Localization and function of $I_h$ channels in a small neural network

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Submitted 18 October 2010; accepted in final form 7 April 2011

Goeritz ML, Ouyang Q, Harris-Warrick RM. Localization and function of $I_h$ channels in a small neural network. J Neurophysiol 106: 44–58, 2011. First published April 13, 2011; doi:10.1152/jn.00897.2010.—Subthreshold ionic currents, which activate below the firing threshold and shape the cell’s firing properties, play important roles in shaping neural network activity. We examined the distribution and synaptic roles of the hyperpolarization-activated inward current ($I_h$) in the pyloric network of the lobster stomatogastric ganglion (STG). $I_h$ channels are expressed throughout the STG in a patchy distribution and are highly expressed in the fine neuropil, an area that is rich in synaptic contacts. We performed double labeling for $I_h$ protein and for the presynaptic marker synaptotagmin. The large majority of labeling in the fine neuropil was adjacent but nonoverlapping, suggesting that $I_h$ is localized in close proximity to synapses but not in the presynaptic terminals. We compared the pattern of $I_h$ localization with Shal transient potassium channels, whose expression is coregulated with $I_h$ in many STG neurons. Unlike $I_h$, we found significant levels of Shal protein in the soma membrane and the primary neurite. Both proteins were found in the synaptic fine neuropil, but with little evidence of colocalization in individual neurites. We performed electrophysiological experiments to study a potential role for $I_h$ in regulating synaptic transmission. At a synapse between two identified pyloric neurons, the amplitude of inhibitory postsynaptic potentials (IPSPs) decreased with increasing postsynaptic activation of $I_h$. Pharmacological block of $I_h$ restored IPSP amplitudes to levels seen when $I_h$ was not activated. These experiments suggest that modulation of postsynaptic $I_h$ might play an important role in the control of synaptic strength in this rhythmogenic neural network.

hyperpolarization-activated inward current; synaptic integration; Shal; transient potassium current; central pattern generator; Crustacea; stomatogastric ganglion

CELL-SPECIFIC DIFFERENCES in ion channel expression help to create a neuron’s unique firing properties and function within a network (Baro et al. 1997; Schulz et al. 2006, 2007). Based on these differences, neuromodulation of subthreshold currents can change the unique input-output properties of individual neurons and alter the resulting network output. One such current is the hyperpolarization-activated inward current ($I_h$). $I_h$ is an inward current carried mostly by Na$^+$ and K$^+$ ions that is slowly activated by subthreshold hyperpolarizations and only slowly deactivates on repolarization. It is well known for its contribution to the depolarizing pacemaker current in the heart (Baruscotti and DiFrancesco 2004; DiFrancesco 2006; DiFrancesco and Ojeda 1980), but $I_h$ is also found in the central and peripheral nervous systems, where it plays a number of roles (Debanne et al. 2006; Harris-Warrick 2002; Johnston et al. 2000; Kaupp and Seifert 2002; Magee 1999; Pape 1996; Robinson and Siegelbaum 2003; Wahl-Schott and Biel 2009).

$I_h$ helps to set the resting potential and contributes to postsynaptic rebound (Harris-Warrick et al. 1995; Pape 1996; Robinson and Siegelbaum 2003), thus playing a supporting role in the generation of plateau potentials and bistability (Kiehn and Harris-Warrick 1992; Robinson and Siegelbaum 2003). In some neurons, $I_h$ also shapes dendritic integration. In layer V pyramidal cells of the somatosensory cortex, $I_h$ activation disconnects somatic and dendritic spike initiation zones and may prevent initiation of dendritic calcium action potentials in the absence of proximal input (Berger et al. 2003). $I_h$ is also involved in the regulation of synaptic transmission, long-term facilitation, and integration of synaptic events through shaping temporal summation as well as spatial normalization of distant synaptic events (Beaumont and Zucker 2000; Berger et al. 2003; Genlain et al. 2007; Harris-Warrick et al. 1995; Magee 1998, 1999; Migliore et al. 2005; Williams and Stuart 2000).

In the pyloric network of the crustacean stomatogastric ganglion, $I_h$ is present in all six identified neuron types; it has different voltage-dependence and kinetic properties in the different neurons and is subject to neuron-specific monoamine modulation (Harris-Warrick et al. 1995; Peck et al. 2006). $I_h$ has also been measured in pyloric neuron axons, where it is strongly modulated by dopamine (Ballo and Bucher 2009; Ballo et al. 2010). The gene for the $I_h$ channel protein PIHH has been cloned from the spiny lobster, Panulirus interruptus (Ouyang et al. 2007). Although there are four isoforms of the homolog mammalian HCN channel genes, only one invertebrate isoform exists, which undergoes a large amount of alternative splicing, generating variants that encode currents with very different voltage-dependence and kinetic properties and cAMP sensitivity. Soma recordings of $I_h$ in pyloric neurons show that the activation and deactivation of this current is most likely too slow to directly impact firing properties on a cycle-by-cycle basis in the pyloric network, and it may act as a slowly changing leak current. However, the large degree of $I_h$ modulation in the stomatogastric ganglion (STG) suggests a functional role for $I_h$, which may vary in a state-dependent way.

In earlier work, we showed that neuronal upregulation of $I_h$ could help to compensate for the artificial overexpression of a transient potassium current ($I_A$) and might be involved in homeostatic regulation of neuronal firing properties (MacLean et al. 2003, 2005; Zhang et al. 2003). Correlations in the normal expression levels of $I_h$ and $I_A$ mRNA have been documented for many neurons in the STG (Schulz et al. 2007) and in the cardiac ganglion of crabs (Tobin et al. 2009). Blocking $I_h$ during ongoing pyloric network activity did not dramatically alter the motor pattern, but this blockade had a more significant effect during application of

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dopamine, which enhances $I_h$ in selected neurons (Peck et al. 2006).

In this article, we further study the possible roles of $I_h$ in the pyloric network. We describe the anatomic distribution of $I_h$ channels in the STG, with a concentration in the fine neuropil where synaptic interactions occur. In addition, we have used electrophysiological experiments to demonstrate that $I_h$ can act to limit the amplitudes of inhibitory synaptic interactions within this rhythmogenic network.

