{\text{sia}} \) and \( I_{\text{ca}} \), only in compartment 3. This reflects the spatial isolation of the SIZ from the soma and the primary localization of calcium currents to regions outside the soma (Hurley and Graubard 1998; Kloppenburg et al. 2000). Analysis of the response of the model cell to changes in \( I_A \) parameters, shown in Figs. 6 and 7, was performed using the software package DTool (Back et al. 1992), running on a Sun Ultra 10 workstation. Rhythmic activity was evoked in the model cell (Fig. 7) by injecting a periodic hyperpolarizing current into the neuropile compartment to mimic rhythmic synaptic inhibition from the AB/PD neurons. The injected current had the form of a rectangular wave of period 2 s, with an initial 1.2-s hyperpolarizing pulse of magnitude 0.25 nA followed by no current injection for 0.8 s. This “cycle frequency” of 0.5 Hz is within the range seen in normal pyloric preparations, as well as those including shal-injected neurons. The calculations used to compare the sensitivity of the firing properties to \( I_A \) parameters in the neuropil and soma (Fig. 8) were carried out using numerical algorithms described in the appendix based on the work of Guckenheimer and Melono (2000). The algorithms were implemented in Matlab 5.3 (The MathWorks, Natick, MA) and used a modified version of the package ADOL-C 1.7 (Griewank et al. 1996). ADOL-C is available by ftp from Argonne National Labs at ftp://info.mcs.anl.gov/pub/ADOLC.

Mathematical model of coupled neurons

We investigated how an increase in \( I_A \) and/or \( I_h \) affects the firing properties of PD neurons using a mathematical model of two single-compartment–coupled cells. The increase in \( I_h \) 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 timescales of oscillations examined in our model simulations. During any simulation with a model that included a voltage-dependent \( I_h \) with its measured time constants, changes in its conductance would be negligible during an oscillation.

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 the 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 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 both chemical and electrical synapses. 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.

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.

The differential equations for the model were numerically integrated using the Radau integrator (Hairer and Wanner 1991) for a time span of 18 s. 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 voltage, and number of spikes were obtained using the same criteria applied to the experimental data.

Results

We studied three pyloric neurons in detail—the Pyloric Dilator (PD), the Lateral Pyloric (LP), and the Ventricular Dilator neuron (VD)—as well as preliminary studies of the Inferior Cardiac (IC) neuron, to examine the effect of overexpression of shal protein after shal RNA injection in the pyloric network. In addition, we studied the voltage dependency and kinetic properties of \( I_A \) in each pyloric neuron type, to determine whether the new protein is modified to generate neuron-specific \( I_A \) current properties, or whether a similar current is generated in all the neuron types. We also studied the homeostatic responses to upregulation of \( I_h \) in each neuron.

Dramatic increase in \( I_A \) 72 h after microinjection of shal-GFP

As described previously for the PD neuron (MacLean et al. 2003), 72 h after injection with shal-GFP RNA, a dramatic increase in \( I_A \) amplitude was observed in all pyloric neuron

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types tested (Fig. 1, Table 3). Representative currents from control pyloric neurons and neurons expressing shal-GFP are superimposed in Fig. 1A. A relatively constant increase of approximately 300–400 nA in the current amplitude (measured at +20 mV) occurred in all the neurons 3 days after injection of shal-GFP RNA, suggesting that the exogenous RNA is translated with relatively equal efficiency in all pyloric neurons. Because of the differences in baseline RNA is translated with relatively equal efficiency in all pyloric shal-GFP–evoked IA relative to the endogenous current through endogenous channels.

Comparison of biophysical properties of the shal-GFP–evoked IA relative to the endogenous current

The voltage dependencies of activation and inactivation of IA vary significantly between pyloric neurons (control neurons, Table 3; Baro et al. 1997). This has been hypothesized to result from posttranslational modifications such as phosphorylation of the shal protein, perhaps in conjunction with alternative splicing of the shal gene to expose new phosphorylation sites (Baro et al. 2001), and/or from different levels of expression of auxiliary subunits that modify the biophysical properties of the shal channel (Zhang et al. 2003). It is of interest then to compare the conductance/voltage (I–V) relationships of each neuron’s endogenous IA before and after expression of shal-GFP. The mean normalized I–V relationships of control neurons and neurons expressing shal-GFP are illustrated in Fig. 1B. In general the activation and inactivation properties of expressing and nonexpressing neurons were similar, as described previously for PD neurons (Zhang et al. 2003). However, two consistent changes were observed in all neuron types after expression of shal-GFP. First, the V1/2 shifted significantly in a depolarized direction in the expressing neurons. With a third-order Boltzmann fit to the data, the V1/2 values of the individual gating particles of all three neurons shifted by a range of 3.9 to 10.7 mV, corresponding to depolarizing shifts of 2.5 to 6.2 mV in the voltage at which half the channels open. The slope of the voltage activation curve was somewhat steeper in the shal-injected cells, although not significantly so. Second, although shal-GFP expression did not change the V1/2 values significantly, the slope of the voltage inactivation curve
became significantly more shallow in the PD and VD neurons (Fig. 1B), as indicated by an increase in the slope parameter for inactivation by 1.4 to 2.5 mV (Table 3; this parameter was not measured in IC neurons). These parameter shifts are similar to those seen earlier after shal RNA injection into PD neurons (Zhang et al. 2003). The fact that these shifts are similar in different neurons suggests that they do not result from cell-specific modifications of the new current.

In general, the shal-GFP–evoked current in the pyloric neurons has properties more similar to lobster I_{shal-GFP} expressed in Xenopus oocytes than the endogenous pyloric currents (Fig. 1C, Table 3). The slope of inactivation in oocytes is more shallow than that in control pyloric neurons, and all three shal-GFP–injected neurons had slope values that were intermediate between the neuronal and oocyte values. Table 3 and Fig. 1C also show that addition of GFP to the shal sequence does not affect the properties of the evoked current in oocytes; shal and shal-GFP also generated currents with identical properties when injected into PD neurons (MacLean et al. 2003).

Under control conditions, all neuron types exhibit quite different properties of endogenous $I_A$ (Table 3; Baro et al. 1997). Using ANOVA to compare the currents evoked after expression of shal-GFP, the $I_A$ exhibited by all neuron types became more homogeneous than their endogenous currents. The variance between the neurons decreased for the $V_{act}$ [$F_{(2,43)} = 2.6, P = 0.9$], $V_{inact}$ [$F_{(2,39)} = 0.7, P = 0.5$], the slope of activation [$F_{(2,18)} = 1.4, P = 2.7$], and the slope of inactivation [$F_{(2,43)} = 2.2, P = 0.12$]; as a result, the neurons' $A$-currents were no longer significantly different from one another. These data suggest that the newly expressed protein is not modified in cell-specific ways in pyloric neurons, but instead retains properties similar to $I_{shal-GFP}$ expressed in oocytes.

