HCN hyperpolarization-activated cation channels strengthen virtual nicotinic EPSPs and thereby elevate synaptic amplification in rat sympathetic neurons

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Running title: h-current modulation of virtual fast EPSPs
Abstract
The influence of h-current ($I_h$) upon synaptic integration in paravertebral sympathetic neurons was studied together with expression of HCN subunit isoforms. All four HCN subunits were detected in homogenates of the rat superior cervical ganglion (SCG) using the polymerase chain reaction to amplify reverse transcribed messenger RNAs (RT-PCR) and using quantitative PCR (qPCR). Voltage clamp recordings from dissociated SCG neurons at 35°C detected $I_h$ in all cells, with a maximum h-conductance of 1.2 ± 0.1 nS, half-maximal activation at -87.6 mV and a reversal potential of -31.6 mV. Interaction between $I_h$ and synaptic potentials was tested with virtual fast nicotinic EPSPs created with dynamic clamp. Blocking $I_h$ with 15 µM ZD7288 hyperpolarized cells by 4.7 mV and increased the virtual synaptic conductance required to stimulate an action potential from 7.0 ± 0.9 nS to 12.1 ± 0.9 nS. In response to stimulation with 40 second long trains of virtual EPSPs, ZD7288 reduced postsynaptic firing from 2.2 to 1.7 Hz and the associated synaptic amplification from 2.2 ± 0.1 to 1.7 ± 0.2. Cyclic nucleotide binding to HCN channels was simulated by blocking native $I_h$ with ZD7288, followed by reconstitution with virtual $I_h$ using a dynamic clamp model of the voltage clamp data. Over a 30 mV range, shifting the half-activation voltage for $I_h$ in 10 mV depolarizing increments always increased synaptic gain. These results indicate that $I_h$ in sympathetic neurons can strengthen nicotinic EPSPs and increase synaptic amplification, while also working as a substrate for cyclic nucleotide-dependent modulation.
**New and Noteworthy:** Using dynamic clamp to create virtual synapses, this study shows that h-current strengthens fast EPSPs and synaptic amplification in sympathetic neurons. Manipulating the voltage-dependence of a virtual h-current also shifted gain. These results reveal a new physiological role for h-channels in the sympathetic motor system.

**Key words:** sympathetic motor system, dynamic clamp, synaptic integration, baroreflex gain, blood pressure

**INTRODUCTION**

This study examines the interaction in rat sympathetic neurons between the hyperpolarization-activated cation current (h-current) and virtual fast EPSPs. The h-current ($I_h$) flows through channels encoded by four genes designated HCN1-4 that belong to the superfamily of cyclic nucleotide gated ion channels (Biel 2009; Craven and Zagotta 2006; Kaupp and Seifert 2001; Pape 1996; Robinson and Siegelbaum 2003). Originally named as the ‘funny’ or f-current because of its unusual voltage-dependence, $I_h$ was discovered in the sino-atrial node and cardiac Purkinje fibers (Brown et al. 1979; DiFrancesco 2010; DiFrancesco and Ojeda 1980), where it plays an essential role in generating the heart’s intrinsic pacemaker activity. Subsequent work detected $I_h$ in a variety of central and peripheral neurons, where it can influence the resting potential, membrane excitability, rhythmic firing and the integration of synaptic potentials (George et al. 2009; Pape 1996; Pape and McCormick 1989; Siegelbaum 2000). In the periphery, $I_h$ has been identified in subtypes of autonomic neurons by its voltage-dependence, together with sensitivity to Cs$^+$, insensitivity to Ba$^{2+}$ and dependence upon Na$^+$. These neuronal groups include AH-type myenteric neurons in the gut (Galligan et al. 1990; Rugiero et al. 2002;
Xiao et al. 2004), parasympathetic neurons in the cardiac ganglion (Cuevas et al. 1997; Edwards et al. 1995; Hogg et al. 2001; Xi-Moy and Dun 1995) and sympathetic neurons in paravertebral ganglia (Jobling and Gibbins 1999; Lamas 1998; Lamas et al. 1997; Tokimasa and Akasu 1990). In addition, \( I_h \) is developmentally regulated in parasympathetic neurons (Hogg et al. 2001) and its modulation mediates some excitatory effects of pituitary adenylate cyclase-activating polypeptide (Merriam et al. 2004; Tompkins et al. 2009). Despite this widespread cellular expression pattern, the implications of \( I_h \) for fast synaptic transmission in autonomic neurons remain largely unexplored.

The possible importance of \( I_h \) in sympathetic neurons became apparent to us during experiments to assess the repetitive firing elicited by long depolarizing current pulses in neurons dissociated from the rat superior cervical ganglion (SCG) (Springer et al. 2015). When hyperpolarizing current pulses were applied to construct steady-state current-voltage (I-V) relations, SCG neurons displayed very prominent sag responses that in some cases were >40 mV in amplitude at negative potentials (see for example Figure 1 in (Springer et al. 2015)). Although sag depolarizations at negative voltages are a hallmark of \( I_h \) activation, rarely have such large responses been reported. Moreover, Springer et al. (2015) observed similar sag responses in whole-cell current-clamp recordings from the intact SCG, as well as in cell culture, thus suggesting that \( I_h \) expression is a normal feature of rat sympathetic neurons and not a consequence of in vitro growth conditions. In preliminary experiments application of ZD7288, a selective blocker of HCN channels (BoSmith et al. 1993; Harris and Constanti 1995), antagonized the sag response, thereby providing additional evidence for activation of inward \( I_h \) at negative potentials.
The present work was undertaken to identify the HCN subunits normally expressed in the SCG and to assess their functional consequences for fast synaptic excitation. To approach this problem, membrane excitability was probed with virtual fast nicotinic EPSPs of defined strength that were created using the dynamic clamp method (Springer et al. 2015). Voltage-clamp measurements were then used to construct a mathematical model of the h-conductance (g_h). Implementing the model under dynamic clamp permitted experiments to reconstitute a virtual I_h after pharmacological blockade of the native g_h. The results confirm that low levels of g_h expression help to regulate the normal resting potential of sympathetic neurons and they reveal for the first time that g_h effectively strengthens the impact of subthreshold virtual fast EPSPs upon postganglionic spike generation. We also found that these effects have the capacity to elevate synaptic amplification in sympathetic ganglia and are modulated by shifts in the voltage-dependence of g_h activation, similar to those produced by binding of cyclic nucleotides to HCN channels.
MATERIALS AND METHODS