Fig. 1. *Panulirus interruptus* hyperpolarization-activated inward current ($I_h$) protein (PIIH)-like immunolabeling is located in the somata and in the neuropil regions throughout the stomatogastric ganglion (STG). A: general anatomy of the STG. The STG contains about 30 motor and interneurons. The somata are situated on the outer surface of the ganglion and send large neurites toward the coarse neuropil in the middle, from where smaller processes branch into the layer of the fine (or synaptic) neuropil, located between the somata and the coarse neuropil. B: PIIH-like immunolabeling is found in STG somata and in the fine neuropil, whereas the larger processes of the coarse neuropil in the middle of the ganglion are much less brightly stained. Single 1-μm-thick optical section of the confocal stack. Scale bar, 150 μm. C: variable PIIH-like immunolabeling in and around the STG somata. Most neurons show strong perinuclear (filled arrowhead) or punctate staining in the soma (open arrowheads), whereas some are apparently lacking PIIH-like immunolabeling (cross). Dense PIIH-like immunolabeling is present in tissue surrounding the somata in the form of strong punctate staining (star) and halo-like layers (small arrows) around cell bodies, which may be glia or connective tissue. Single 1-μm-thick optical section of the confocal stack. Scale bar, 30 μm. D: PIIH-like immunolabeling reveals large fibrous structures (arrows) of unknown origin, which enter the STG from the stomatogastic nerve (stn) and dorsal ventricular nerve and branch within the ganglion. Maximum intensity projection of 7 consecutive confocal sections. Scale bar, 30 μm. E: specificity of the PIIH antibody was determined with Western blots of *Panulirus* central nervous system (CNS) tissue and from *Xenopus* oocyte, expressing PIIH RNA. The antibody labeled a strong (~76 kDa) band in protein extracted from ganglia and from stomatogastric nervous system nerves (left). A larger band in the range of 116 kDa was found in *Panulirus* brain tissue and may be phosphorylated, glycosylated, or oligomeric forms, whereas the faint smaller band (~64 kDa) may be the result of protein breakdown or smaller splice variants. Comparison of protein extracted from *Xenopus* oocytes without (control) and with injection of PIIH RNA shows a strong PIIH-positive band of similar weight in the PIIH-expressing oocytes. The *Panulirus* tissue and the oocyte extracts were run separately with different molecular mass ladders.
METHODS

Preparation. Adult California spiny lobsters, *P. interruptus*, were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in artificial seawater at 16°C until use. All procedures were in accordance with the guidelines established by the National Institutes of Health and, where applicable, were approved by the Institutional Animal Care and Use Committees at Cornell University. Lobsters were anesthetized on ice for 30 min before dissection. The STG, along with its motor nerves and associated commissural and esophageal ganglia, was dissected and pinned in a silicone elastomer (Sylgard)-coated dish, as described by Mulloney and Selverston (1974). The physiological saline solution consisted of (in mM) 479 NaCl, 12.8 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10.0 MgSO₄, 2 glucose, and 11.1 Tris base, pH 7.4 (Mulloney and Selverston 1974). Neurons were identified during intracellular recordings (3 M KCl or 0.6 M K₂SO₄/H₂SO₄ 20 mM KCl, 10–25 MΩ) by their typical membrane potential oscillation shapes and synaptic inputs (Kloppenburg et al. 1999) and by coincidence of action potentials recorded intracellularly with extracellular recordings using suction or pin electrodes on the respective motor nerves.

Immunocytochemistry. Identified neurons were labeled with 4% neurobiotin (NB) in 50 mM Tris and 0.5 M KCl. For NB injection, tips of low-resistance electrodes were backfilled with the NB solution for 10 min. The shaft was then filled with 2 M KCl, leaving a 1-cm gap between the NB in the tip and the KCl in the shaft to avoid mixing. The resistance of the filled electrode was 25–90 MΩ. NB was injected for about 40 min with 500-ms, +5-nA pulses at 1 Hz. Lucifer yellow (LY) was injected for 10–40 min with 500-ms, 1- to 14-nA pulses at 1 Hz. Preparations were left for 1 h for individual neuron staining.

STGs were fixed in 2 or 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 50–90 min at room temperature. The fix was