$I_A$ kinetics

The time course of inactivation of $I_A$ in pyloric neurons is best fit with a double-exponential relation. In control neurons, the majority of the current inactivates with a slower time constant ($\tau_{slow}$ around 80–110 ms) in PD, LP, and IC neurons, whereas a minority (<50%) inactivates rapidly ($\tau_{fast}$ varying between 7 and 25 ms). The VD neuron has exceptionally rapid inactivation kinetics, with both $\tau_{fast}$ and $\tau_{slow}$ four- to fivefold more rapid than in PD and LP (Baro et al. 1997). After expression of shal-GFP, the inactivation kinetics of $I_A$ were accelerated so that the majority of the expressed current inactivated with a fast time constant instead of a slower time constant (Table 3). Figure 2A shows the amplitude-normalized current during a voltage step to $+20$ mV in both the control neurons and neurons expressing shal-GFP. Clearly the amount of current that inactivates slowly is greater in the control PD neurons and neurons expressing shal-GFP. For these neurons, the time constants do not change after shal-GFP RNA injection (Table 3). However the percentage of current inactivating rapidly increases from 34–42 to 53–57% after expression of shal-GFP. The VD neuron undergoes a much more dramatic shift: the two time constants for inactivation assume slower values, similar to those seen in the other neurons after shal-GFP expression, and the additional current dominates the total $I_A$ in this cell. Again, the rapidly inactivating component is the majority of the current. This shift in the percentage of current inactivating during each time constant is similar to the inactivation properties of lobster $I_{shal-GFP}$ expressed in oocytes (Table 3). ANOVA reveals that the variance between neurons for the values of $\tau_{slow}$ and $\tau_{fast}$ significantly decreases, as VD becomes more similar to IC, LP, and PD [$F_{(1,33)} = 0.02, P = 0.8$],
suggested that the expressed protein is not completely modified to the neuron-specific parameters.

$I_A$ is mostly inactivated when the neuron is voltage clamped at $-50$ mV, and the neuron must be hyperpolarized to remove this inactivation. Using hyperpolarizing voltage steps to $-120$ mV of increasing duration (range 40–800 ms) the kinetics of recovery from inactivation of $I_A$ can be examined (Fig. 2, B and C). The curve generated by measuring maximal current amplitude against time of hyperpolarization is best fit with a single exponential time constant (Fig. 2C). In the noninjected PD, LP, and VD neurons, the recovery from inactivation is rapid, and essentially complete after 200 ms. After shal-GFP expression, complete removal of inactivation is significantly slower and takes >500 ms at $-120$ mV (Fig. 2, B and C, Table 3; PD, P = 0.001; LP, P = 0.004; and VD, P = 0.03; this parameter was not measured for IC neurons). The time constants shifted in the direction of $I_{shal-GFP}$ measured in oocytes, although recovery is even slower in oocytes (Table 3). This further suggests that $I_{shal-GFP}$ is not modified to neuron-specific parameters.

**Increase in $I_A$ is accompanied by an increase in $I_h$**

As previously seen for PD neurons (MacLean et al. 2003), LP, VD, and IC neurons, which expressed $shal-GFP$ and had an increased $I_A$, showed a significant increase in $I_h$ amplitude when measured by voltage clamp (Fig. 3A, Table 4). The increased $I_h$ was slowly activated during long hyperpolarizing voltage steps from $-40$ mV and was completely blocked by 5–10 mM Cs$^+$ (data not shown), characteristic features of $I_h$. We measured the amplitude of $I_h$ as the difference between the values reached at the end of the instantaneous initial step (representing the leak current) and at the end of the voltage step. Occasionally there were unexpected large jumps in inward current during long hyperpolarizing steps below $-110$ mV; these were seen in neurons injected with both $shal-GFP$ and $GFP$ RNA, were not blocked by Cs$^+$, and could not be fit

### Table 3. Properties of $I_A$ in control and $shal-GFP$—expressing neurons

<table>
<thead>
<tr>
<th>Cell Type (number)</th>
<th>$I_{max}$ nA</th>
<th>$G_{max}$ μS</th>
<th>Act $V_{1/2}$ mV</th>
<th>Act slope factor, mV</th>
<th>Inact $V_{1/2}$, mV</th>
<th>Inact slope factor, mV</th>
<th>Inact $\tau_{max}$ ms</th>
<th>$%$ peak $I_{max}$ (Vmax)</th>
<th>$%$ peak $I_{max}$ (Vhold)</th>
<th>Release from Inact $\tau$, ms</th>
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</thead>
<tbody>
<tr>
<td>PD control n = 37</td>
<td>405.1 ± 97.4</td>
<td>4.2 ± 1.1</td>
<td>-41.9 ± 6.3</td>
<td>-17.7 ± 4.5</td>
<td>-67.2 ± 4.9</td>
<td>4.2 ± 0.3</td>
<td>20.6 ± 5.8</td>
<td>101.9 ± 51.5</td>
<td>33.6 ± 1.5</td>
<td>66.4 ± 1.5</td>
</tr>
<tr>
<td>expressing n = 38</td>
<td>696.3 ± 156.5**</td>
<td>6.8 ± 1.7**</td>
<td>-39.0 ± 4.3*</td>
<td>-18.4 ± 2.7</td>
<td>-68.6 ± 7.6</td>
<td>5.6 ± 1.3**</td>
<td>23.7 ± 5.8</td>
<td>101.0 ± 20.2</td>
<td>53.2 ± 10**</td>
<td>46.8 ± 10**</td>
</tr>
<tr>
<td>LP control n = 9</td>
<td>201.8 ± 49.6</td>
<td>2.0 ± 0.4</td>
<td>-41.5 ± 5.2</td>
<td>-21.4 ± 8.2</td>
<td>-62.1 ± 8.0</td>
<td>7.0 ± 1.3</td>
<td>24.2 ± 3.6</td>
<td>107.6 ± 29.5</td>
<td>41.9 ± 10</td>
<td>58.1 ± 10</td>
</tr>
<tr>
<td>expressing n = 9</td>
<td>491.2 ± 101.7**</td>
<td>5.1 ± 1.3**</td>
<td>-34.4 ± 5.6**</td>
<td>-19.4 ± 4.5</td>
<td>-70.0 ± 10.9</td>
<td>8.2 ± 1.2</td>
<td>28.1 ± 13.0</td>
<td>104.6 ± 27.5</td>
<td>56.9 ± 2.8*</td>
<td>43.1 ± 10*</td>
</tr>
<tr>
<td>VD control n = 9</td>
<td>82.6 ± 33.3</td>
<td>0.8 ± 0.3</td>
<td>-45.2 ± 8.0</td>
<td>-20.7 ± 6.2</td>
<td>-71.8 ± 6.1</td>
<td>4.2 ± 0.6</td>
<td>4.7 ± 2.4</td>
<td>41.4 ± 33.1</td>
<td>46.9 ± 2.0</td>
<td>53.1 ± 20</td>
</tr>
<tr>
<td>expressing n = 12</td>
<td>358.9 ± 124**</td>
<td>3.6 ± 1.1**</td>
<td>-35.6 ± 7.1**</td>
<td>-19.1 ± 2.5</td>
<td>-72.7 ± 7.7</td>
<td>6.7 ± 1.7**</td>
<td>22.4 ± 11.4**</td>
<td>103.5 ± 39.2**</td>
<td>59.3 ± 10</td>
<td>40.7 ± 10</td>
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<tr>
<td>IC control n = 4</td>
<td>68.1 ± 28.2</td>
<td>0.7 ± 0.3</td>
<td>-40.6 ± 3.8</td>
<td>-18.0 ± 1.8</td>
<td>NA</td>
<td>NA</td>
<td>7.5 ± 1.2</td>
<td>83.7 ± 37.8</td>
<td>50.4 ± 4.6</td>
<td>49.6 ± 4.6</td>
</tr>
<tr>
<td>expressing n = 2</td>
<td>358.5 ± 205.7</td>
<td>3.8 ± 1.9</td>
<td>-33.6 ± 4.8</td>
<td>-17.3 ± 7.3</td>
<td>NA</td>
<td>NA</td>
<td>15.3 ± 3.8</td>
<td>80.9 ± 9.6</td>
<td>64.7 ± 20.7</td>
<td>35.3 ± 20.7</td>
</tr>
<tr>
<td>Lobster $I_{shal}$ oocyte</td>
<td>NA</td>
<td>NA</td>
<td>-42.9 ± 2.3</td>
<td>-21.1 ± 2.7</td>
<td>-68.6 ± 2.5</td>
<td>7.4 ± 2.2</td>
<td>37.7 ± 5.0</td>
<td>184.9 ± 117.4</td>
<td>53.5 ± 1.8</td>
<td>46.7 ± 1.8</td>
</tr>
<tr>
<td>Lobster $I_{shal}$-GFP oocyte</td>
<td>NA</td>
<td>NA</td>
<td>-41.6 ± 5.4</td>
<td>-20.1 ± 1.5</td>
<td>-68.9 ± 6.1</td>
<td>8.2 ± 1.0</td>
<td>56.9 ± 19.2</td>
<td>192.0 ± 54.1</td>
<td>62.4 ± 9.9</td>
<td>37.6 ± 10.3</td>
</tr>
</tbody>
</table>

