Activation state of the hyperpolarization-activated current modulates temperature-sensitivity of firing in locus coeruleus neurons from bullfrogs

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Santin JM, Hartzler LK. Activation state of the hyperpolarization-activated current modulates temperature-sensitivity of firing in locus coeruleus neurons from bullfrogs. Am J Physiol Regul Integr Comp Physiol 308: R1045–R1061, 2015. First published April 1, 2015; doi:10.1152/ajpregu.00036.2015.—Locus coeruleus neurons of anuran amphibians contribute to breathing control and have spontaneous firing frequencies that, paradoxically, increase with cooling. We previously showed that cooling inhibits a depolarizing membrane current, the hyperpolarization-activated current (Ih) in locus coeruleus neurons from bullfrogs, Lithobates catesbeianus (Santin JM, Watters KC, Putnam RW, Hartzler LK. Am J Physiol Regul Integr Comp Physiol 305: R1451–R1464, 2013). This suggests an unlikely role for Ih in generating cold activation, but led us to hypothesize that inhibition of Ih by cooling functions as a physiological brake to limit the cold-activated response. Using whole cell electrophysiology in brain slices, we employed 2 mM Cs+ (an Ih antagonist) to isolate the role of Ih in spontaneous firing and cold activation in neurons recorded with either control or Ih agonist (cyclic AMP)-containing artificial intracellular fluid. Ih did not contribute to the membrane potential (Vm) and spontaneous firing at 20°C. Although voltage-clamp analysis confirmed that cooling inhibits Ih, its lack of involvement in setting baseline firing and Vm precluded its ability to regulate cold activation as hypothesized. In contrast, neurons dialyzed with cAMP exhibited greater baseline firing frequencies at 20°C due to Ih activation. Our hypothesis was supported when the starting level of Ih was enhanced by elevating cAMP because cold activation was converted to more ordinary cold inhibition. These findings indicate that situations leading to enhancement of Ih facilitate firing at 20°C, yet the hyperpolarization associated with inhibiting a depolarizing cation current by cooling blunts the net Vm response to cooling to oppose normal cold-depolarizing factors. This suggests that the influence of Ih activation state on neuronal firing varies in the poikilothermic neuronal environment.

temperature; Q10; neuronal excitability; hyperpolarization-activated current; respiratory control

The American bullfrog, Lithobates catesbeianus, has a broad geographical distribution within North America and undergoes rapid and variable temperature changes throughout a single day (21, 49). Owing to the temperature sensitivity of neurophysiological mechanisms, changes in temperature pose a challenge to regulating neurally controlled behaviors, like breathing, in the bullfrog and other poikilothermic animals. Despite rate increases of neurally controlled, rhythmic behaviors typically associated with temperature (41), aspects of the respiratory pattern are maintained across temperatures in amphibians and other ectothermic vertebrates. Specifically, breathing frequency has low temperature dependence (Q10 ~ 1.7) during changes at higher temperatures (2, 42), and tidal volume (i.e., the volume of air consumed in a breath) is insensitive to variations in temperature (27, 51). Consistent with these observations in vivo, respiratory-related cranial nerve activity of the isolated bullfrog brain stem in vitro maintains burst frequency across higher temperatures, and the duration and amplitude of each burst (i.e., neural correlate of tidal volume) do not depend on temperature (32). Therefore, the central respiratory control system may be equipped with mechanisms that offset the effects of temperature to preserve respiratory effort across temperatures relevant for lung ventilation in amphibians.