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Fig. 2. PIIH-like immunolabeling on a ventricular dilator (VD) neuron. A: extended focus projection of PIIH-like immunoreactivity (Ai) and a dye-filled VD neuron (Aii) in a whole mount STG (z-stack of 89 0.7-μm-thick optical sections, adjusted to show an ~35-μm-thick midsection of stack). A colocalization channel (yellow) was generated and overlaid with both channels (Aiii). The brightness and density of the colocalization channel were enhanced to reveal PIIH-like immunolabeling relative to the VD neuron. B: 3-dimensionally (3-D) rendered transparency projection of z-stack of 89 0.7-μm-thick optical sections. A colocalization channel was generated to emphasize PIIH-like immunolabeling in or in contact with the VD neuron. Nonuniform and patchy distribution of PIIH-like immunolabeling along the VD neuron (yellow) was visualized. Opacity was adjusted to show an ~35-μm-thick midsection of stack. Most PIIH-like immunoreactivity was found on and around medium-size and small branches. The larger diameter processes in the coarse neuropil, which consist of the primary neurite and the 2 axon precursors (left), show very little PIIH. See D and E for details of boxed regions. Scale bar, 50 μm. C: most of the smaller PIIH-like immunolabeled processes originated from a few single branches, which split off the primary neurite just below the soma. Continuous branches are individually marked with arrowheads and stars to help with visual separation; D: although most of the primary neurite is PIIH negative, a short, stubby structure that branches into several very thin processes from the primary neurite has patches of PIIH-like immunolabeling (arrows). E: patchy appearance of PIIH-like immunolabeling on branch points in the fine neuropil (arrows) and on flat paddle- or handlike structures (arrowhead), which are often found at or toward the end of very thin, long processes.
washed out with 8 changes of PBST (PBS + 0.3–1% Triton X-100) over 2–8 h. The tissue was then blocked for 3 h with 5% normal goat serum and 1% BSA in PBST at room temperature and incubated overnight in a rabbit anti-Shal (1:2,000) (Baro et al. 2000), rabbit anti-synaptotagmin (1:1,000; generated by the laboratory of Dr. N. Reist), or mouse anti-penta-His (1:20–1,000; Abcam) primary antibody in PBST + 5% normal goat serum and 0.1% BSA. The primary antibody was washed out with PBST for 2 h. The tissue was then incubated for 2 h with the respective Alexa Fluor-conjugated secondary antibodies (Molecular Probes) at 1:500 dilution in PBST + 5% normal goat serum and 0.1% BSA. The secondary antibody was washed out with PBS for 2 h. All incubations were performed at room temperature with constant shaking. NB was visualized by addition of Alexa Fluor 568-conjugated StreptAvidin (1:250; Molecular Probes) during secondary antibody incubation. The LY signal was amplified with a rabbit anti-LY antibody (1:500; Molecular Probes) and a secondary Alexa Fluor 488-conjugated anti-rabbit antibody. The STG was mounted and cleared on a slide with Vectashield mounting medium (Vector Laboratories). For measurements on sectioned ganglia, fixed ganglia were imbedded in 4% low-melting point agarose (Sigma) in Panulirus saline. Slices (40–70 μm) were made with a vibrating microtome (Leica Microsystems; speed setting 4, frequency setting 9) and transferred to PBST-filled wells. Antibody treatment was performed on the floating agarose sections or individual ganglion slices on a slide. Antibody staining in images of x-y planes or series of z-stacks was visualized and collected with a Leica TCS SP2 confocal system. For multiple staining, sequential imaging and narrow emission settings were used to prevent bleed-through effects. Image analysis and three-dimensional (3-D) reconstructions were performed with Volocity visualization and classification software. The “Colocalization” feature in Volocity was used to evaluate colocalization as described by Manders et al. (1993), using automatic thresholding (Costes et al. 2004) to generate colocalization channels. To improve visibility of colocalization in transparency projections, the brightness and opacity of the filled neuron and the colocalization channel were adjusted (see Fig. 2, B–E, Fig. 4B, and Fig. 5, B and C). Gamma values of the channels were not changed. For one preparation (see Fig. 2), confocal stacks were acquired as tiles at ×63 magnification and subsequently aligned and stitched with a GUI-based MALTAB tool, written by Ted Brookings.

Synaptic transmission measurements. The pyloric dilator (PD) and lateral pyloric (LP) neurons were impaled with two electrodes each to allow independent current injection and voltage recording in each cell (Fig. 1A). Action potentials and transient potassium currents \( I_{Na(V)} \) and \( I_A \) were blocked with 0.1 μM tetrodotoxin and 4 mM 4-aminoypyridine to isolate \( I_h \). Although only little \( I_h \) should be active in the voltage ranges that were tested, the long hyperpolarization of the PD neuron causes a significant removal of \( I_h \) inactivation, which could interfere with the inhibitory postsynaptic potential (IPSP) measurements, especially at or near physiological membrane potentials. Ideally, we would have preferred to block all outward currents; however, the drug of choice (tetraethylammonium) can block graded synaptic transmission and therefore was not used. Synaptic measurements were recorded under current-clamp conditions with step depolarizations of the presynaptic LP neuron to evoke graded IPSPs in the PD cell (see Fig. 7, A and B). LP-evoked IPSPs were recorded while the PD was depolarized or hyperpolarized to activate or deactivate \( I_h \) to differing extents (see RESULTS). Current injection protocols (see Fig. 7B) were generated by Clampex software (Molecular Devices, Sunnyvale, CA). The PD membrane potential was changed by a series of 5-s current injecting steps in 0.5- to 2-nA increments at 1-min intervals to allow full recovery of \( I_h \). If necessary, a bias current was injected into the PD cell to hold the membrane potential at −58 mV, the average PD membrane potential after blocker application. IPSPs were elicited by 200-ms depolarizing steps to −30 mV in the LP cell at the beginning or the end (after 7.8 s) of the PD polarization. The LP cell was held at −58 to −60 mV between steps. To avoid Cl⁻ loading during the current steps, which would alter the reversal potential of the PD IPSP, we used relatively high-resistance (20 MΩ or higher) electrodes filled with 0.6 M K₂SO₄ + 20 mM KCl. IPSP amplitudes were measured and plotted against the membrane potential immediately before the IPSP.

Statistics. All values are means ± SD. Statistical significances were determined using ANOVA and Student’s t-test after testing for normality (\( P < 0.05 \)) and using the Mann-Whitney rank sum test when the normality test failed. Regression lines were plotted, and R values were determined using SigmaPlot and SigmaStat 10.0 (Systat Software).

RESULTS

Immunocytochemical detection of \( I_h \) channels. In our first attempt to map the distribution of \( I_h \) channels in the STG of \( P. 
interruptus, we used a polyclonal antibody raised against PAIH, the $I_h$ protein in the related species *P. argus*, kindly provided to us by Dr. B. Ache. This antibody has been successfully used in *P. argus* to localize $I_h$ channel protein to the transduction compartment of olfactory receptor neurons (Gisselmann et al. 2005). Unfortunately, the specificity of this antibody was not adequate for use in our species, because the strongest band in Western blots was of lower molecular mass (~60 kDa) than what would be expected for PIIH, based on the sequences of previously identified splice variants (77–82 kDa). Furthermore, incubation of the Western membrane with pre-immune serum still produced bands. Thus we could not rule out the possibility of nonspecific binding of this PAIH antibody to a highly abundant unidentified protein. Instead, we took