Animal Use

All experiments used Sprague-Dawley rats (CD strain; Charles River Laboratories, Wilmington, MA). The Institutional Animal Care and Use Committee at the University of Pittsburgh approved all animal protocols for this study.

Standard RT-PCR analysis

Superior cervical ganglia were dissected from adult female and male rats that had been killed by CO₂ inhalation and then homogenized in 200 µl glass micro tissue grinders (# 885470-0000 Kimble Chase, Vineland, NJ). Total RNA was extracted using the RNeasy Micro kit (#74004, Qiagen, Valencia, CA) that contained homogenization buffer and genomic DNA elimination buffer. RNA was reverse transcribed to cDNA using the RT² First Strand kit (#330404, Qiagen). PCR primers were designed (Table 1) to span exon boundaries with GC content close to 50% and melting temperatures in the range of 55-60°C and then synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Amplification reactions (25 µl) contained 1 µl cDNA, 5 µl 1.125 mM dNTP, 2 µl 20 µM forward primer, 2 µl 20 µM reverse primer, and 0.25 µl DreamTaq DNA polymerase (#EP0702, ThermoFisher, Grand Island, NY). The thermocycling protocol consisted of 94°C for 2 minutes, 36 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 90 seconds, followed by 72°C for 7 minutes and cool down to 4°C. PCR products for each of the four HCN subunits and neuropeptide Y (NPY) were run on 2% agarose gels with 0.01% ethidium bromide, visualized on a UV trans-illuminator and photographed using a Kodak EDAS 290 Electrophoresis Analysis System.
Quantitative PCR

Total RNA was extracted from SCGs removed from four postnatal day 42 male rats using the same methods as for RT-PCR. RNA from each sample was reverse-transcribed into cDNA using iScript Reverse Transcription Supermix (#1708840 BioRad, Hercules, CA). The RT reaction contained 4µl of iScript supermix, 1µg RNA and nuclease free water to a 20 µl final volume. The RT thermal cycling protocol consisted of priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C followed by RT inactivation for 1 minute at 95°C. qPCR primers for each HCN isoform and for GAPDH (Table 1) were designed to span exon boundaries, with GC content near 50% and melting temperatures ranging from 55-60 °C and were synthesized by IDT. Amplification reactions (20 µl) were run in triplicate and contained 1µl undiluted cDNA, 10 µl SsoAdvanced™ Universal SYBR® Green Supermix, (#172-5271 BioRad), 0.25 µl each of sense and anti-sense primers (final concentrations 250nM) and 8.5 µl of water. The qPCR protocol (BioRad CFX96 qPCR system) was: 30 second at 95°C followed by 40 cycles of 5 seconds at 95°C and 1 minute at 60°C. Melt curves were obtained by increasing the sample temperature from 65°C to 95°C in 0.5°C steps.

The efficiency (E) of primer pairs was determined in duplicate qPCR measurements of the threshold cycle (Ct) for increasing volumes of cDNA (0.5, 1 and 2 µl). The slopes of linear fits of log10 (µl cDNA) vs Ct were used to calculate the primer efficiency (E = 10 [-1/slope]). E values were 1.855 for GAPDH, 2.022 for HCN1, 2.1136 for HCN2, 2.007 for HCN3 and 1.985 for HCN4. Ct’s were corrected by multiplying them by log2(E). Relative HCN expression levels were calculated from corrected Ct’s as \( R_{HCN/GAPDH} = 2^{-\Delta Ct} \), where \( \Delta Ct = Ct_{GAPDH} - Ct_{HCN} \) (Pfaffl 2001).
**Cell culture**

Primary cultures of dissociated sympathetic neurons were prepared as previously described (Springer et al. 2015). Pups were anaesthetized with isoflurane (Piramal Critical Care, Bethlehem, PA, USA) and killed by thoracotomy and cutting the heart. For each dissociation, SCGs were dissected from a litter of 15-day-old rat pups and placed into chilled L-15 media (#SH30525.01, GE Healthcare Hyclone, Logan, UT). Ganglia were desheathed, cut into pieces and enzymatically digested at 37°C for 30 minutes in 2 mg/ml collagenase type 4 (#LS004186, Worthington Biochemical Corp, Lakewood, NJ) in L-15, followed by 30 minutes in 0.25% trypsin (#15050-057 GIBCO), with gentle agitation every 10 minutes. Trypsin was neutralized by diluting 1:10 with growth media that contained minimal essential medium (Richter’s modification with L-glutamine, #SH30601.01 Hyclone), 10% fetal bovine serum (#S11150 Atlanta Biologicals, Norcross, GA), 1% penicillin-streptomycin (#B21210Atlanta Biologicals, Cat. No.), 10 ng/ml nerve growth factor (#BT-5017 Harlan Bioproducts, Indianapolis, IN) and 0.4 µM cytosine arabinoside hydrochloride (C6645 Sigma-Aldrich, St. Louis, MO). Digested tissue was pelleted by centrifugation at 100 x g for 1 minute, suspended in 1 ml of growth medium, and triturated using 3 flame-polished Pasteur pipettes with graded tips. Cells were plated onto 8 to 12, 12mm poly-D-lysine/laminin-coated glass coverslips (#354087 Corning, Corning, NY) in a multi-well plate and incubated at 37°C in 5% CO₂ for 1 hour for cell attachment. Then 0.5 ml of growth media was added and changed daily.