Values indicate averages ± SD. *Significantly different (P < 0.05). ** Significantly different (P < 0.01).
with the less hyperpolarized steps by a single Boltzmann relation. To avoid contaminating our measurements of $I_h$ with this current of unknown origin, we limited our analysis of $I_h$ to steps down to $-100$ mV; examples of currents during steps to $-90$ mV are shown in Fig. 3A. During a voltage step to $-100$ mV (Fig. 3B) there was an average 392% increase in $I_h$ amplitude in shal-GFP–expressing PD neurons ($-43.3 \pm 33.9$ nA, $n = 42$ vs. $8.8 \pm 3.8$ nA, $n = 22$ in control neurons; $P = 0.000000004$), a 235% increase in shal-GFP–expressing VD neurons ($-40.3 \pm 29.5$ nA, $n = 6$, vs. $12.9 \pm 2.7$ nA, $n = 5$, in control neurons; $P = 0.03$), a 245% increase in shal-GFP–expressing LP neurons ($-25.6 \pm 27.8$ nA, $n = 3$, vs. $7.4 \pm 0.8$ nA, $n = 3$, in control neurons; $P = 0.15$), and a 337% increase in shal-GFP–expressing IC neurons ($-35.9 \pm 32.6$ nA, $n = 2$, vs. $8.2 \pm 0.6$ nA, $n = 4$, in control neurons; $P = 0.2$). The mean normalized $I-V$ relationships of control neurons and neurons expressing shal-GFP are illustrated in Fig. 3B. As can be seen, the current is not approaching saturation of activation.

### TABLE 4. Properties of $I_h$ in control and shal-GFP–expressing neurons

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$I_{\text{max}}, \text{nA}-100$ mV</th>
<th>$G_{\text{max}}, \mu S$</th>
<th>Act $V_{1/2}$, mV</th>
<th>Act Slope Factor, mV</th>
<th>Act $\tau$, s-100 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD control (42)</td>
<td>$-8.8 \pm 3.8$</td>
<td>$-0.19 \pm 0.1$</td>
<td>$-88.9 \pm 5.8$</td>
<td>8.5 $\pm 1.6$</td>
<td>4.2 $\pm 0.9$</td>
</tr>
<tr>
<td>expressing (22)</td>
<td>$-43.3 \pm 33.9^{**}$</td>
<td>$-1.9 \pm 1.8^{**}$</td>
<td>$-88.7 \pm 4.4$</td>
<td>9.6 $\pm 2.5$</td>
<td>6.8 $\pm 1.9^{*}$</td>
</tr>
<tr>
<td>VD control (6)</td>
<td>$-12.9 \pm 2.7$</td>
<td>$-0.27 \pm 0.17$</td>
<td>$-85.2 \pm 6.4$</td>
<td>8.8 $\pm 0.4$</td>
<td>1.6 $\pm 0.4$</td>
</tr>
<tr>
<td>expressing (5)</td>
<td>$-40.3 \pm 29.5^{*}$</td>
<td>$-1.4 \pm 1.2^{*}$</td>
<td>$-83.9 \pm 2.3$</td>
<td>6.9 $\pm 1.5$</td>
<td>5.8 $\pm 4.2$</td>
</tr>
<tr>
<td>LP control (3)</td>
<td>$-7.4 \pm 0.8$</td>
<td>$-0.12 \pm 0.01$</td>
<td>$-85.6 \pm 1.9$</td>
<td>9.3 $\pm 0.9$</td>
<td>2.6 $\pm 0.6$</td>
</tr>
<tr>
<td>expressing (3)</td>
<td>$-25.6 \pm 27.8$</td>
<td>$-0.7 \pm 0.9$</td>
<td>$-90.7 \pm 10.8$</td>
<td>9.1 $\pm 2.7$</td>
<td>2.6 $\pm 0.9$</td>
</tr>
<tr>
<td>IC control (4)</td>
<td>$-8.2 \pm 0.6$</td>
<td>$-0.13 \pm 0.02$</td>
<td>$-85.6 \pm 1.8$</td>
<td>6.3 $\pm 1.0$</td>
<td>1.8 $\pm 0.8$</td>
</tr>
<tr>
<td>expressing (2)</td>
<td>$-35.9 \pm 32.6$</td>
<td>$-1.1 \pm 0.09$</td>
<td>$-102.5 \pm 10.1$</td>
<td>7.9 $\pm 1.4$</td>
<td>3.1 $\pm 1.1$</td>
</tr>
</tbody>
</table>

Values indicate averages $\pm$ SD with number of cells in parentheses. *Significantly different ($P < 0.05$). **Significantly different ($P < 0.01$).
at these more depolarized voltages. The data were fit with a first-order Boltzmann relation, but the absolute values obtained should be read with caution because of the difficulty in making this fit. However, we were able to make preliminary assessments of the effects of shal overexpression on the Boltzmann parameters we obtained. In general, despite the large increase in maximal conductance, there was no significant change in the channel properties at the voltages tested in PD, LP, and VD neurons. \( V_{\text{act}} \) in these neurons and the slope of activation were not significantly modified after the expression of shal-GFP (Table 4). In the IC cell, overexpression of \( I_A \) appeared to induce an \( I_h \) with somewhat more hyperpolarized \( V_{\text{act}} \) (Table 4, Fig. 3), but we have only a very small sample size for this cell.