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**Fig. 4.** PIIH-like immunoreactivity in the pyloric dilator (PD) fine neuropil. *A*: overlay of PIIH-like immunolabeling in the fine neuropil of a sectioned ganglion (*Ai*) with a Lucifer yellow-labeled PD neuron (*Aii*) revealed relatively sparse PIIH-like immunolabeling of the PD neuropil (arrowheads in *Aiii*). Scale bar, 50 µm. *B*: a minority of branches of the PD neuron showed more extensive PIIH-like immunolabeling, as shown in a 3-D rendered transparency projection from 25 1-µm-thick optical slices from a different preparation. Scale bar, 30 µm. *C*: clusters of PIIH-like immunolabeling in the fine neuropil (*Ci*) occurred in close proximity to the dye-labeled PD neuropil (*Cii*), with occasional overlap visible (filled arrowheads in *Ciii*). Single optical section (0.45 µm) of a sectioned ganglion. Scale bar, 10 µm.
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advantage of the fact that the carboxy-terminal region of the PIIH gene from *P. interruptus* has an unusual string of eight continuous histidine residues. This sequence could be specifically recognized by a commercially available monoclonal antipenta-His antibody, which is usually used to help purify proteins that have been artificially tagged with a sequence of five histidines. We confirmed the anti-penta-His recognition of the *Iₚ* protein using Western blots of proteins extracted from PIIH-expressing and control noninjected *Xenopus* oocytes. Figure 1E shows a clear band at ~76 kDa, near the predicted range of 77–82 kDa, as calculated from the sequences, showing that the anti-penta-His antibody does recognize the PIIH protein. A major band of the same size was labeled in protein extracts from *P. interruptus* brain and nervous tissue (Fig. 1E).

A minor band in the range of 116 kDa was found in *Panulirus* brain tissue and may be phosphorylated, glycosylated, or oligomeric forms, whereas a faint smaller band (~64 kDa) may be a consequence of protein breakdown or smaller splice variants. However, we emphasize that these bands may also represent other proteins recognized by the antibody. A National Center for Biotechnology Information BLASTp search of arthropod sequences restricted to Crustacea (search for: short and nearly exact matches; expect threshold: 200,000; Word size: 2; SEG filters: off; Score restricted to Crustacea (search for: short and nearly exact matches; expect threshold: 200,000; Word size: 2; SEG filters: off; Score restricted to Crustacea) did not find any bands on the Western blot. E75, a nuclear receptor found in *Daphnia pulex* and *D. magna* with a molecular mass of 104 and 102 kDa, respectively, could be of concern with regard to our 116-kDa band. However, the repetitive histidine sequence in this receptor is not evolutionarily conserved: it is not found in any of its arthropod homologs. It therefore seems unlikely, although not entirely dismissible, that the Cancer borealis E75 receptor would also contain this 5+His repeat and be recognized by the antibody. In addition, this nuclear protein would be unlikely to be highly expressed in distal neuropil (see below). The other two proteins of concern are doublesex-Mab related 99B (BAG12873.1) and a transcription cofactor vestigial protein (BAJ05330.1), both from *D. magna*, with calculated molecular masses of 63.9 and 59.5 kDa, similar to the smaller 64-kDa band on our Western blot. Since a band of this size was also recognized by the polyclonal anti-PAIH antibody, we feel it unlikely to be caused by homologs of these proteins. Thus we used the anti-penta-His antibody to study the distribution of PIIH protein in the STG.

*Iₚ* protein is expressed in the soma and neuropil regions of STG neurons. In the lobster STG, the majority of the 30 neuronal somata are located on the ventral surface, surrounded by glial cells and neuropil (Fig. 1A); some of the neurons are on the dorsal surface. These unipolar neurons send a primary neurite into the central core of the ganglion. This region, called the coarse neuropil, contains primarily large processes of the STG neurons as well as the axons of neurons in other ganglia and sensory neurons; there are no synaptic contacts in this region. The primary neurite then divides repeatedly into smaller branches, which in turn ramify in a more superficial area under and in between the somata called the fine or synaptic neuropil. This is where all the synapses in the ganglion are located (Fig. 1A).

In the superficial soma compartment, we found immunolabeling consistent with *Iₚ* protein expression in the somata of pyloric neurons (Fig. 1B). In this article, we refer to this labeling as PIIH-like immunoreactivity. The density of soma labeling varied between different neurons of the same ganglion, with some somata very strongly labeled, whereas others showed weak or no label (Fig. 1C). Among the pyloric neurons, the somata of the anterior burster, ventricular dilator (VD), and inferior cardiac (IC) neurons were usually more strongly labeled, whereas the PD neurons often exhibited weaker staining; however, due to the variability in staining between ganglia, there was not a statistically significant correlation between neuron type and somatic PIIH-like labeling intensity. The membranes of the somata did not show stronger PIIH-like staining than the cytosolic label; however, often a concentric pattern of higher intensity was located intracellularly around the nucleus (Fig. 1C), likely arising from intracellular protein still bound in the Golgi apparatus or endoplasmic reticulum (ER). There was significant labeling in between the somata, reflecting intense labeling in the synaptic fine neuropil (see below) as well as putative glial cells and connective tissue located there (Fig. 1C). There was also sometimes PIIH-like labeling in large fibrous structures of unknown origin, which enter the STG from the stomatogastric nerve and dorsal ventricular nerve and branch on the surface and within the neuropil of the ganglion (Fig. 1D).

Figure 2 shows a double-label experiment of a VG neuron filled with LY (*Aii*) and stained for PIIH-like immunoreactivity (*Ai*). A colocalization channel was generated to emphasize PIIH-like staining in or in contact with the VG neuron, generated from 89 0.7-μm optical sections (Fig. 2, *A* and *B*). A nonuniform and patchy distribution of PIIH-like immunoreactivity along the VG neuron (yellow) was visualized using a 3-D rendered transparency projection of the colocalization channel and the VG fill. Most of the PIIH-like...
immunolabeling was found in a patchy distribution on and around the medium-size and small branches, often close to branch points (arrows in Fig. 2, D and E). Most of the smaller PIIH-positive processes originated from a few single branches, which split off the primary neurite just below the soma (Fig. 2C). The large central neurites in the coarse neuropil and the two processes that lead to axons showed very little PIIH-like immunoreactivity; one exception was a short, stubby structure off the primary neurite, which gave rise to several very thin processes and showed patches of PIIH-like staining (Fig. 2D). A distinguishing feature of PIIH-like labeling was its localization on branch points in the fine neuropil and on flat paddle- or handlike structures, which were often found at or toward the end of very thin, long processes (Fig. 2E).