**Electrophysiology**

Whole-cell perforated patch clamp recordings at 35-36°C were made from SCG neurons after 2 to 4 days in vitro. External solution contained (in mM): 146 NaCl, 4.7 KCl, 20 HEPES, 0.6
MgSO₄, 1.6 NaHCO₃, 0.13 NaH₂PO₄, 2.5 CaCl₂, 7.8 dextrose, adjusted to pH 7.4 with NaOH and bubbled with 100% O₂. Internal solution contained (in mM): 94 K⁺-gluconate, 30 KCl, 10 phosphocreatine di tris salt, 10 HEPES, 0.2 EGTA, 4 Mg₂ATP, 0.3 Na₂GTP, adjusted to pH 7.3 with KOH. Stock solution of amphotericin B (40 mg/ml DMSO) was added to the internal solution for a final concentration of 200 µg/ml. Measurements were started once patch perforation had reduced access resistance to <15 MΩ. No corrections were made for a calculated tip potential of 14 mV, except when noted. Chemicals were purchased from Sigma-Aldrich.

**Measurement of Iₜₜ**

Voltage clamp recordings were made using an Axopatch 200 amplifier, DigiData 1440A interface and pClamp 10 software (Molecular Devices, Sunnyvale, CA). Cell size was determined from the whole-cell capacitance compensation of the amplifier. Series resistance was monitored throughout the experiment and compensated 85-90%. Current recordings were low-pass filtered at 1 kHz and digitized at 10 kHz. To isolate Iₜₜ, voltage protocols were executed before and after bath application of 10-15 µM ZD7288, an Iₜₜ-specific channel blocker (#ab120102 abcam, Cambridge, MA).

**Dynamic clamp implementation of virtual synapses and virtual Iₜₜ**

Dynamic current clamp recordings were made using an Axoclamp 2B amplifier (Molecular Devices) and a G-clamp interface and software running at 20 kHz (Kullmann et al. 2004). After establishing a stable recording in current clamp mode the series resistance was fully compensated using the amplifier bridge circuitry. Voltage recordings were low pass-filtered at 3 kHz. Two types of conductance were implemented with the dynamic clamp: a nicotinic...
conductance to produce virtual synaptic activity and an h-conductance to study its role in synaptic integration.

Virtual synaptic current was modeled by equation 1,

\[ I_{syn}(t) = g_{syn}(t) \times S \times (V_m - E_{rev}) \]  

where synaptic conductance \( g_{syn}(t) \) as a function of time was calculated as the sum of two exponentials, with time constants of 1 ms \( (\tau_{rise}) \) and 7 ms \( (\tau_{decay}) \) (Springer et al. 2015; Wheeler et al. 2004). \( V_m \) is the membrane potential at a given time, \( E_{rev} = 0 \text{ mV} \) and \( S \) is a scaling factor used to adjust the strength of individual synapses.

The strengths of virtual synapses were calibrated in each neuron to the threshold synaptic conductance \( \text{thresh-}g_{syn} \), defined as the minimum \( g_{syn} \) needed to elicit an action potential (Kullmann and Horn 2006; Schobesberger et al. 2000). \( \text{thresh-}g_{syn} \) was found by an automated binary search algorithm that delivered virtual EPSPs of varying strength at 0.5 Hz and settled on a solution within 10 trials (Kullmann et al. 2004). The stability of each cell recording was monitored throughout experiments by repeatedly measuring \( \text{thresh-}g_{syn} \).

To measure synaptic gain, 40 second conductance command templates for the dynamic clamp were designed to mimic the noisy bursts of EPSPs characteristic of baroreceptor-entrained synaptic activity in rat vasomotor neurons (Springer et al. 2015). Presynaptic convergence was mimicked by implementing one strong suprathreshold synapse set to 300% \( \text{thresh-}g_{syn} \) and eight weak subthreshold synapses set to 60% \( \text{thresh-}g_{syn} \). We assumed an average heart rate of 5 Hz and a 20% on/off duty cycle for bursts of synaptic activity during each heartbeat. To achieve an average presynaptic firing rate \( f_{pre} \) of 1 Hz per synapse, they each fired randomly at 5 Hz during the active 40 ms period within the 200 ms cardiac cycle. The method for calculating exponentially distributed random intervals between synaptic events was as previously described.
Templates were constructed at 50 µs resolution so that the dynamic clamp could operate at 20 kHz. Synaptic gain was calculated as the frequency of postsynaptic action potentials ($f_{post}$) divided by $f_{pre}$ (Wheeler et al. 2004). The same approach has previously been shown to produce average postsynaptic firing rates in the range of 2.3 to 2.6 Hz when secondary synapses were set to 90% thresh-$g_{syn}$ (Springer et al. 2015). This is only slightly lower than the 2.9 Hz average firing rate reported for rat vasomotor sympathetic neurons in vivo (Bratton et al. 2010). Further comparison of the two approaches reveals the limitations of each. Bratton et al. (2010) estimated that individual synapses fired at 1.38 Hz and that 2 or 3 synapses were active in most cells. Dividing $f_{post}$ by $f_{pre}$ indicates that the in vivo synaptic gain was 2.1, which is slightly lower than estimated by Springer et al. (2015). However, the microelectrode damage associated with in vivo recording would lead to an underestimate of gain (Springer et al. 2015). In addition, counting active synapses in vivo relied on distinctions in EPSP amplitude, which can be hampered by the fact that they fluctuate from one response to the next and sometimes straddle threshold. By contrast our model for this study may overestimate the number of active synapses and underestimate their strength by holding them fixed at a value well below threshold. Despite these uncertainties concerning the exact number of active synapses in living animals and their precise strength, our approach successfully approximated in vivo postsynaptic firing rates and estimates of synaptic gain.