The locus coeruleus (LC) is one brain stem region involved in control of breathing (12) that contains neuronal activity consistent with a role in temperature compensation of respiratory parameters. Thus far, the LC of adult amphibians is the only discrete brain nucleus described with an unequivocal role in modulation of breathing in vivo (34), specifically, as a central chemoreceptor. Stimulation of the LC by focal acidification leads to increases in breathing, while ablation results in reduced ventilatory response to hypercarbia (34). Furthermore, LC neurons of bullfrogs appear to directly sense CO2/pH (46). We recently demonstrated that CO2/pH chemoreceptive neurons within the LC (~90% of neurons) counterintuitively exhibit intrinsic spontaneous firing rates that vary inversely with temperature (i.e., neurons increase firing frequencies when cooled and decrease firing frequencies when warmed) (47). Intriguingly, only chemoreceptors show these responses to temperature changes, but CO2/pH-insensitive neurons within or outside the LC do not. Because chemosensitive LC neurons are part of the respiratory network of amphibians (34), firing frequencies that vary inversely with temperature present a plausible neuronal mechanism to oppose temperature-proportional rate effects that presumably act on other aspects of the respiratory control system. Understanding the mechanisms that underlie and/or modulate cold-enhanced/warm-depressed LC activity could provide insight into how temperature interacts with the respiratory control system to regulate breathing across temperatures in amphibians and other ectothermic vertebrates.

Two ionic mechanisms have been proposed to determine cold activation in LC neurons from bullfrogs, which include the activation of a putative cold-induced depolarizing current (Icold) and the inhibition of a hyperpolarization-activated current (Ih) (47). Here we investigate Ih. Ih is an inward (depolarizing) Na+/K+ current carried by hyperpolarization-activated cyclic nucleotide gated (HCN) channels. Consistent with evidence that cooling inhibits Ih (11, 37, 40), we found cooling eliminated the depolarizing voltage “sag” caused by activation of Ih during negative current injection (47). Inhibition of an excitatory membrane current associated with pacemaking is seemingly at odds with our findings that LC neurons intrinsically increase spontaneous discharge at cold temperatures. This suggests that a role for Ih in the generation of cold activation is...
 unlikely, but implies that inhibition of this depolarizing current by cooling may function to dampen or limit the magnitude of cold activation in LC neurons. For this hypothesis to be supported, Ih would have to, first, be inhibited by cooling and, second, contribute to spontaneous firing in LC neurons under resting conditions. We therefore tested three specific hypotheses: 1) cooling inhibits Ih in LC neurons from bullfrogs; 2) Ih contributes to spontaneous firing; and 3) inhibition of Ih due to cooling negatively regulates the magnitude of cold-induced firing. To address hypothesis 1, we used whole cell voltage clamp to characterize the electrical properties, pharmacology (antagonists and agonists), and temperature sensitivity of Ih. Using whole cell current clamp combined with a pharmacological inhibitor approach, we next tested hypothesis 2 by elucidating the role of Ih in generation of spontaneous firing by measuring action potential firing frequency in two experimental groups of LC neurons: one group recorded with control artificial intracellular fluid (ICF) in the patch pipette, and the other containing elevated cAMP to study Ih in the activated state. Finally, we tested hypothesis 3 using the same approach to assess the ability of Ih to influence cold activation.

METHODS

Preparation of Brain Stem Slices

Adult bullfrogs, Lithobates catesbeianus (N = 38), of either sex were kept in plastic tanks containing 22°C water with access to wet and dry areas, exposed to 12:12-h light-dark cycles, and consumed a diet consisting of crickets. All experiments performed were approved by the Wright State University Institutional Animal Care and Use Committee. Bullfrogs were euthanized by rapid decapitation posterior to the tympanic membranes, and the head was placed in ice-cold artificial cerebral spinal fluid (aCSF; see Solutions section below for composition) bubbled with 97.5% O2 and 2.5% CO2. Following removal of the frontoparietal bone, the brain stem was carefully dissected. Dissection time ranged between 5 and 10 min. One-half of the forebrain was removed and then the brain was attached to an agar block (ventral surface attached to block; rostral side facing down) and cut into 400-μm cross sections using a Vibratome tissue slicer (Leica Microsystems, Buffalo Grove, IL). Brain stem slices containing the LC (45) were given 1 h to recover from slicing in aCSF equilibrated with 80% O2, 1.3% CO2, balance N2 (pH 7.9) at room temperature. Before electrophysiology experiments, the slice containing the LC was transferred to the 1-mL recording chamber, stabilized with a nylon grid, and superfused with 20°C aCSF at rate of ~1–2 ml/min.