Similar PIIH-like labeling was seen in double stains of other pyloric neurons. Consistently, the strongest PIIH-like immunoreactivity was observed in the fine neuropil, where it often appeared in clouds at the ends of very fine branches, in bulbous or fingerlike structures (Fig. 3). The fine neuropil of an IC neuron was labeled nonuniformly with patches of PIIH-like staining; not all branches were labeled (Fig. 3A). The fine neuropil of the LP neuron showed bulbous varicosities that were strongly positive for PIIH-like immunoreactivity (Fig. 3B). In a different, sectioned VD neuron, which had been filled with NB, many spiny and bulbous processes showed overlap with PIIH-like staining (Fig. 3C). The physiological properties of $I_h$ have previously been studied in detail using voltage clamp in the pyloric pacemaker neurons, specifically the PD neurons. After filling a PD neuron with LY (a dye that does not cross gap junctions in the STG of $P$. interruptus), we sectioned the ganglion into 40-µm slices and labeled for PIIH-like immunoreactivity. Overall, PIIH-like labeling of the PD neuropil was somewhat sparser than in the other neurons. However, we found a number of branches that showed PIIH-like immunolabeling (Fig. 4, A and B). Clusters of strong PIIH-like signal were found in close proximity to the dye-labeled PD fine neuropil, with many overlapping regions (Fig. 4C).

$I_h$ expression in the synaptic neuropil. The fine neuropil has been shown by electron microscopy to be the site of synaptic interactions within the STG (King 1976), raising the question of whether $I_h$ channels are selectively located at or near synapses. To answer this question, we performed double-labeling experiments for PIIH and a $Drosophila$ anti-synaptotagmin antibody, which labels both synaptic vesicles and dense-core vesicles in Crustacea (Skiebe and Wollenschlaeger 2002). Anti-synaptotagmin labeling was seen in large clusters with punctate staining throughout the fine neuropil; several areas showed concentrated staining in the ganglion (Fig. 5A). Coselecting for PIIH-like immunoreactivity revealed an increased density of $I_h$ protein in areas of strong synaptotagmin labeling. A colocalization channel was generated and overlaid with the PIIH-like and synaptotagmin channels to reveal overlap in a single 1-µm optical section of a sectioned STG (Fig. 5Aii). We found some spots of overlap between PIIH-like staining and synaptotagmin immunoreactivity; however, the majority of PIIH-like patches did not colocalize with synaptotagmin immunoreactivity, and many synaptotagmin-positive spots did not overlap with PIIH-like staining. To better understand PIIH-like and synaptotagmin immunoreactivity distribution, we performed triple labeling of a NB-filled IC neuron with both antibodies. Again, colocalization channels were generated and manual thresholding was used to show only protein in or within immediate proximity to the surface of the filled cell (Fig. 5E).}

*Fig. 6. PIIH-like and Shal immunoreactivity in the STG. A: overview of PIIH-like (Ai) and Shal immunoreactivity (Aii) in the same STG whole mount shows a distinctly different labeling pattern. Whereas PIIH-like immunoreactivity was found throughout the STG in neurons and the surrounding tissue, Shal immunoreactivity was concentrated in neurons with high concentrations in the primary neurites. Scale bar, 150 µm. AB, anterior burster neuron; AM, anterior median neuron; DG, dorsal gastric neuron; GM, gastric mill neuron; LG, lateral gastric neuron; MG, median gastric neuron; PY, pyloric neuron; S, STG. B: PIIH-like and Shal immunolabeling overlap in the soma of STG neurons. Both show a perinuclear distribution of labeling (arrow), but only Shal was highly concentrated in the soma membranes (open arrowhead), and only PIIH-like immunolabeling was found in the tissue surrounding the soma (filled arrowhead). Scale bar, 30 µm. C: PIIH-like (Ci) and Shal immunolabeling (Cii) in the coarse neuropil. Transparency projection of 7 1.3-µm optical slices reveals cloudy and patchy PIIH-like immunoreactivity throughout the coarse neuropil (Ci), whereas anti-Shal distinctly labeled large processes of the primary neurites (Cii). Overlay shows that large processes in the coarse neuropil generally lack PIIH-like immunoreactivity while showing strong Shal immunolabeling (open arrowheads in Cii). Some PIIH-like immunolabel was also found in or in close vicinity of axons, shown for anterior lateral nerve (arrows, right) on leaving the STG. Scale bar, 70 µm. D: single optical section shows area of the fine neuropil, which shows intense PIIH-like (Di) and Shal labeling (Dii). At a superficial level, both signals occurred within the same regions of the fine neuropil (Dii). Note the complete absence of PIIH-like immunoreactivity in the first stretch of the primary neurites, where the most intense Shal immunolabeling was found (open arrowhead). In the neuropil, PIIH-like staining is punctate (flat arrowheads) and only occasionally appears to overlap with Shal immunolabeling (filled arrowheads). Scale bar, 30 µm. E: at high magnification, PIIH-like labeling (Ei) and Shal immunolabeling (Eii) are frequently found in close apposition throughout the fine neuropil but rarely overlap and colocalize. At this level of magnification, PIIH-like immunolabeling still appears concentrated in a patch or punctate pattern (flattened arrowheads in Eii), which is found in the close vicinity of Shal-positive structures (open arrowheads in Eii), but the overlay shows that both signals rarely overlap at the pixel range (Eii). Scale bar, 20 µm."

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to show the double label at the single-pixel level. The virtual absence of membrane-bound double labeling for PIIH-like and synaptotagmin immunoreactivity, despite the large occurrence of synaptotagmin-labeled structures (Fig. 5B), might indicate a primarily postsynaptic distribution of PIIH. However, without the higher gain analysis using electron microscopy, we cannot determine with certainty whether \( I_h \) channels are localized at pre- or postsynaptic sites.