Virtual $I_h$ as a function of voltage and time was implemented by equation 2,

$$I_h(V,t) = \overline{g_h} * m_t * (V_m - E_{rev})$$  (2)

where $\overline{g_h}$ was the maximum h-conductance, which could be set to any value through the G-clamp software, and $m_t$ was the level of activation at a given time, which varied between 0 and 1. The value of $m_t$ was initialized by calculating it over a 10 second period using the resting
potential and the steady-state activation ($m_\infty$) relation for $g_h$ and then connecting the output to the
current injection circuitry of the recording amplifier. Thereafter, $m_t$ was updated during each 50
$\mu$s time step ($\Delta t$) of the dynamic clamp feedback loop with the following logic. If $m_\infty$ at time
step $t$ was greater than $m_\infty$ at time step $t-1$, then $m_t$ was calculated as $m_t = m_{t-1} + (\Delta t \times (m_\infty - m_{t-1})$
/ $\tau_{activation}$), where $\tau_{activation}$ is the time constant for activation. Alternatively, if $m_\infty$ at time step $t$
was less than $m_\infty$ at time step $t-1$, then $m_t$ was calculated as $m_t = m_{t-1} + (\Delta t \times (m_\infty - m_{t-1}) /$
$\tau_{deactivation}$), where $\tau_{deactivation}$ is the time constant for deactivation. Functions for $m_\infty$, $\tau_{activation}$ and
$\tau_{deactivation}$ are presented along with the underlying data in the Results section.

Analysis

Curve fitting was done using Igor Pro 6.3 (Wavemetrics, Lake Oswego, OR) and statistical
comparisons were made using GraphPad InStat (LaJolla, CA). Grouped data are expressed as
the mean ± SEM.
RESULTS

Expression of HCN subunits in the SCG

Standard RT-PCR detected mRNAs for all four HCN isoforms in the SCG of adult rats (Fig. 1A). NPY was included in the experiment as a positive control. Quantitative PCR using a second set of primers (Table 1) also detected all four HCN isoforms. Melt curves for qPCR reactions confirmed that each amplified a single reproducible peak. Correcting the Ct data for amplification efficiency and normalizing to GAPDH expression suggested that HCN1 and HCN2 were present in higher abundance than HCN3 and HCN4 (Fig. 1B). However, none of the differences in HCN levels were statistically significant (ANOVA).

Voltage clamp analysis of $I_h$

$I_h$ was observed in all neurons where stable perforated patch recordings were obtained. Figure 2A illustrates currents recorded from one cell at 35° C. Jumping the holding potential ($V_{hold}$) from -40 to -120 mV in 10 mV hyperpolarizing steps activated an inward current that became faster and larger with increasing hyperpolarization. The protocol employed shorter steps at more negative potentials because they were sufficient to achieve steady state activation of $I_h$ and avoided the instability of currents sometimes engendered during prolonged large hyperpolarizations. The inward relaxation currents produced by hyperpolarization were blocked by bath application of 10 to 15 µM ZD7288. $I_h$ was taken as the difference between currents measured in the absence and presence of ZD7288. During wash in of ZD7288 the resting membrane potential measured under current clamp hyperpolarized from $-55.7 \pm 1.4$ mV to $-60.4 \pm 1.6$ mV (n=16; P=0.0002, paired t-test), thus indicating that $I_h$ is active in resting sympathetic
neurons. The action of ZD7288 did not reverse during 1 hour of washing. The magnitude of $I_h$ was reduced >50% when measured at room temperature (22° C) (not shown).

After conditioning voltage steps to activate $I_h$, the membrane potential was jumped to -100 mV in order to obtain a family of tail currents having the same driving force (Fig. 2A). To determine the voltage dependence of steady-state activation ($m_\infty$), the amplitudes of individual tail currents were measured, normalized (equation 3) and plotted as a function of voltage (Fig. 2B).

$$m_\infty = (I - I_{\text{min}}) / (I_{\text{max}} - I_{\text{min}})$$  \hspace{1cm} (3)

$I_{\text{min}}$ is the tail current amplitude after stepping from -120 to -100 mV and $I_{\text{max}}$ is the tail current after stepping from -40 to -100 mV. The voltage dependence of $m_\infty$ was then fit to a Boltzmann relation (equation 4, Fig. 2B).

$$m_\infty(V_m) = 1 / (1 + \exp((V_m - V_{1/2}) / s))$$  \hspace{1cm} (4)

where $V_{1/2}$, the voltage for half-maximal activation, was found to be -73.6 mV and $s$, the slope factor, was found to be 11.7 ($n=18$ neurons). Taking the calculated tip potential of 14 mV into account, we estimate the true $V_{1/2}$ was -87.6 mV.

Time constants for $I_h$ activation ($\tau_{ activation}$) were determined by fitting exponential functions to inward current relaxations during initial steps to different voltages (Fig. 2A). Although two components of activation were seen in a few cells at -100 to -120 mV, this was not the case in the majority of cells. We therefore used a single exponential function to fit all the data and then built an empirical model for $I_h$ activation kinetics as a function of voltage (equation 5, Fig. 2C).

$$\tau_{ activation}(V_m) = 53.5 + 67.7 * \exp((V_m + 120) / -22.4)$$  \hspace{1cm} (5)
Extrapolating this relation to the vicinity of normal resting potentials, $\tau_{activation}$ is 1 second at -60 mV and 1.3 seconds at -55 mV.