Temperature of the chamber was manipulated using a Warner Instruments bipolar in-line temperature controller (model CL-100; Hamden, CT). Because we controlled the temperature with an in-line heater-cooler, the temperature of the solution varied up to 1°C along the longitudinal axis of the chamber. Temperatures (including kinetics of temperature changes) at the location of the slice within the recording chamber were, therefore, determined before experiments under identical experimental conditions, but without the slice present in the chamber to ensure accurate experimental temperatures.

Solutions

aCSF was composed of the following (in mM): 104 NaCl, 4 KCl, 1.4 MgCl2, 7.5 glucose, 40 NaHCO3, 2.5 CaCl2, and 1 NaH2PO4, and equilibrated with 80% O2, 1.3% CO2, and balance N2 (pH = 7.9 at 20°C and 7.8 at 10°C). Although cooling resulted in a minor acidification, firing increases during cooling are not explained by this small pH decrease (46, 47). This pH and CO2/HCO3− combination was chosen because it closely mimics arterial composition in vivo (13). We added either 2 mM cesium chloride (CsCl) (Sigma-Aldrich, St. Louis, MO) or 50 μM ZD-7288 (Tocris, Bristol, UK) to the aCSF to inhibit the Ih. H-89 dihydrochloride hydrate (10 μM) (H-89; Sigma-Aldrich, St. Louis, MO) was included in the aCSF to inhibit protein kinase A (PKA) and possibly several other protein kinases (28). Inhibitors H-89 and ZD-7288 were reconstituted in water and stored in 20 and 40 mM stock solutions, respectively, at −20°C. For experiments using ZD-7288 and H-89, stock solutions were diluted to final concentrations in aCSF.

Electrophysiological Recordings

Whole cell current- and voltage-clamp recordings were acquired as previously described (47). Briefly, 4–7 MΩ pipettes were backfilled with mock ICF containing the following (in mM), 110 potassium-glucuronate, 2 MgCl2, 10 HEPES, 1 Na2-ATP, 0.1 Na2-GTP, 2.5 EGTA, pH 7.2, with KOH, and placed over an AgCl2-coated Ag wire. For experiments that assessed the effects of increased intracellular cAMP (cAMPi) on whole cell currents and voltage, the artificial ICF included 100 μM cAMP. cAMP was provided to the neurons by diffusion through the pipette to ensure that a near-uniform amount of cAMP entered each neuron across experiments. The slice was visualized at ×4 magnification using a Nikon Cool Snap camera and NIS Elements Imaging Software (Nikon, Elgin, IL). The LC was identified by its bilateral location adjacent to the fourth ventricle (45). Neurons within the LC were then visualized and selected for recording at ×60 magnification. A syringe was connected to the headstage by a tube to apply positive pressure through the pipette to keep the tip free of debris. The pipette was positioned adjacent to the soma of the neuron of interest using a micromanipulator (Burleigh PCS 5000; Thorlabs, Newton, NJ). Before entry into the on-cell configuration, the pipette offset was zeroed. Negative pressure was applied until a >1 GΩ seal formed. Rapid, but light suction then was applied by mouth to rupture the seal and enter the whole cell configuration. Changes in membrane potential (Vm) were measured in “current-clamp mode” using an Axopatch 200B amplifier, Digidata 1440A A/D converter, and Molecular Devices P10 Clampex software (Molecular Devices).

Voltage-clamp protocols and analysis. Whole cell voltage-clamp recordings were performed to measure properties of Ih and outward K+ currents also using an Axopatch 200B amplifier and Digidata 1440A A/D converter. To study properties of Ih, a hyperpolarizing step protocol was applied from either −52 mV or −92 mV, depending on the experiment, to −132 or −142 mV [change (Δ) = −10 mV]. To measure outward K+ currents, Vm was held at −82 mV and stepped from −92 to +28 mV (Δ = 10 mV). The reversal potential of Ih (Eh) was estimated as previously described (30). Briefly, we plotted the current-voltage relationship of the instantaneous component of Ih elicited from holding potentials, where Ih is relatively activated (−112 mV) and less activated (−72 mV). The instantaneous component of the current elicited from holding potentials of −72 and −112 mV was fitted with a linear regression. The Vm where these extrapolated linear regressions of the instantaneous components of Ih intersect indicates the Vm where no current passes through the hyperpolarization-activated conductance and thus represents Eh (see Fig. 1, C and D).