\( I_h \) and \( I_A \) protein localization. We previously described a homeostatic interaction between \( I_h \) and \( A \)-type potassium channels such that artificial upregulation of \( I_h \) by Shal RNA injection led to a compensatory neuronal upregulation of \( I_h \) to retain normal firing activity (MacLean et al. 2003, 2005). The molecular mechanisms for this coregulation remain unknown. One possibility is that the proteins are physically coupled and are trafficked together to the cell surface. \( G \) protein receptors and ion channels have previously been shown to physically interact with scaffold proteins and other regulatory proteins in multiprotein complexes (Cooper 2003; Ma and Jan 2002; Mathie et al. 1998). If this were so, one might expect to find overlapping patterns of PIIH-like and Shal immunoreactivity localization. We therefore used a rabbit polyclonal antibody to lobster Shal, which encodes \( I_A \) in the STG, that was previously designed and tested in our laboratory (Baro et al. 2000). Costaining of Shal and PIIH-like immunoreactivity revealed distinctly different patterns of labeling of the STG (Fig. 6).

Whereas PIIH-like immunolabeling was found throughout the STG in neurons and the surrounding tissue, Shal immunolabeling was primarily concentrated in neurons, with a high level of immunostaining in the primary neurites (Fig. 6A).

As previously described, Shal protein is trafficked to the cell surface of STG somata and their primary neurites; this was seen as bright ringlike staining in cross sections of the soma. However, such ringlike staining was not observed with the PIIH antibody (Fig. 6, A and B). Both antibodies showed strong intracellular perinuclear immunolabeling in the soma of STG motoneurons (Fig. 6B). In addition, anti-Shal strongly labeled the primary neurites arising from the somata, whereas PIIH-like immunolabeling did not (Fig. 6, A, C, D). Strong Shal immunoreactivity was also seen in the coarse neuropil (Fig. 6C) (Baro et al. 2000), whereas PIIH-like labeling was patchy and sparse in this region (Fig. 6C). PIIH-like immunoreactivity was also found in or in close vicinity to axon precursors (Fig. 6C), but we did not find convincing PIIH-like immunolabeling in the axons of the nerves (not shown).

In the fine neuropil, PIIH-like and Shal immunolabeling revealed similar distribution patterns at a superficial level, with high concentrations in the densest part of the synaptic neuropil (Fig. 6D). However, at high magnifications it was apparent that the PIIH-like and Shal-immunoreactive structures in the fine neuropil were not identical but in close apposition (Fig. 6E). Shal immunolabeling in the membranes of the fine branches was usually more homogenous than PIIH-like immunolabeling, which, as stated above, often showed distinct punctate staining patterns.

\( I_h \) activation reduces the strength of synaptic transmission. The strong PIIH-like immunolabeling in the synaptic fine neuropil suggested that this channel might function to regulate synaptic transmission in the pyloric network. To test this, we studied the effect of \( I_h \) activation on IPSP amplitude at the glutamatergic LP → PD synapse. For this purpose, we used the two-electrode current-clamp technique to control the membrane potential of the presynaptic LP cell and recorded LP-evoked graded IPSPs in the PD neuron while it was stepped to different membrane potentials (Fig. 7A). A range of 8-s hyperpolarizing and depolarizing current steps were injected into the PD neuron to activate or deactivate \( I_h \) to differing extents. \( I_h \) activation was monitored by the expression of a slow depolarizing sag potential in the PD neuron (Fig. 7B). At the beginning of a PD hyperpolarization, the \( I_h \) channels have only just begun to open, so \( I_h \) activation is low. We compared LP-evoked IPSP amplitudes at this point with those recorded after 7.8 s of PD hyperpolarization, when \( I_h \) channels were more completely activated to their steady-state level for that voltage (Fig. 7, B and C). Comparisons were made at the same voltages early and late in the current step (Fig. 7, B and C); thus larger initial steps had to be made to correct for the depolarizing sag to obtain comparable voltages at the end of the current step as at the beginning.

We consistently found that the amplitude of IPSPs at the same voltage after \( I_h \) activation at the end of PD hyperpolarization was smaller than that at the beginning, before \( I_h \) was significantly activated. This effect was strong for all voltages hyperpolarized below −75 mV (Fig. 7, B–E) and could be quite large. For example, when the PD neuron was hyperpolarized to −110 mV at the beginning of the step, the IPSP amplitude was 12.3 ± 1.3 mV, whereas when the PD was at −110 mV at the end of the step, the IPSP amplitude was only 5.6 ± 1.8 mV (62% decrease, \( n = 6, P < 0.01 \)). Overall, the difference between IPSPs before and after activation of \( I_h \) was statistically significant at all PD membrane potentials of −80 mV and below (see stars below x-axis in Fig. 7E). With depolarizing current steps, when little \( I_h \) was activated by the step, there was not a large difference between IPSP amplitude measured at the beginning and the end of the step. At the most depolarized membrane potentials between −40 and −35 mV, the IPSPs began to decline in amplitude, most likely due to shunting by other voltage-activated currents. With these depolarizing current steps, the IPSP amplitude at the beginning of the PD polarization tended to be smaller than at the end, although this difference did not reach statistical significance. This could be a consequence of slow inactivation of voltage-dependent currents (including \( K^+ \) and \( Ca^{2+} \) currents) or deactivation of resting \( I_h \) itself (see below).

IPSP amplitude during block of \( I_h \) channels. If activation of \( I_h \) channels during hyperpolarizing voltage steps were shunting synaptic input, then \( I_h \) blockers should reduce or eliminate the difference between the IPSP amplitudes at the beginning and at the end of the PD current steps. To block \( I_h \), we used 5 mM CsCl or 100 μM ZD7288, both of which cause comparable block of \( I_h \) in this system (Peck et al. 2006). Blockade of \( I_h \) dramatically changed the amplitudes of LP-evoked IPSPs in the PD neuron. In the presence of either 5 mM CsCl (Fig. 8, A and B) or 100 μM ZD7288 (Fig. 8C), the late IPSP elicited at the end of a PD hyperpolarization was significantly increased in amplitude at all membrane potentials below −65 mV. This effect partially reversed for CsCl after 30–45 min of washout (Fig. 8B), but not for ZD7288 (data not shown). There was also a trend with both blockers toward larger late IPSP amplitudes at more depolarized potentials between −65 and −45 mV, although it did not reach statistical significance for CsCl (Fig. 8, B and C).
If the late IPSP attenuation after long hyperpolarizing steps under control conditions was entirely due to activation of Ih, the difference between early and late IPSP amplitudes should be eliminated during Ih block. Indeed, after application of 5 mM CsCl, IPSPs at the beginning of the PD polarization were not significantly larger than those at the end of the pulse (n = 5, P > 0.05, Fig. 8D).