The kinetics of $I_h$ deactivation and its reversal potential ($E_{rev}$) were assessed with a protocol (Fig. 3A) that first fully activated $I_h$ by stepping to -120 mV for 1.5 s followed by 10 mV steps to less hyperpolarized potentials (-110 to -40 mV). Unlike the steep voltage dependence of $I_h$ activation kinetics, the voltage dependence of deactivation was very shallow in the range from -90 to -50 mV, with time constants typically between 50 to 100 ms (Figure 3B). The kinetics of $I_h$ deactivation were therefore fit to a simple linear relationship (equation 6).

$$\tau_{deactivation}(V_m) = 40.9 - 0.45 * V_m \quad (6)$$

$E_{rev}$ was estimated from the peak tail current amplitudes, normalized for each cell to the tail current amplitude at -80 mV. The pooled data were fit to a straight line and extrapolated to the x-intercept at -17.6 mV (Fig. 3C). Taking the calculated tip potential of 14 mV into account, we estimate the true $E_{rev}$ of $I_h$ was -31.6 mV.

The fully activated $I_h$ measured at -120 mV showed little dependence upon cell size, as reflected by whole cell capacitance (Fig. 4A). These values were then used to calculate the maximum $h$-conductance (equation 7).

$$\overline{g_h} = \frac{I_h(-120mV)}{(V_m - E_{rev})} \quad (7)$$

In 18 neurons, $\overline{g_h}$ was $1.2 \pm 0.1 \text{ nS}$ (range 0.7 to 3.1 nS) (Figure 4B).

$I_h$ enhances synaptic strength

After completing the voltage-clamp analysis, additional experiments were done to test whether $I_h$ alters the efficacy of fast EPSPs. Using the dynamic clamp method we measured the effect of ZD7288 upon thresh-$g_{syn}$. Figure 5A illustrates an example from a cell where blocking $I_h$ with
15µM ZD7288 doubled the amount of virtual synaptic conductance required to initiate an action potential. This indicates that h-channels normally act to enhance the strength of subthreshold EPSPs. In a group of 16 neurons (Fig. 5B) adding 10 to 15µM ZD7288 increased thresh-gsyn from 7.0 ± 0.9 nS to 12.1 ± 2.0 nS (P<0.0001, Wilcoxon matched-pairs signed-ranks test). As in the initial experiments (previous section), ZD7288 hyperpolarized Vrest in this group of cells from -54.4 ± 1.0 mV to -59.2 ± 0.9 mV (Fig. 5C; n=16; P<0.0001, paired t-test). There was a strong negative correlation (r = -0.829, P<0.0001) between the relative increase in thresh-gsyn produced by blocking gh and the associated hyperpolarization of Vrest (Fig. 5D). One might imagine that the increase in total membrane conductance produced by tonic activation of gh in a resting cell would create a shunt that reduces synaptic efficacy by reducing the size of fast EPSPs, but this not the case. Because gh produces a depolarizing shunt that is excitatory due to its reversal potential being positive to spike threshold, the resulting inward Ih depolarizes Vrest. By lowering the amount of synaptic conductance needed to fire an action potential, gh increases the efficacy of fast EPSPs

Ih enhances synaptic amplification

One would expect that strengthening of subthreshold secondary synapses by Ih should also enhance summation of subthreshold EPSPs, enabling them to drive more spikes and thereby to increase synaptic gain. To test this hypothesis using dynamic clamp, we stimulated neurons for 40 seconds with a conductance template that contained a pattern of virtual synaptic events designed to mimic the physiological convergence of strong and weak synapses onto rat SCG neurons (Springer et al. 2015) and to produce a realistic postsynaptic firing rate (Bratton et al. 2010). Thresh-gsyn was measured in each cell and used to calibrate the template so that it
contained one primary synapse at 300% thresh-$g_{\text{syn}}$ and eight secondary synapses, each at 60% thresh-$g_{\text{syn}}$. Every synapse fired at an average rate of 1cHz in a noisy bursting pattern entrained to the cardiac cycle (see Methods). Figure 6A illustrates a 5 second sample from one such experiment. The lower trace shows the bursty synaptic conductance template and the upper traces show the postsynaptic responses measured before and after blocking $I_h$ with 15 µM ZD7288. An important feature of this approach is that the identical complex conductance template can be applied repeatedly and changes in the response can be assessed for each synaptic event. In this case, inspection of the records revealed a clear example of a secondary EPSP triplet that generated an action potential under control conditions, but failed to do so in ZD7288 (Fig. 6A, right panel). This contrasted with an adjacent primary EPSP that remained suprathreshold in strength when $I_h$ was blocked. The overall reduction in postsynaptic spike output during the entire 40 second trial was quantitated and used to measure synaptic gain ($f_{\text{post}}/f_{\text{pre}}$). In 7 neurons tested this way, blocking $I_h$ reduced synaptic gain (Fig. 6B) from 2.2 ± 0.1 to 1.7 ± 0.2 (P=0.0008, paired t-test). This corresponds to a reduction of the average postsynaptic firing rate from 2.2 to 1.7 Hz and it indicates that postsynaptic h-channels have the capacity to elevate use-dependent synaptic gain in sympathetic ganglia.