Similar to the methods of others (20, 36, 43), steady-state voltage dependence of Ih activation and maximal conductance was determined by eliciting tail currents from a series of prepulse steps (−52 to −142 mV) for 3 s and then measured immediately following a step to −92 mV (see Fig. 3). Measurement of the tail current at the instant of the step to −92 mV was selected because it is near the predicted Nernst potential for K+ (equilibrium potential approximately −86 mV) and negative to activation voltages of other voltage-sensitive channels; therefore, voltage-dependent activation and conductance of Ih is presented with little contamination from other ion channels. Maximal steady-state conductance (Gh,max) under each condition was determined by converting the tail current recorded immediately following the transition to −92 mV from a conditioning pulse at −142 mV into a conductance value using the equation:

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where \( I_{\text{max tail}} \) and \( I_{\text{min tail}} \) are the maximum and minimum tail currents, respectively, measured at \(-92\ \text{mV}\) immediately following the conditioning pulse at \(-142\ \text{mV}\); and \( V_m \) \((-92\ \text{mV})\) subtracted from the average \( E_h \) \((-40.75\ \text{mV};\ \text{determined in this study})\) is the driving force. All tail currents were then normalized \((I_{\text{normalized tail}})\) according to the equation:

\[
I_{\text{normalized tail}} = \frac{I_{\text{tail}} - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}}
\]

where \( R \) is the tail current measured at \(-92\ \text{mV}\) following the step from the prepulse potential; \( R_{\text{min}} \) is the minimum inward tail current; and \( R_{\text{max}} \) is the maximum inward tail current. Normalized tail currents were then fitted to the Boltzmann equation using Clampfit software in the form:

\[
\text{Normalized } G(V_m) = \frac{1}{1 + e^{(V_m - V_{0.5})/k}}
\]

where \( G(V_m) \) represents the fraction of activated \( G_h \) at \( V_m\); \( V_{0.5} \) is the voltage at which the steady-state conductance is half-maximal, and \( k \) is the Boltzmann slope factor.

Series resistance \((R_s)\) was typically \(-20\ \text{M} \Omega\) and was routinely compensated by \(50-70\%\) using the circuitry of the Axopatch 200B amplifier. The largest steady-state \( I_h \) recorded at \(-142\ \text{mV}\) was approximately \(-400\ \text{pA}\); therefore, in our study, a recording with an \( R_s \) of \(20\ \text{M} \Omega\) that underwent \(50-70\%\) compensation \((i.e., \text{effective } R_s = 6-10\ \text{M} \Omega)\) and contained a steady-state \( I_h \) of \(-400\ \text{pA}\) will produce a maximum voltage error of \(2.4-4\ \text{mV}\). These small voltage errors that occurred as a result of the uncompensated \( R_s \) were not corrected. In contrast, maximum outward \((K^+)\) currents were larger \((1-6\ \text{nA} \text{recorded at a command potential of } +28\ \text{mV})\) and will, therefore, suffer from large voltage errors as a result of the \( R_s \). The goal of these experiments was to qualitatively assess the effect of \(2\ \text{mM Cs}^+\) or \(50 \mu\text{M ZD}-7288\) on outward currents to select the most suitable antagonist of \( I_h \) in our preparation. Since \( R_s \) underwent only small and generally acceptable increases throughout the experiment \((\text{mean } \Delta = 1.68 \pm 0.33\ \text{M} \Omega \text{ or } 14.06 \pm 2.75\% \text{ after compensation})\), voltage errors as a result of \( R_s \) were consistent across the control and antagonist treatments in the same neuron; therefore, we did not correct for errors due to \( R_s \). To accurately represent these data in the face of probable voltage errors due to \( R_s \), normalized outward currents are presented as a function of the command potential rather than \( V_m \). All neurons used in experiments had an interspike \( V_m \) more negative than \(-50\ \text{mV}\) and action potential amplitudes \(>60\ \text{mV}\) upon entry into the whole cell configuration. Data were filtered at \(2\ \text{kHz}\) and collected at \(10\ \text{kHz}\). Current and voltage-clamp recordings were analyzed offline using pCLAMP software (Molecular Devices). All voltages from voltage- and current-clamp experiments were corrected for a liquid junction potential of \(12\ \text{mV}\).