A fraction of Ih channels might be open at the resting potential. To test this hypothesis, we blocked Ih by bath application of either 5 mM CsCl or 100 nM ZD7288 for at least 20 min. Both drugs slightly but significantly hyperpolarized the resting potentials of the PD and LP neurons. On average, bath application of 5 mM CsCl caused the PD neurons...
to hyperpolarize by 4.1 ± 1.4 mV (from −56.2 to −60.3 mV, $P = 0.04$, $n = 7$). Similarly, 100 μM ZD7288 hyperpolarized PD neurons by 2.2 ± 1.6 mV (from −55.6 to −59.0 mV, $P = 0.03$, $n = 5$).

To further study whether some $I_h$ was active at more depolarized membrane potentials and could affect synaptic transmission within the normal voltage range of the PD neurons, we compared IPSPs at the beginning of the PD voltage steps under control conditions and after blockade of $I_h$. We found that blockade of $I_h$ by either 5 mM CsCl (Fig. 8E) or 100 μM ZD7288 (Fig. 8F) increased the early IPSP amplitude at most membrane potentials, which in the case of ZD7288 was noticeable (up to 2-fold in individual experiments) even in the physiological range of −60 to −45 mV (Fig. 8F). At these potentials the increase approached significance ($P < 0.2$), but it was only statistically significant ($P < 0.05$, $n = 5$) at more hyperpolarized PD voltages (Fig. 8F). CsCl had a similar though smaller effect (Fig. 8E), perhaps due to the voltage dependence of $I_h$ block by CsCl (DiFrancesco 1982). It should be emphasized that these experiments were performed in the absence of modulatory inputs, which can shift the voltage dependence of $I_h$ and alter its contribution to the resting potential (Peck et al. 2006).

**DISCUSSION**

We have demonstrated that PIIH-like immunoreactivity, consistent with expression of the protein encoding $I_h$, is present in the lobster STG. Although only low membrane labeling is detected in the somata or coarse neuropil, areas in the fine neuropil, which is the site of synaptic interactions, are strongly labeled. This led us to test whether $I_h$ can regulate synaptic strength; our results suggest that it can shunt inhibitory synaptic events when it is activated.

**Distribution of $I_h$ protein in the STG.** The distribution of $I_h$ channels is not uniform in STG neurons. The somata of individual neurons revealed a wide range of PIIH-like immunoreactivity; this varied between different ganglia, and overall, there was not a statistically significant correlation between neuron type and somatic PIIH-like immunolabeling intensity. In the somata, the majority of PIIH-like immunoreactivity was seen in a concentric ring around the nucleus, probably reflecting protein still bound in the ER/Golgi system and trafficking vesicles. We saw no strong rings of label around the somata or parallel “railroad track” labeling of the membranes of neurites, as were previously seen for membrane-bound potassium channels (Fig. 6) (Baro et al. 2000; French et al. 2004). Tissue in between and around the somata on the dorsal and ventral surfaces of the ganglion usually revealed strong PIIH-like immunoreactivity. This could arise in part from the superficial fine neuropil (see below) but could also reflect the presence of PIIH in connective tissue or glial cells. Glial cells express a number of voltage-sensitive channels encoding potassium and sodium currents, where they can affect the glial resting potential and, indirectly, the activity of nearby neurons (Janigro 1997; Kang et al. 1998; Yamazaki et al. 2005).

In the neuropil, we found sparse and patchy PIIH-like immunolabeling on larger primary and secondary neurites of the coarse neuropil; this contrasts with the relative smooth distribution of Shal potassium channel immunolabeling on these neurites (Baro et al. 2000). The fine neuropil had the most intense, cloudy PIIH-like immunolabeling with localized punctate staining at higher magnifications. Labeling often occurred close to branching points or on very thin, long branches. Thus a significant amount of PIIH protein appears to be localized to the fine neuropil, where synaptic interactions are localized.

Synaptotagmin, a vesicular protein thought to be the calcium sensor that triggers exocytosis, is associated with release sites for both clear and dense-core vesicles in Crustacea (Skiebe and Wollenschläger 2002). Our double-labeling experiments for PIIH-like and synaptotagmin immunoreactivity showed a strong proximity of PIIH-like and synaptotagmin labeling in the branches of the fine neuropil; however, the two labels typically were not close enough to show pixel-to-pixel colocalization at the light microscopic level. This suggests that $I_h$ channels are not primarily localized at the presynaptic terminal itself and provides some evidence for a postsynaptic localization of the channel. However, more detailed electron microscopy studies are needed to verify this. The close proximity of PIIH-like immunolabeling to synapses suggests that $I_h$ activation may act to modulate synaptic strength, which we discuss below.

$I_h$ and $I_A$ protein localization. We earlier showed that artificial upregulation of $I_h$ (by Shal RNA injection) evoked a homeostatic compensatory upregulation of $I_h$ to maintain the normal firing properties of the neuron (MacLean et al. 2003, 2005); even under normal conditions, PD neurons maintain a fixed ratio of expression of $I_h$ and $I_h$ (MacLean et al. 2005; Schulz 2006, 2007). One possible explanation for this coupled coregulation could be a coordinated surface expression of $I_h$ and $I_h$ channels, perhaps mediated by joint binding to surface scaffold proteins. We tested this with double-labeling studies to look for colocalization of Shal and PIIH-like immunoreactivity. These studies revealed similarities and differences in their immunolabeling patterns. Shal immunoreactivity showed a smooth distribution of labeling along the membranes of the cell bodies and primary neurites, which showed much less and more patchy PIIH-like labeling, which was mostly intracellular in these compartments. The fine neuropil exhibited equally strong labeling for PIIH-like and Shal immunoreactivity. However, at high magnifications, the sites of the most intense staining in the fine neuropil usually showed Shal and PIIH-like immunoreactivity adjacent to each other but rarely overlapping. This lack of colabeling makes direct interactions of the channels in a multiprotein complex less attractive hypothesis to explain the homeostatic response. It appears that the compensa-
ory response can occur even though the proteins are physically separate, although both are highly enriched in the synaptic neuropil. However, our light-level study cannot conclusively rule out the possibility of colocalization of a subset of channels, which would lead to some overlap and some differential staining.