A notable feature of our model used to create dynamic clamp templates was that it generated synaptic activity in a subset of cardiac cycles (Fig. 6A). Since only some virtual EPSPs reached threshold, action potentials were triggered in an even smaller subset of cardiac cycles. Occasionally 2 spikes were seen during a single cardiac cycle (Fig. 6A, right panel). These observations recapitulate aspects of intracellular recordings from vasoconstrictor neurons in anesthetized rats (Bratton et al. 2010) and extracellular single unit recordings from awake healthy human subjects (Macefield 2011). Indeed, changes in vasoconstrictor sympathetic
activity are often characterized in the human microneurography literature in terms of the proportion of cardiac cycles where spikes are generated. We therefore analyzed our data to determine the effect of I\textsubscript{h} blockade and to compare it with human data from 33 vasoconstrictor neurons (see Fig 3F in (Macefield 2011)). Each of the 7 cells used to measure synaptic gain had been stimulated with 40 second templates containing 200 cardiac cycles. We found that spikes occurred in just under 40% of the cycles (Fig. 6C) and that double spikes occurred in <5% of the cycles. We only observed one instance of a triple spike and never saw a quadruple spike. Consistent with the reduction of synaptic gain produced by ZD7288 (Fig. 6A,B), blocking I\textsubscript{h} caused an increase in the proportion of cardiac cycles with no spikes and decreases in the proportion of cycles with 1 or 2 spikes. Perhaps more interesting, the distribution of spikes per cycle that we observed was quite similar to the human data described by Macefield (2011), which are replotted in Figure 6C.

**Shifting the voltage dependence of g\textsubscript{h} modulates synaptic gain**

In other systems, cyclic AMP (cAMP) modulates h-current by binding directly to HCN channels and shifting the voltage-dependence (V\textsubscript{1/2}) of activation (Biel et al. 2009; Craven and Zagotta 2006). Raising cAMP can shift V\textsubscript{1/2} by up to 20 mV in the depolarizing direction (Chen et al. 2001; DiFrancesco and Tortora 1991; He et al. 2014; Ishii et al. 1999; Ludwig et al. 1998) and lowering cAMP can shift V\textsubscript{1/2} by up to 10 mV in the hyperpolarizing direction (DiFrancesco and Tromba 1988). To test whether such a mechanism could possibly alter synaptic gain in sympathetic neurons we designed an experiment in which native g\textsubscript{h} was blocked with ZD7288 and then reconstituted with a virtual g\textsubscript{h} using dynamic clamp (Methods).
The ability of virtual $g_h$ to mimic native $g_h$ was verified by simulating h-currents in real time using the G-clamp system in an open loop configuration. After setting $g_h$ to 1.5 nS, voltage clamp protocols were applied to the dynamic clamp through the $V_m$ input and the calculated current outputs were monitored. Figure 7 illustrates the simulated currents obtained using the same protocols that had been employed to measure activation and tail currents (Fig. 2A) and deactivation (Fig. 3A). Apart from the noise associated with physiological currents, the simulations reproduced all salient features of $I_h$ in SCG neurons.

In recordings from 8 neurons, it required 1 to 2.5 nS of virtual $g_h$ ($V_{1/2} = -73.6$ mV) to restore $V_{rest}$ to its initial value prior to addition of ZD7288. Importantly, the virtual $g_h$ also restored thresh-$g_{syn}$ ($6.0 \pm 0.8$ nS with native $g_h$, $6.0 \pm 1.1$ nS with virtual $g_h$; n=8; P=0.7422, Wilcoxon matched-pairs signed-ranks test), thus providing further validation of the model $g_h$.

The value of thresh-$g_{syn}$ was then used to scale the same 40 second long noisy bursting pattern of virtual synaptic activity described in the previous section. With this stimulus template, we first measured synaptic gain with $V_{1/2} = -73.6$ mV and then with $V_{1/2}$ shifted in 10 mV steps to -63.6 mV, -53.6 mV, and -83.6 mV. Figure 8A illustrates 6.5 second segments from one cell showing that shifting $V_{1/2}$ to -63.6 mV increased the spike output and shifting it to -83.6 mV decreased the spike output. Regardless of the starting point, depolarizing 10 mV shifts in $V_{1/2}$ always increased synaptic gain in 18 of 18 comparisons made in 8 neurons. Normalizing the synaptic gain data from each cell to the value at $V_{1/2} = -73.6$ mV and combining the results showed that depolarizing $V_{1/2}$ by 10 and 20 mV increased synaptic gain by $19.2 \pm 3.2\%$ (n=7) and $40.6 \pm 6.1\%$ (n=8), respectively (Fig. 8B). Hyperpolarizing $V_{1/2}$ by 10 mV reduced synaptic gain by $11.0 \pm 1.5\%$ (n=5). Together, these results support the possibility that signaling through cyclic
nucleotides in sympathetic neurons may fine tune synaptic amplification by modulating the excitatory strength of nicotinic synapses that are normally subthreshold in strength.
DISCUSSION

Results from this study show that the rat SCG expresses all four HCN subunit isoforms (Fig. 1A) and that functional expression of $I_h$ enhances postsynaptic excitability in a way that strengthens fast EPSPs (Fig. 5A,B) and enhances synaptic amplification (Fig. 6). Our results (Figs. 2,3) also confirm previous observations of $I_h$ in paravertebral sympathetic neurons (Jobling and Gibbins 1999; Lamas 1998; Lamas et al. 1997; Tokimasa and Akasu 1990) and the conclusion that $g_h$ is partially active in cells at rest and thereby serves to depolarize $V_{rest}$ (Fig. 5C) by almost 5 mV (Lamas 1998). Unlike the previous studies of sympathetic neurons, the present experiments were performed at physiological temperature and focused on synaptic integration.

**Heterogeneity of HCN subunit expression**

All four HCN isoforms were detected by standard RT-PCR and by qPCR (Fig. 1) using two sets of primers directed at different exon spanning domains (Table 1). This result is similar to an earlier analysis that also detected all four subunits in myenteric neurons of the mouse distal colon (Xiao et al. 2004). The earlier study concluded that HCN3 was barely detectable and that HCN1 and HCN2 were present at higher levels than HCN4. More detailed studies of subunit expression at the single cell level will be required in order to identify the subunits that encode $I_h$ in sympathetic SCG neurons, to determine whether the channels are homomeric or heteromeric and to identify possible differences between h-channels in functional subtypes of sympathetic neurons.