\( I_h \) kinetics

Although the voltage dependency of channel gating of the shal-GFP–evoked increased \( I_h \) is unmodified, we found significant differences in the kinetics of channel activation (Table 4). The enhanced current in shal-injected PD, VD, and IC neurons had somewhat slower activation parameters: \( \tau_{\text{act}} \) showed a trend to be slower when measured at steps to \(-100 \) mV, increasing by one- to twofold in PD (6.8 ± 1.9 vs. 4.2 ± 0.9 s in control neurons; \( P = 0.04 \)), two- to threefold in VD (5.8 ± 4.2 vs. 1.6 ± 0.4 s in control neurons; \( P = 0.07 \)), and twofold in IC (3.1 ± 1.1 vs. 1.8 ± 0.8 s in control neurons; \( P = 0.09 \)). However, we did not observe any change in LP neurons. Activation is extremely slow (essentially not measurable) at physiological voltages more depolarized than \(-60 \) mV, and becomes slower still in most shal-injected neurons. Deactivation after returning to the holding potential also appears to be slowed in the shal-injected neurons (see tail currents in Fig. 3A). The normal period of the pyloric rhythm is about 2 s; the neurons are hyperpolarized for less than half of this time, which appears too short to significantly alter the conductance of the new \( I_h \) in the normal voltage range. As a result, we propose that the additional \( I_h \) may function by acting as a tonic depolarizing leak conductance.

Subtle effects of overexpression on rhythmic activity

The heterogeneity of \( I_A \) amplitude and properties among the different pyloric neurons has been proposed to determine, in part, their functional roles in the pyloric network because subtle changes of 10–25\% in \( I_A \) cause strong alterations in neuronal firing (Baro et al. 1997; Hartline 1979; Kloppenburg et al. 1999; Tierney and Harris-Warrick 1992). Accordingly, increasing \( I_A \) by 125–400\% after overexpression of shal-GFP should dramatically alter the firing properties of the neurons. However, as described previously for the PD neurons (MacLean et al. 2003), we found only small and subtle changes in neuronal activity after shal-GFP expression. Figure 4 compares the rhythmic activity of representative control and shal-GFP–expressing PD, LP, and VD (Fig. 4A, PD (Fig. 4B), and LP (Fig. 4C) neurons. Nearly all of the activity parameters in VD, PD, and LP were unchanged despite large increases in \( I_A \) after shal-GFP microinjection (Fig. 4, Table 5). The cycle frequency, oscillation amplitude, membrane potential at the trough of the oscillation, and spikes per burst were not significantly changed, although there were some subtle, nonsignificant trends, especially in LP and VD neurons that could be explained by an increase in \( I_h \). Only two statistically significant changes were found (Table 5). In the PD neuron, increased expression of \( I_{\text{shal-GFP}} \) led to a significant (\( P = 0.02 \)) decrease in the slope of the rise phase from 0.1 ± 0.02 to 0.06 ± 0.01 mV/ms (Table 5). Second, after expression of exogenous \( I_A \), the PD neuron’s overall spike amplitude decreased significantly from 9.7 ± 0.5 to 7.3 ± 1.7 mV (\( P = 0.008 \)) (Table 5). These changes, although subtle, are consistent with an increase in \( I_A \).

Localization of \( I_A \) and the implications for neuronal cycling

We have previously hypothesized that the homeostatic compensatory increase in \( I_h \) accounts for the failure of the firing

![FIG. 4. Rhythmic activity of neurons expressing shal-GFP is only slightly modified. A: comparison of activity during the pyloric rhythm in a control PD (thick) and a PD expressing shal-GFP (thin) 72 h after injection of shal-GFP RNA. Single cycles of activity, as indicated by the dashed line box, are overlaid in A(ii). Note the slower rise time of the oscillation in the neuron expressing shal-GFP (gray). B: comparison of activity during the pyloric rhythm in a control PD (thick) and a PD expressing shal-GFP 72 h after injection of shal-GFP RNA (thin). Single cycles of activity, as indicated by dashed line box, are cycle normalized and overlaid (shal-GFP–expressing, gray).](image-url)
properties of PD neurons to change after *shal*-GFP overexpression (MacLean et al. 2003). However, we also observed an inappropriate mistargeting of the new *shal* channels in the injected neurons, which may also contribute to the lack of effect of the increased $I_A$. In all cases when we observed fluorescence in neurons expressing *shal*-GFP, whether by GFP fluorescence or *shal* immunocytochemistry, intense new labeling was localized only to the soma and the proximal initial neurite (e.g., Fig. 5); increased label disappeared before or near the initial neurite’s first branch in the neuropil. This is in marked contrast to the normal pattern of *shal* expression in uninjected neurons: the protein is found in both the soma and all the neurites within the STG, where synaptic integration and spike initiation occur (Baro et al. 2000). To verify that the labeling procedure did not discriminate against central neuropil labeling, we coinjected two separate RNAs, one encoding the cytoplasmic GFP and the other encoding *shal* (with no GFP tag). As illustrated in Fig. 5, GFP labeling in two injected pyloric neurons is found in the soma, throughout the neurites and in the axon leaving the STG. In contrast, antibody labeling of *shal* in the same neurons shows that the new intense labeling is localized only to the soma and initial neurite of the injected neurons (Fig. 5).

**Response to changes in distribution of $I_A$ in a threecompartment mathematical model**

In pyloric neurons, processing of synaptic inputs and spike initiation occur in the neurites or at the beginning of the axon, which are electrically distant from the soma (Hartline and Graubard 1992). To examine whether the inappropriate somatic localization of the overexpressed *shal* protein was sufficient to explain why we saw only modest effects on neuronal activity, we analyzed the sensitivity to targeted changes in $I_A$ of the firing properties in a three compartment mathematical model of a pyloric neuron, based on a previous model of the LP neuron (Harris-Warrick et al. 1995a). The three compartments in our model represent the soma (labeled compartment 1, Table 6), the primary neurite coming out from the soma (compartment 2), and the neuropil/axon (compartment 3). The compartments are coupled by equivalent resistances with coupling constants of 0.1, which was previously shown to best fit the observed spatial decay of voltage (Guckenheimer, unpublished data). Values for the capacitance in each compartment were chosen to reflect the relative volumes of each region. The set of equations used to describe the model are shown in Table 6, where the index $j$ in the equations for $I_A$ and $I_{K(V)}$ corresponds to the compartment numbers. The parameter values used for the equations are shown in Table 7. With an injected current of $I_{ext} = 1.5$ nA, the model neuron is tonically active with a period of 196 ms (5.1 Hz) and a trough resting potential of $-52.0$ mV (Fig. 6A). These characteristics are similar to those seen in isolated LP neurons (Flamm and Harris-Warrick 1986a).