Current-clamp protocols and analysis. Action potential firing frequency from current-clamp recordings was analyzed by integrating the trace into 10-s bins. Firing rates for control \((n = 24)\) and \(100 \mu\text{M cAMP} \((n = 24)\) pipette solution were determined by averaging 1 min of integrated firing after an \(-3\-min\) stabilization period upon entry into the whole cell configuration. The 3-min window was provided to allow adequate diffusion of the cAMP into the neuron. Action potential properties of neurons containing control and elevated-cAMP pipette solution were analyzed as follows: threshold voltage, \( V_{\text{th}} \) at the instant of rapid depolarization; depolarization rate, the slope of the rising phase of the action potential from the threshold voltage to the peak, excluding the top and bottom \(10\%\) of the upstroke; repolarization rate, the slope of the falling phase of the action potential from the peak to the threshold, excluding the top and bottom \(10\%\) of the downstroke; half-width, the time elapsed at the \( V_m \) halfway between threshold and peak during depolarization and repolarization; after-hyperpolarization, the most negative \( V_m \) reached following repolarization subtracted from the interspike \( V_m \) and height, voltage difference from the threshold to the peak. After recording the initial firing frequency at \(20\ °\text{C}\), the in-line heater-cooler was used to change the temperature of the bath from \(20\) to \(10\ °\text{C}\). Temperature changes took \(-4\ \text{min}\), and the firing rate of each neuron at \(10\ °\text{C}\) was measured for \(-1\ \text{min}\) once the bath temperature reached \(10\ °\text{C}\).

Recordings of control \((n = 8)\) and cAMP-containing \((n = 6)\) neurons upon exposure to \(\text{Cs}^+\) was measured for \(-1\ \text{min}\) after \(-6\ \text{min}\) of exposure. Each neuron exposed to \(2\ \text{mM Cs}^+\) was then cooled from \(20\) to \(10\ °\text{C}\). In current- and voltage-clamp experiments that used \(\text{Cs}^+\), neurons were not returned to control aCSF. Although the effects of \(\text{Cs}^+\) are often reported to wash out following the return to control solution, pilot experiments revealed that, even \(20\ \text{min}\) after washout of \(\text{Cs}^+\) with control aCSF, \( I_h \) amplitude measured in voltage clamp only recovered to \(-50\%\) of initial values. This reduction was not a result of rundown, because \( I_h \) amplitude remained stable for at least \(20\ \text{min}\) \(\text{(data not shown)}\). A series of experiments was also conducted using the PKA inhibitor, \(\text{H-89}\), to determine any \( I_h \)-independent effects of cAMP on the firing responses we observed. These experiments assessed both firing rates at \(20\ °\text{C}\) and firing rates after the transition to \(10°\text{C}\) in three separate neurons undergoing different treatments from the same slice preparation \((n = 5\ \text{slices})\); neuron 1, control pipette solution with control aCSF; neuron 2, elevated cAMP, in the absence of the PKA inhibitor; and neuron 3, elevated cAMP, following \(-20\)- to \(-30\-min\) incubation of the slice with the PKA inhibitor, \(10 \mu\text{M H-89}, dissolved in the aCSF.