$\overline{g_h}$ is small, but powerful

We found that $\overline{g_h}$ ranged from 0.7 to 3.1 nS (Fig. 4), did not vary with cell size and generated currents that were typically no more than one or two hundred pA when fully activated (Figs. 2,
3). The small size of $I_h$ compared to other voltage-gated currents in sympathetic neurons, which can be nA in magnitude, helps to explain why they have not drawn much attention in the past. Most studies of sympathetic ganglia have employed microelectrodes, which introduce shunts on the order of 3 to 10 nS that can distort excitability and mask the influence of $I_h$ (Springer et al. 2015). Giga-seal patch recordings like those employed here result in much higher input resistances than in microelectrode recordings and thereby allow small currents, like $I_h$, to exert powerful effects on excitability. This enabled us to observe the increase in thresh-$g_{syn}$ when $g_h$ was blocked with ZD7288 (Fig. 5A,B).

**Implications for synaptic amplification, neuromodulation and blood pressure control**

The ability of $g_h$ to enhance synaptic amplification is a natural consequence of the reduction in thresh-$g_{syn}$, as predicted by synaptic gain theory (Karila and Horn 2000; Kullmann and Horn 2006; Springer et al. 2015; Wheeler et al. 2004). When synaptic amplification is greater than 1 (Fig. 8), it signifies that the number and frequency of action potentials leaving the ganglion is greater than that of spikes entering the ganglion. Under the conditions of our experiments gain resulted in an approximate doubling of the firing rate. Blocking $I_h$ reduced gain from 2.2 to 1.7. This amplification may be especially important in baroreceptor entrained sympathetic neurons because they are embedded in a negative feedback loop that uses gain to control blood pressure.

Future examination of synaptic amplification in sympathetic ganglia should recognize that many gaps remain in our understanding of this system. Bratton et al. (2010) have suggested that the synaptic activity they observed in anesthetized rats may be driven by only 2 or 3 synapses, not the full complement of 8 or 9 synapses in our model of synaptic convergence. However, the *in vivo* analysis was constrained by the need to infer convergence from indirect
measures such as EPSP amplitudes. Such efforts are complicated by the fluctuations that naturally occur in EPSP size. In addition, our model used synapses of fixed strength – how would their efficacy change if they fluctuated in size? Despite the simplifying assumptions built into our model, it was capable of generating realistic postsynaptic firing rates and realistic distributions of spikes per cardiac cycle. Might it be possible in future to design stronger mechanistic predictions using our experimental approach to synaptic amplification that could then be tested in single unit studies of human vasoconstrictor neurons?

Finally, the present study suggests that HCN channels may contribute to the regulation of ganglionic synaptic gain through their expression level and through modulation by binding of cyclic nucleotides. When cyclic nucleotide mediated shifts in $g_h$ activation were incorporated into our dynamic clamp model of virtual $g_h$, it produced clear shifts in synaptic gain (Fig. 8). Additional experiments will be needed to determine whether catecholamines and neuropeptides that stimulate and inhibit cyclic nucleotide accumulation in sympathetic neurons produce significant modulation of synaptic gain that results from changes in $g_h$. 


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DISCLOSURES

No conflicts of interest are declared by the authors

AUTHOR CONTRIBUTIONS

J.P.H. designed the project. K.M.S., K.L.C. and I.A. did the PCR analysis. P.H.M.K. and M.G.S. did the electrophysiology experiments. All authors contributed to experimental design, data analysis, to preparation of figures and to writing the manuscript. All authors approved the final version of the manuscript.
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Tompkins JD, Lawrence YT, and Parsons RL. Enhancement of Ih, but not inhibition of IM, is a key mechanism underlying the PACAP-induced increase in excitability of guinea pig intrinsic cardiac neurons. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R52-59, 2009.


Figure Legends

Fig. 1. Detection of all four HCN isoforms in SCG homogenates. A Standard RT-PCR included NPY as a positive control. 100 bp size ladder in the left lane. B Normalized qPCR data from SCGs taken from four rats (mean ± S.E.). Samples from different animals were used in A and B.

Fig. 2. Steady-state activation and activation kinetics of Ih in rat SCG neurons. A Families of membrane currents recorded under voltage clamp from one SCG neuron. The same voltage protocol, illustrated in the lower left of the panel, was used to measure currents in normal saline (left) and after addition of 15 µM ZD7288 (middle). Ih was isolated by subtracting the two sets of records (right). B The inverse sigmoidal relation that describes steady-state activation (m∞) as a function of voltage was determined by plotting the amplitudes of normalized tail currents measured at -100 mV (equation 3 in text). The data from 18 neurons were fit to a Boltzmann relation (solid line, equation 4) with V1/2 = -73.6 mV. C Exponential time constants for activation as a function of voltage from -80 to -120 mV were determined by single exponential fits at the onset of current relaxations. The voltage dependence of τactivation data were then fit (solid line) to an exponential function (equation 5). Open diamonds in panels B and C are data from individual cells. Filled diamonds are the means.

Fig. 3. Deactivation kinetics and reversal potential of Ih in rat SCG neurons. A Each family of membrane currents was in response to the voltage protocol shown at the lower left of the panel. This is a different neuron than shown in Fig. 1. Currents were measured in normal saline (left) and after addition of 15 µM ZD7288 (middle). Difference currents (right) are taken as Ih (right).
The time constant for deactivation (\(\tau_{\text{deactivation}}\)) was determined by fitting a single exponential to the current relaxations after stepping from -120 mV to test voltages ranging from -50 to -90 mV. The voltage dependence of \(\tau_{\text{deactivation}}\) was fit to a straight line (equation 6). The reversal potential of \(I_h\) was determined by plotting instantaneous current amplitudes after stepping from -120 mV to test voltages ranging from -40 to -110 mV. Data in each neuron was normalized to the response at -80 mV. A straight line fit to the data predicts an extrapolated reversal potential of -17.6 mV. Open diamonds in B and C are data from 18 neurons. Filled diamonds show the mean ± SEM.