We investigated whether the model could provide evidence that the firing properties of the pyloric neuron are not sensitive to increases in $I_A$ localized to the soma and primary neurite. The voltage traces in Fig. 6 illustrate the difference between making corresponding changes in the A current in the neuropil/axon versus the soma and primary neurite. A model cell with control $I_A$ is shown firing in Fig. 6A. To mimic the *shal*-GFP-expressing cells, we modified three $I_A$ parameters to those seen in the injected neurons (Table 3): the maximal conductance ($g_A$), the half-activation voltage ($V_{1/2}$), and the inactivation slope factor ($s_A$). In Fig. 6B, these three parameters were changed only in the soma and primary neurite. The results of corresponding changes in neuropil/axon $I_A$ are shown in Fig. 6C. Clearly, modification of neuropil/axon $I_A$ has a much more dramatic effect on the frequency of firing, but modification

### Table 5. Comparison of neuronal cycling between control and expressing cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Slope of Rise Phase, mV/ms</th>
<th>Oscillation Amplitude, mV</th>
<th>Membrane Potential, mV</th>
<th>Spike Number</th>
<th>Spike Amplitude, mV</th>
<th>Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD control (6)</td>
<td>0.03 ± 0.01</td>
<td>9.5 ± 2.5</td>
<td>-59.1 ± 8.3</td>
<td>5.1 ± 0.9</td>
<td>9.7 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>expressing (21)</td>
<td>0.03 ± 0.04</td>
<td>9.7 ± 3.0</td>
<td>-53.8 ± 7.6</td>
<td>5.7 ± 1.2</td>
<td>7.3 ± 1.7**</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>LP control (4)</td>
<td>0.05 ± 0.03</td>
<td>7.6 ± 5.4</td>
<td>-49.5 ± 10.4</td>
<td>7.9 ± 4.1</td>
<td>3.1 ± 1.3</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>expressing (3)</td>
<td>0.07 ± 0.04</td>
<td>6.4 ± 1.5</td>
<td>-53.4 ± 9.6</td>
<td>4.9 ± 1.6</td>
<td>4.1 ± 1.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>VD control (5)</td>
<td>0.1 ± 0.02</td>
<td>6.0 ± 2.6</td>
<td>-47.9 ± 13.4</td>
<td>8.6 ± 2.3</td>
<td>8.4 ± 1.7</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>expressing (6)</td>
<td>0.06 ± 0.01*</td>
<td>5.8 ± 1.7</td>
<td>-48.9 ± 8.5</td>
<td>6.7 ± 1.8</td>
<td>6.6 ± 1.8</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values indicate average ± SD with number of cells in parentheses. *Significantly different (P < 0.05). **Significantly different (P < 0.01).

**FIG. 5.** Shal protein is localized to the somata of injected neurons. Confocal images of neurons coinjected with *shal* RNA and GFP RNA. GFP fluorescence (green) extends throughout the neurites and the axons of injected neurons, whereas shal antibody labeling (red) is localized only to the somata and initial segments of the injected neurons.
amplitude was also reduced by I

TABLE 7. Parameters for model of the LP neuron

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Meaning</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_A$</td>
<td>2.1</td>
<td>$I_A$ conductance</td>
<td>$\mu$S</td>
</tr>
<tr>
<td>$g_K$</td>
<td>0.35</td>
<td>Delayed rectifier $K^+$ conductance</td>
<td>$\mu$S</td>
</tr>
<tr>
<td>$g_{K(Ca)}$</td>
<td>0.4</td>
<td>Calcium-activated $K^+$ conductance</td>
<td>$\mu$S</td>
</tr>
<tr>
<td>$g_{Na}$</td>
<td>0.005</td>
<td>Sodium conductance (neuropil only)</td>
<td>$\mu$S</td>
</tr>
<tr>
<td>$g_l$</td>
<td>0.15</td>
<td>Leak current conductance</td>
<td>$\mu$S</td>
</tr>
<tr>
<td>$k_A$</td>
<td>8.93</td>
<td>$I(A$ current inactivation time constant)</td>
<td>(ms)$^{-1}$</td>
</tr>
<tr>
<td>$k_{Ca}$</td>
<td>5.0</td>
<td>$I(Ca$ delay time constant)</td>
<td>(ms)$^{-1}$</td>
</tr>
<tr>
<td>$c_{Ca}$</td>
<td>0.04</td>
<td>$I(Ca$ concentration time constant)</td>
<td>(ms)$^{-1}$</td>
</tr>
<tr>
<td>$E_{Na}$</td>
<td>-140</td>
<td>Sodium reversal potential</td>
<td>mV</td>
</tr>
<tr>
<td>$E_K$</td>
<td>-86</td>
<td>Potassium reversal potential</td>
<td>mV</td>
</tr>
<tr>
<td>$E_{K_a}$</td>
<td>50</td>
<td>Sodium reversal potential</td>
<td>mV</td>
</tr>
<tr>
<td>$e$</td>
<td>-45</td>
<td>Leak reversal potential</td>
<td>mV</td>
</tr>
<tr>
<td>$C_s$</td>
<td>0.0005</td>
<td>Capacitance of soma</td>
<td>nF</td>
</tr>
<tr>
<td>$C_p$</td>
<td>0.0001</td>
<td>Capacitance of primary neurite</td>
<td>nF</td>
</tr>
<tr>
<td>$C_N$</td>
<td>0.0025</td>
<td>Capacitance of neuropil compartment</td>
<td>nF</td>
</tr>
</tbody>
</table>

limited to the soma and primary neurite still has a marked effect to reduce spike frequency by $>25\%$, and the spike amplitude was also reduced by $>25\%$.

We then explored the effect of modifying the properties of somatic $I_A$ on a bursting pyloric cell. To mimic the bursting in LP driven by rhythmic synaptic inhibition in the intact, cycling pyloric network, we evoked bursting in the model neuron using a periodic inhibitory external current injected into the neuropil/axon compartment. The model cell bursts at a cycle frequency of 0.5 Hz, with four spikes per burst and spike amplitude (measured in the soma) of 4.4 mV (Fig. 7A). The slope of the rise phase was 5.9 mV/s. To represent the cells expressing shal-GFP, we increased the parameters $g_{A_r}$, $V_A$, and $s_h$ to 5.5 $\mu$S, -34.1 mV, and 8.4 mV, respectively, but only in the soma and primary neurite. We see a large decrease in the slope of the rise phase to 1.1 mV/s, causing a significant delay in the onset of spiking after the inhibitory input. There was also a reduction in the number of spikes per burst from four to two and a decrease in spike amplitude to 3.1 mV (Fig. 7B).

To consolidate these effects on model activity, we studied how the frequency of tonic firing changes when $I_A$ parameters are selectively modified in the soma versus the dendritic compartments. To do this, we used the numerical algorithms described in the appendix to compute the sensitivity of the tonic firing period to each of the $I_A$ parameters in the soma/primary neurite and in the neuropil/axon. If the sensitivity of the period to a parameter is large and positive (or negative), then a small increase in this parameter will give a large increase (or decrease) in the period of firing. We then calculated the ratio of the sensitivity of the firing properties to each parameter of $I_A$ when altered in the neuropil/axon relative to the soma (Fig. 8). This shows that the sensitivity to each of the $I_A$ parameters in the neuropil/axon was larger than for the corresponding soma/primary neurite $I_A$ parameters because the ratio of the sensitivity in the neuropil/axon to that in the soma/primary neurite for each $I_A$ parameter was greater than one in all cases. We can conclude that changing the properties of the neuropil/axon $I_A$ will have a more significant effect than a corresponding change in the properties of the soma/primary neurite $I_A$. In particular, the period of firing is 3.1-fold more sensitive to changes in the maximal A-conductance in the neuropil/axon than in the soma/primary neurite (Fig. 8).

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The results from this simplified model neuron indicate that the mislocalization of shal could contribute modestly to the failure of the injected neurons to change their firing properties because changes in \( I_A \) properties limited to the soma/primary neurite cause more subtle effects than those limited to the neuropil/axon. However, the model neuron is still considerably more sensitive to increases in soma/primary neurite \( I_A \) than the shal-GFP–expressing neurons, which showed almost no change in properties at all. This suggests that the mislocalization alone cannot explain the failure of the neurons to change their firing properties after shal-GFP overexpression; additional factors must be involved.