$I_h$ activation reduces the size of synaptic input. Since $I_h$ channels are strongly expressed in the synaptic regions of the fine neuropil, we tested whether $I_h$ activation can affect synaptic strength. Our experimental design at the graded LP → PD synapse measured the postsynaptic effects of $I_h$ activation by comparing the LP-evoked IPSP amplitude at varying PD voltages under conditions where $I_h$ was either weakly or strongly activated. We found that activation of $I_h$ dramatically decreased the size of the postsynaptic responses. This effect could be abolished by the $I_h$ blockers ZD7288 and CsCl, confirming that our voltage-step protocols were monitoring the selective effect of activating $I_h$. It should be noted that both CsCl and ZD7288 can have nonspecific effects. CsCl blocks other potassium currents at higher concentrations (Llinás 1988), and ZD7288 has been known to affect synaptic transmission in a non-$I_h$-dependent way (Chevaleyre and Castillo 2002). The fact that both blockers had approximately the same effect on the membrane potential-IPSP amplitude curves, despite their very different nonspecific effects, supports our argument that this change is indeed due to the block of $I_h$.

Studies in other systems have suggested that $I_h$ can both directly and indirectly increase synaptic transmission (Beaumont and Zucker 2000, 2002; Boyes et al. 2007; Genlain et al. 2007), but it can also have indirect effects to reduce postsynaptic integration (Macaferrie et al. 1996; Magee 1998, 1999; McCormick and Pape 1990). Activation of $I_h$ channels will reduce the neuron’s membrane resistance and thus shunt synaptic events by reducing the effective length constant (Berger et al. 2003; Magee 1998, 1999; Williams and Stuart 2000). Migliore et al. (2004) showed that $I_h$ could also selectively block temporal summation of unsynchronized input. We propose that an increase in $I_h$ in the postsynaptic PD neuron significantly reduces the synaptic amplitude both by reducing the postsynaptic input resistance near the synapse and by providing an inward counter-current to shunt the inhibitory IPSPs.

In the lobster, the voltage range for $I_h$ activation is relatively hyperpolarized ($V_{1/2}$ of the various splice forms ranges from $-83$ to $-107$ mV; Ouyang et al. 2007). Thus the shunting effects of $I_h$ activation were most predominant at rather hyperpolarized potentials, where $I_h$ activation is high. However, when we compared the amplitude of PD IPSPs at the beginning of the PD polarization (with low $I_h$ activation) before and after $I_h$ block with 100 µM ZD7288, we observed an increase in IPSP amplitude at all membrane potentials, including large effects (up to 2-fold) in the physiological range of $-70$ to $-35$ mV. In addition, both ZD7288 and low concentrations of CsCl, which also blocks $I_h$ in the STG, caused a slight hyperpolarization of the resting potential. These data indicate that a small fraction of $I_h$ channels are open at physiological membrane potentials and can regulate normal synaptic transmission.

Two possible mechanisms could make $I_h$ an important regulatory current in the pyloric network, despite its hyperpolarized voltage dependence of activation (Ouyang et al. 2007). First, the hyperpolarizing inhibitory synaptic responses in distal parts of the neuron will be larger when they reach the soma, which would enhance the distal activation of $I_h$ and thus its role in regulating synaptic strength. Second, and more importantly, $I_h$ activation kinetics and voltage dependence are significantly affected by cAMP binding, which can be changed by neuromodulatory inputs (Ballo et al. 2010; Ouyang et al. 2007; Robinson and Siegelbaum 2003; Rosenkranz and Johnston 2006; Santoro et al. 2004). In the absence of additional modulatory inputs, the contribution of open $I_h$ channels to pyloric activity may be small, because we previously showed that blockade of $I_h$ with CsCl had only subtle effects on the ongoing rhythmic pyloric motor pattern (Peck et al. 2006). However, cAMP binding at the CN-binding site depolarizes the voltage dependence of activation, which would increase the resting active $I_h$ in the physiologically relevant voltage range. Consistent with this possibility, in the STG, dopamine shifts the activation curve of $I_h$ in the AB cell by 10–15 mV in the depolarizing direction (Peck et al. 2006). Using optical methods to monitor cAMP levels, Hempel et al. (1996) showed that different neuromodulators caused unique and local elevations of cAMP transients in the fine neuropil of STG neurons. Thus dendritic $I_h$ channels near synaptic sites are a likely target for cAMP-dependent modulation, which would regulate synaptic strength in a cell- and possibly even compartment-specific manner. Modulatory activation of postsynaptic $I_h$ channels close to synaptic inputs could effectively shunt these synapses. This modulation could provide a potentially important and powerful mechanism to regulate the strength of synaptic input and thus modulate the rhythmic activity of the entire circuit in a context-dependent manner.

We examined the functional implications of $I_h$ specifically for the LP → PD synapse. All the neurons within this network receive inhibitory input, most of them with very similar timing, and therefore the role of $I_h$ in modulating or shunting the IPSP could be similar in the other pyloric neurons. This might be specifically important during different behavioral states in the presence of different modulators, which can dramatically change the output of this seemingly rigid network (Harris-Warrick and Marder 1991). On the other hand, it is certainly possible that this is not the only function of $I_h$ and that its function may vary among the different neurons of the STG networks.

ACKNOWLEDGMENTS

We thank Ted Brooks for providing the GUI-based MATLAB alignment and stitching tool, Carol Bayles for technical assistance during confocal image acquisition, and Pat Rivlin for giftng the syntapotagmin antibody.

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grant R01-NS17323 to R. Harris-Warrick.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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