**Fig. 4.** Summary of maximum \(I_h\) and \(g_h\) in 18 SCG neurons. A The maximum \(I_h\) measured at -120 mV varied little as a function of whole cell capacitance. B Values for \(g_h\) were tightly clustered between 0.7 and 1.7 nS per cell. Diamonds denote individual cells with the mean ± SEM plotted to the right.

**Fig. 5.** \(I_h\) strengthens virtual nicotinic synapses by lowering thresh-\(g_{\text{syn}}\). A An example of virtual EPSPs that straddle threshold before and after adding ZD7288 to block \(I_h\). Thresh-\(g_{\text{syn}}\) doubles in the absence of \(I_h\). B Similar data from 16 neurons. C Blocking \(I_h\) also hyperpolarizes \(V_{\text{rest}}\). D The reduction of thresh-\(g_{\text{syn}}\) by ZD7288 is proportional to the change in \(V_{\text{rest}}\).

**Fig. 6.** \(I_h\) increases synaptic gain. A Neurons were stimulated with a pattern of virtual synaptic conductance (\(g_{\text{syn}}\)) that reflected the convergence of strong and weak synapses firing in a noisy burst pattern (see text and Methods for details). Lower panels illustrate the \(g_{\text{syn}}\) command.
template applied to the dynamic clamp. Upper panels illustrate the neuronal response before and
after addition of ZD7288. The traces at left show a 5 second segment from a longer 40 second
stimulation sequence. Asterisks at the top denote 4 action potentials that disappeared when I_h
was blocked. Each of these spikes was driven in control saline by summation of weak
subthreshold EPSPs (60% thresh-gsyn). By contrast spikes initiated by individual strong synapses
(300% thresh-gsyn) were impervious to I_h blockade. The dashed box at left of panel A highlights
a sequence whose time course has been expanded at the right. In it one can see a triplet of virtual
secondary EPSPs that reach threshold and fire an action potential in control saline, but fail to do
so in ZD7288. B Synaptic gain was calculated by measuring the average rate of postsynaptic
firing (f_post) during 40 seconds of stimulation and dividing it by the average presynaptic firing
rate used to construct the presynaptic template (f_pre = 1 Hz). In 7 of 7 neurons tested this way,
inhibiting I_h reduced synaptic gain. C In each of the 7 cells used to measure synaptic gain,
spikes in each cardiac cycle were counted and used to plot the distribution of spikes per cardiac
cycle. Comparison human data for vasoconstrictor neurons is replotted from Macefield (2011).

Fig. 7. Families of h-currents simulated in real time using the G-clamp dynamic clamp system
with G_h set to 1.5 nS and 20 kHz temporal resolution. A h-currents elicited by the same protocol
used in Fig. 2 to measure the voltage dependence of steady-state activation (m_infty) from tail
currents and the voltage dependence of activation kinetics (tau_activation). B h-currents elicited by
the same protocol used in Fig. 3 to measure the voltage dependence of deactivation (tau_deactivation)
and the reversal potential (E_rev) for I_h. As expected the calculated currents replicated the form of
physiological currents.

Fig. 8. Shifting the voltage dependence of steady-state I_h activation modulates synaptic gain.
Example of an experiment where native gh was blocked with ZD7288 and then reconstituted with virtual gh at three different levels of V_{1/2}. Each set of records shows the same 6.5 second segment from a longer 40 second sequence. The dynamic clamp command signal (bottom row) was constructed to contain converging strong (300% g_{syn}) and weak (60% g_{syn}) synapses firing in a noisy burst pattern at average frequencies of 1 Hz. The inward virtual I_h generated by the dynamic clamp (middle row) became larger as V_{1/2} was moved from -83.6 mV to -63.6 mV. Note that inward currents have a positive sign because they follow the standard convention for current clamp data. As a consequence of activating more I_h at rest, shifting V_{1/2} in the depolarizing direction also depolarized V_{rest} by a few mV. This can be seen in the membrane potential records (top row). In these data one can also see changes in the number of action potentials driven by summation of weak EPSPs (asterisks) – 3 spikes when V_{1/2} = -83.6 mV, 5 spikes when V_{1/2} = -73.6 mV and 8 spikes when V_{1/2} = -63.6 mV. This indicates that shifting V_{1/2} in the depolarizing direction has the effect of increasing synaptic gain.

Grouped data summarizing the effect of shifting V_{1/2} upon synaptic gain. In each cell, the gain values were normalized to the gain observed when V_{1/2} = -73.6 mV. The n for each measurement is denoted in the graph.
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Table 1 – Primer sets used for PCR detection of HCN and control mRNAs.
A control ZD7288 control - ZD7288
deactivation

B

C

-17.6 mV
evaculated $E_{rev}$
A

$\text{cell size (pF)}$

$\text{maximum } g_h (\text{nS})$

$I_{\text{h},-20\text{mV}}$ (pA)

B

$\text{maximum } g_h (\text{nS})$
Figure A shows the changes in membrane potential ($V_m$) and synaptic conductance ($g_{syn}$) for control and ZD7288 treatments. Figure B illustrates the relationship between threshold synaptic conductance ($thresh-g_{syn}$) and control compared to ZD7288. Figure C displays the variation in resting potential ($V_{rest}$) with control and ZD7288. Figure D presents the ratio of threshold synaptic conductance ($ZD7288 / control$) against the change in resting potential ($\Delta V_{rest}$).
A

\[ V_{1/2} = -83.6 \text{ mV} \]

\[ V_{1/2} = -73.6 \text{ mV} \]

\[ V_{1/2} = -63.6 \text{ mV} \]

B

<table>
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