Compensatory interactions between \( I_A \) and \( I_h \) in a two-cell bursting model

Because the mislocalization of the exogenous shal channels to the soma and initial neurite does not fully explain the lack of effect on firing properties in the injected cells, we carried out a second modeling exercise to determine whether a compensatory increase in \( I_h \) alone was sufficient to explain the ob-

FIG. 6. Mathematical model shows that the frequency of tonic firing in the LP cell is much more sensitive to changes in neuropil \( I_A \) than changes in \( I_A \) in the soma and primary neurite. A–C: tonic activity in control conditions and with modified \( I_A \) in the model cell. A: control has \( g_A = 2.1 \mu S, V_a = -43.9 \) mV, \( s_b = 7.0 \) mV in all compartments. B: modified \( I_A \) so that \( g_A = 5.5 \mu S, V_a = -34.1 \) mV, \( s_b = 8.4 \) mV in soma and primary neurite. Neurip values are unchanged from control. C: modified neuropil \( I_A \) so that \( g_A = 4.85 \mu S, V_a = -34.1 \) mV, \( s_b = 8.4 \) mV in neuropil. Soma and primary neurite values are the same as control.

The results from this simplified model neuron indicate that the mislocalization of shal could contribute modestly to the failure of the injected neurons to change their firing properties because changes in \( I_A \) properties limited to the soma/primary neurite cause more subtle effects than those limited to the neuropil/axon. However, the model neuron is still considerably more sensitive to increases in soma/primary neurite \( I_A \) than the shal-GFP–expressing neurons, which showed almost no change in properties at all. This suggests that the mislocalization alone cannot explain the failure of the neurons to change their firing properties after shal-GFP overexpression; additional factors must be involved.

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FIG. 7. Mathematical model shows that the slope of the rise phase, number of spikes, and spike amplitude in a bursting LP cell decrease when somatic \( I_A \) is modified to mimic the \( I_A \) seen in cells expressing shal-GFP. A: control has \( g_A = 2.1 \mu S, V_a = -43.9 \) mV, \( s_b = 7.0 \) mV in all 3 compartments. B: modified parameter values are \( g_A = 5.5 \mu S, V_a = -34.1 \) mV, \( s_b = 8.4 \) mV in the soma and primary neurite. Corresponding values for the neuropil are left unchanged.

FIG. 8. Frequency of tonic firing in a 3-compartment mathematical model of the LP neuron is more sensitive to changes in neuropil \( I_A \) than soma \( I_A \). Each bar represents the ratio of the sensitivity in the neuropil to that in the soma for a particular \( I_A \) parameter. For example, the frequency of firing is 3.1-fold more sensitive to changes in the maximal \( A \)-conductance \( g_A \) in the neuropil than in the soma. Other bars show the same information for other \( I_A \) parameters: \( V_{act} \), the half-activation voltage; \( s_{act} \), the slope of the steady-state activation curve; \( V_{inact} \), the half-inactivation voltage; \( s_{inact} \), the slope of the steady-state inactivation curve. These data were generated using the algorithms described in the APPENDIX.
served compensation. We chose to model the increased $I_h$ as a depolarizing leak conductance, based on the arguments made earlier that the current’s kinetics in the physiological voltage range are very slow relative to the neuronal oscillations. Other groups have reported a similar contribution of $I_h$ to the tonic resting membrane potential in both mammalian neurons (Lamas 1998; Kiehn et al. 2000) and lobster neurons (Corotto and Michel 1998).

This second model was developed to reflect the experimental conditions in which the effects of shal-GFP injection into a cycling PD cell were measured. The model consists of two coupled single-compartment model neurons, so we could eliminate any potential contribution of the spatial mislocalization of $I_A$ and determine whether the compensatory upregulation of $I_h$, by itself, could occlude the effects of shal overexpression. The first model neuron is a “follower” PD neuron in which the effects of shal-GFP injection are mimicked by altering the parameters of $I_A$ and $I_h$. The second model neuron is a composite reflecting the electrically coupled oscillating AB and the second PD neuron, as well as inputs from other pyloric neurons, that do not have altered channel properties. This “driver” neuron drives the PD neuron with a realistic oscillatory waveform to fire rhythmic bursts at a frequency of about 0.75 Hz and firing four spikes per burst with a duty cycle of 0.15 (Fig. 9A). The follower neuron is slightly phase delayed compared with the driver neuron, but they fire the same number of action potentials per burst (Fig. 9A).

If we alter the properties of $I_A$ alone in the follower PD neuron to those that were seen in shal-GFP–injected PD neurons, the cell shows dramatic alterations in firing properties (Fig. 9B). It stops firing spikes, dependent of course on the size of the increase in maximal conductance. Fluctuations in the membrane potential are smaller than in control conditions: the driving cell has a stronger postburst hyperpolarization than the follower neuron, but the follower neuron has an average potential that is significantly more hyperpolarized than that of the driving cell.

On the other hand, if we alter the properties of $I_h$ alone in the follower PD neuron to those seen in the shal-GFP–injected neuron, with no change in $I_A$ properties, we get a very different response (Fig. 9C): the neuron is depolarized compared with the driver, and is phase advanced in its bursts, firing an extra action potential per burst. These are qualitatively similar to the effects of upregulating $I_h$ through injection of the PAIH gene, which does not cause a compensatory upregulation of $I_A$ (Zhang et al. 2004).

In contrast, if we alter both $I_A$ and $I_h$ parameters in the follower PD cell to those seen in the shal-GFP–injected PD neuron, we obtain rhythmic bursting that is similar to that seen in the control cell (Fig. 9D). The cell returns to firing four spikes per burst, has a cycle frequency of 0.75 Hz, and a duty cycle of 0.15, as it did under control conditions. The only discrepancy with the experimental results is that the follower cell is somewhat depolarized during the interburst interval relative to the driver cell.

Finally, to further examine the full parameter space of the combined effect of altering the maximal conductance of $I_h$ and $I_A$ on this model, we produced a coarse plot of the number of spikes per burst as a function of the conductance parameters of these currents, $g_h$ and $g_A$, respectively (Fig. 10). This figure illustrates the opposite effects that $I_A$ and $I_h$ have in the number of spikes fired by the PD neuron: higher $g_A$ decreases the number of spikes, whereas higher $g_h$ increases them. The most remarkable aspect of this figure is the “bands” of identical spikes/burst in the plot; these demonstrate the compensatory

![Figure 9](https://example.com/figure9.png)

**FIG. 9.** In a 2-cell model, the increase in $I_A$ can be compensated by an increase in $I_h$. Each panel shows the activity of one PD neuron with varying $I_A$ and $I_h$ parameters (red), and one driver neuron that represents the oscillatory input to the PD neuron from the rest of the pyloric network (green). A: control coupled pair. Follower PD neuron lags slightly behind the driver neuron ($g_h = 0.00, g_a = 2.6$). B: an increase in $I_A$ alone in the PD neuron ($g_h = 0.00, g_a = 10.0$) hyperpolarizes the neuron and abolishes active spiking; the passive depolarizations are electronically spread from spikes in the driver neuron. C: an increase in $I_h$ alone results in a tonic depolarization in the follower neuron ($g_h = 0.12, g_a = 2.6$) and an increase both in the rate of rise and spike number; in this case the PD neuron precedes the driver in firing. D: an increase in both currents in the ratio seen after shal RNA injection reestablishes the cycling to control conditions, except that the PD neuron has a depolarized interburst interval ($g_h = 0.12, g_a = 10.0$).
Positive correlation between $I_A$ and $I_h$ in noninjected PD neurons

We have significantly increased our experimental database of control PD neurons that were not injected with $shal$ RNA. The maximal $I_A$ and $I_h$ vary considerably among PD neurons in different animals; similar variability in $I_A$ and other currents within a cell type has been previously described by Golowasch et al. (1999). However, analysis of the relationship between maximal $I_A$ and maximal $I_h$ in these neurons shows a statistically significant positive relationship: neurons that have a higher baseline level of $I_A$ also tend to have a higher baseline level of $I_h$ ($r = 0.608$ for a linear relationship, $P < 0.001$; Fig. 11). The cell capacitance and thus size do not vary greatly between PD neurons; Baro et al. (1997) reported that the PD input capacitance was $1.2 \pm 0.07$ nF ($n = 10$). Thus the variability of current amplitudes between the neurons arises from the different current densities rather than size. These results suggest that the upregulation of $I_h$ after overexpression of $I_A$ is not simply an experimental artifact, but reflects a natural interaction between $I_A$ and $I_h$ in pyloric neurons that could normally coregulate the expression levels of these two channels within a defined ratio.

DISCUSSION

Evidence from single-cell RT-PCR and immunocytochemical studies suggests that the $shal$ gene encodes $I_A$ in pyloric neurons (Baro et al. 1997, 2000). To examine this hypothesis further, we overexpressed shal protein by injecting $shal$ RNA into identified pyloric neurons. Interestingly, the $shal$ RNA was translated with approximately equal efficiency in all the pyloric neurons examined. Because of the dramatically different baseline current amplitudes in the different neurons, this constant...
increase in $I_A$ amplitude resulted in a markedly different percentage increase in each neuron. The expressed shal current in neurons was a fairly good phenocopy of the endogenous $I_A$; however, it was not identical. With an increasing contribution of exogenous current to total current there was an increased similarity of $I_A$ in neurons expressing shal-GFP to lobster $I_{\text{shal-GFP}}$ expressed in *Xenopus* oocytes, and the variability of $I_A$ between the neurons was significantly decreased. Thus the shal-GFP–evoked current did not completely adopt the cell-specific parameters of $I_A$, which differ between the pyloric neurons. Recent results in PD neurons from our laboratory (Zhang et al. 2003) suggest that for the exogenous shal current to possess normal baseline properties, coexpression and coassembly with proteins in the KChIP family, a set of proteins that modify Kv4 expression (An et al. 2000) is required. Because a very high density of shal-GFP protein was manufactured after exogenous RNA injection it appears that the neuron is unable to synthesize sufficient quantities of this auxiliary subunit and, as a consequence, the exogenous channels may be unmodified homomultimers similar to those made in *Xenopus* oocytes. The shift in biophysical parameters from cell-specific toward oocyte $I_{\text{shal-GFP}}$ values is consistent with this interpretation. Although KChIP proteins appear to be essential for normal $I_A$ function in PD neurons, other regulatory proteins, or additional posttranslational modifications, may be needed in the other pyloric neurons because their $I_A$ properties differ from the PD neurons.

The role of shal in the pyloric network

A-type potassium channels have been shown to be critical in determining neuronal activity during the pyloric cycle. When reduced by as little as 25% by 4-AP, the cycle frequency, spike frequency, and slope of the rise phase of the oscillation are all increased, leading to marked phase changes in firing of the neurons within the pyloric cycle (Tierney and Harris-Warrick 1992). Because of the heterogeneity of $I_A$ between neurons, these effects are quantitatively different in each of six pyloric neurons. $I_A$ is also a major target for dopamine (DA) modulation, which dramatically alters the pyloric cycle (Harris-Warrick et al. 1995a,b, 1998; Kloppenburg et al. 1999). For example, the $I_A$ amplitude in the PD neuron is increased by only 10% during bath application of DA (Kloppenburg et al. 1999). As a result there is a decrease in the slope of the rise phase, and a decrease in spike number and spike amplitude; sometimes the PD stops firing altogether (Flamm and Harris-Warrick 1986a,b). However as we previously demonstrated (MacLean et al. 2003), the changes in firing properties after an artificial increase in $I_A$ were surprisingly small. Only two statistically significant changes were seen: a decrease in spike amplitude in PD and a decrease in the slope of the rise phase of the oscillation in VD. These are consistent with an increased $I_A$; but such modest changes were unanticipated after an increase of 125–400% in $I_A$ amplitude. We have studied two potential explanations for the failure of shal-GFP overexpression to modify firing properties. The first is a failure to target the exogenous channels to the neuropil, where synaptic integration and spiking occur; instead the new protein is selectively targeted to the soma and initial neurite (Fig. 5). Using a three-compartment mathematical model, we examined whether this inappropriate targeting of the expressed channel could fully account for the absence of strong effects on neuronal firing properties. In this model, spike activity was 3.1-fold more sensitive to increases in $I_A$ amplitude in the neuropil/axon compartment compared with the soma and primary neurite compartments. This is consistent with the fact that both synaptic integration and spike initiation occur in the neuropil, electrically remote from the soma. However, when $I_A$ parameters were adjusted to those observed in the shal-GFP–injected neurons only in the soma and primary neurite compartments, the model neuron’s spike activity and spike amplitude were still very significantly reduced. The effect was much greater than we observed after overexpression of shal-GFP. These results suggest that additional compensatory processes must be activated to maintain normal firing properties in the injected neurons despite the increased $I_A$.

A second possible explanation is the compensatory increase in $I_h$. Even in noninjected PD neurons, a positive relationship exists between the amount of $I_A$ and $I_h$ (Fig. 11), suggesting that under normal conditions the expression of these channels is in some way coregulated. Upregulation of $I_h$ after shal RNA injection occurs in all the pyloric neurons tested to date, suggesting that a linkage between these two competing channels may be a general property among pyloric neurons and not unique to any one cell type. Like $I_A$, the increased $I_h$ has somewhat different properties than those of the endogenous currents, perhaps also a result of lack of sufficient quantities of auxiliary subunits or use of new alternative splice variants of the $I_h$ gene. A recent report suggests that the Min-K–related peptide can act as an auxiliary protein for HCN channels (Decher et al. 2003; Yu et al. 2001), specifically accelerating the kinetics of channel activation and deactivation.

We have limited our examination of $I_h$ properties to voltage steps down to only −100 mV, as we occasionally observed very large jumps in inward current at more hyperpolarized voltages in neurons injected with either GFP or shal-GFP RNA. Similar large inward currents that are observed only at very negative, nonphysiological voltages, have been observed after injection of RNA for other membrane proteins in *Xenopus* oocytes (Kuruma et al. 2000). The source of this current is at present unknown; however, in *Xenopus* oocytes, the current was not detectable at voltages more depolarized than −130 mV. In contrast, the $I_h$ enhanced by shal RNA injection into pyloric neurons becomes active at physiologically significant subthreshold voltages and is easily seen at voltages more depolarized than −100 mV (Fig. 3). As we reported previously (MacLean et al. 2003), overexpression of the lobster *shaker* gene, which encodes an $I_A$ selectively targeted to the axons of STG neurons, or of GFP did not produce an increase in $I_h$ in the relevant voltage range. These results suggest that the link between shal and $I_h$ is not simply an artifact of channel RNA injection, and thus may be specific. However, it will be necessary to test other ion channel genes before this can be confirmed.

Interestingly we have not found a reciprocal homeostatic interaction between the two channel proteins. We previously reported that artificial overexpression of $I_h$ does not lead to a compensatory increase in $I_A$ (Zhang et al. 2004). The reasons for this apparent unidirectionality are not clear, although it suggests that the neuron monitors $I_A$ more carefully and compensates for variation in $I_A$ to a greater extent than it does $I_h$. This may arise because $I_h$ is probably a component of the leak.
current that helps maintain the resting potential, and could in theory be compensated by a large number of other currents. In other systems, $I_h$ has more rapid kinetics of activation and more depolarized voltage activation curves; in these systems, $I_h$ may serve as a pacemaker current that can help to regulate tonic firing or oscillatory bursting behavior (Funahashi et al. 2003; Kocsis and Li 2004; Luthi and McCormick 1999).

**Physiological mechanisms of neuronal homeostasis**

This activity-independent homeostasis coregulates two currents that are active in the critical voltage range below spike threshold; they have opposing effects and their ratio has been previously demonstrated to regulate neuronal spike and pacemaker activity (Angstadt and Calabrese 1989; Harris-Warrick et al. 1995b). We previously showed in pyloric neurons that the rate of postsynaptic rebound and the initial spike interval (ISI) after inhibition are coregulated by $I_h$ and $I_A$ (Harris-Warrick et al. 1995b); pharmacologically induced decreases in $I_A$ led to more rapid postsynaptic rebound and shorter first ISIs, whereas pharmacologically induced decreases in $I_h$ had the opposite effect. Our new results suggesting that $I_A$ and $I_h$ are expressed in a constant ratio in normal PD neurons (Fig. 11) imply that this coregulation is organized at a molecular level of interaction between the channels. In the shal-expressing neurons, both $I_A$ and $I_h$ are significantly increased, once again by a constant ratio, such that their balance of activity is retained, and the cell shows no significant change in firing activity. Blocking either current alone can uncover the hidden effects of the other current. Injection of mutant, nonfunctional shal RNA still upregulates $I_h$, demonstrating that this is an activity-independent homeostatic event (MacLean et al. 2003). We were able to model these interactions in a simple two-cell model (Fig. 9). In this model we chose not to use a multicompartment model because we did not wish to introduce additional unknown variables such as the distribution of $I_h$ current in the different compartments. Selectively increasing $I_A$ in one of the model neurons caused it to hyperpolarize and fall silent, showing only electrotonic coupling depolarizations from action potentials in the other neuron. On the other hand, selectively raising $I_h$ caused the neuron to depolarize, to phase advance, and to fire additional spikes per burst. Finally, when both $I_A$ and $I_h$ were raised to the extents and in the ratio seen in the experimentally injected neurons, the firing properties of the neuron were largely unchanged, just as in the experiments. A detailed study of the interaction between $I_A$ and $I_h$ in the control of spike number per burst shows a set of “stripes” in the parameter space, which are linear regions where correlated increases in both $I_A$ and $I_h$ in a constant ratio do not alter the firing properties of the neuron (Fig. 10). This model suggests that the compensatory upregulation of $I_h$ is by itself sufficient to cancel the effects of the shal-GFP–induced upregulation of $I_A$, retaining normal firing properties in the injected neurons. By itself, the model of course cannot prove that this is also necessary, nor can it eliminate additional compensatory changes that might contribute to the homeostatic response. However, both experimental and modeling approaches (Goldman et al. 2001; Prinz et al. 2004) have indicated that similar parallel changes in linked channels can maintain normal activity over a broad range of channel expression.

**APPENDIX**

**Sensitivity calculations for the LP model**

The mathematical model for the LP neuron defined in Tables 6 and 7 is a conductance-based three-compartmental model. The variables in each compartment evolve in time according to Hodgkin–Huxley-type differential equations, and the compartments are electrically coupled. The variables in the model define a multidimensional phase space so that any given state of the cell will correspond to a point $x$ in the phase space. Because we are interested in periodic behavior in the neuron (bursting or tonic firing), we seek regular or periodic orbits in phase space. These are solutions [$\psi(x, t \tau)$] of the differential equations for which there exists a period $T$ such that $\psi(x, t + T) = \psi(x, t)$, for all $t$ (Guckenheimer and Holmes 1983). We sought to accurately compute a periodic orbit and investigate how $T$ changes as a function of the A-current parameters $\lambda$.

Guckenheimer and Melono (2000) developed robust algorithms for computing periodic orbits in dynamical systems. The algorithms formulate defining equations $F(x, t, \lambda) = 0$ for a discrete periodic orbit, which are then solved numerically using Newton’s method. The software package ADOL-C (Griewank et al. 1996) is used to compute high-order Taylor series expansion of trajectories using automatic differentiation in C++. ADOL-C can also compute the Jacobian of the flow map (Guckenheimer and Holmes 1983), and a modified version can compute the derivative of the flow map with respect to parameters. Using ADOL-C in this way allows us to accurately compute all the quantities needed to solve the defining equations $F = 0$ using the Newton algorithm.

Having computed a periodic orbit with period $T$, we set out to determine the sensitivity of the orbit to the parameters in the model. More precisely, we want to calculate the partial derivative of $T$ with respect to the parameters $\lambda$. On a periodic orbit, the equations $F(x, t, \lambda), t(\lambda), \lambda = 0$ are satisfied. Differentiating these with respect to $\lambda$ gives

$$\partial_T(x, t) = -[D_x F]^T D_\lambda F$$

so we can calculate the vector $\partial_T$ of partial derivatives.

It is straightforward to use these periodic orbit algorithms together with a continuation algorithm to compute a family of periodic orbits by varying some active parameter (Guckenheimer and Melono 2000). To produce Fig. 8, we computed a family of six periodic orbits in the LP cell representing tonically firing cells with periods between 196 and 243 ms. The slope of the steady-state activation curve for sodium was used as the active parameter in our continuation algorithm. This range of periods simulates natural variation that might be seen in shal-GFP–injected preparations of isolated LP neurons in Panulirus interruptus. The sensitivity of the period $T$ to each $I_A$ parameter was computed as described above, and ratios were taken between the corresponding parameters in the soma and neuropil. Thus a ratio of $>1$ means the period of firing is more sensitive to changes in the neuropil parameter than in the soma parameter. The bar graph in Fig. 8 represents a mean of these ratios over the six orbits. For the purposes of the sensitivity calculations, the $I_A$ parameters in the primary neurite (compartment 2) were assumed to be the same as those in the soma (compartment 1). The mean ratios and SDs for $g_{a}, V_c, s_{\alpha}, V_{\alpha}$, and $s_{h}$ are $3.11 \pm 0.03$, $2.95 \pm 0.03$, $2.17 \pm 0.02$, $2.97 \pm 0.01$, and $2.59 \pm 0.10$, respectively.

**REFERENCES**


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