\( V_m \) was determined by measuring the average \( V_m \) of the interspike interval. Input resistance \((R_{in})\) was determined by injecting a series of hyperpolarizing current injections \((-15\text{ to } -60\ \text{pA}; \Delta = -15\ \text{pA})\) into the soma of the neuron for \(500\ \text{ms}\) and measuring the voltage deflection. Since LC neurons contain \( I_h \), depolarizing “sag” occurred during negative current injection; thus we measured the voltage change at steady state and used Ohm’s law to calculate \( R_{in} \) for each current injection. We averaged the \( R_{in} \) determined by each of the four current injections as an estimation of the \( R_{in} \) for the neuron under each condition.

Statistical Analysis

Data are presented as means \(\pm\ \text{SE}\). Statistical analyses were run, and figures were constructed using Graph-Pad Prism 6.01 (GraphPad Software, San Diego, CA). Unless otherwise indicated, \( n \) represents the number of cells included in each analysis. Means between two groups collected from separate neurons were analyzed using an unpaired, two-tailed \( t \)-test. If comparisons were made between treatments on the same neuron, a paired \( t \)-test was used. These statistical tests assume equal standard deviations. Some analyses in this study had \( F \)-statistics that rejected the null hypothesis that populations were heterogeneous. These situations are indicated in the RESULTS and had Welch’s correction applied to the \( t \)-test. In some situations, as indicated in the RESULTS section, a two-way ANOVA was used to determine the effect of treatment group \((\text{e.g., control, cAMP-dialyzed})\) on firing rate under different conditions \((\text{e.g., changing temperature or application of Cs}^+)\). Within-group comparisons were then made using Holm-Sidak’s multiple-comparisons test. Means of three or more groups were analyzed using a one-way ANOVA with Tukey’s post hoc test for multiple comparisons. Pertinent statistical information including, \( P \) values, \( T \) values, \( F \) values, and degrees of freedom are presented in the RESULTS section. Statistical significance was accepted when \( P < 0.05\).

RESULTS

Characterization of the \( I_h \) in Bullfrog LC Neurons

These experiments were undertaken to characterize \( I_h \) and to confirm its similarity to \( I_h \) carried by HCN channels as described in other excitable cells. Figure 1A shows that voltage
relationships of applied a hyperpolarizing step protocol to activate tentative current recordings from a voltage-clamp experiment that typically characterized by its sensitivity to ZD-7288 and low inward rectification as the membrane becomes increasingly steady-state component (solid circle in Fig. 1). The hyperpolarized (Fig. 1A) shows that locus coeruleus (LC) neurons from bullfrogs contain a slowly activating, noninactivating, inwardly rectifying current that slowly activates upon a 3-s step from −52 to −142 mV in voltage clamp. The solid circle in A indicates the time point where the \( I_{\text{steady-state}} \) was measured for each voltage, as plotted in B. C: two families of current recordings elicited from a more positive (−72 mV) and more negative (−112 mV) holding potential (\( V_{\text{hold}} \)) to determine the reversal potential \( (E_h) \) of \( I_h \). \( E_h \) can be estimated by extrapolating the intersection of the instantaneous component of the current evoked from each \( V_{\text{hold}} \). The time point of the instantaneous component used for estimation of \( E_h \) is indicated for currents elicited from −72 mV (○), and for currents elicited from −112 mV (□) \( V_{\text{hold}} \). D: the mean \( E_h \) (−40.7 ± 3.2 mV) as determined by the described method. Values are means ± SE.

We wanted to use these antagonists to determine the role of \( I_h \) in spontaneous firing and cold sensitivity in current-clamp experiments. Unfortunately, in addition to its block of \( I_h \), 2 mM Cs\(^{+}\) may also inhibit certain K\(^{+}\) channels (31). Additionally, ZD-7288 inhibits T-type Ca\(^{2+}\) channels (44), voltage-gated Na\(^{+}\) channels (60), and outward currents (unpublished observation) (10). To assess whether or not Cs\(^{+}\) and ZD-7288 inhibited outward (K\(^{+}\)) currents and select the best blocker of \( I_h \) in LC neurons, we measured their effects on outward currents with depolarizing steps from −92 to +28 mV in voltage clamp. As demonstrated in the representative trace [Fig. 2D (top; representative trace) and Fig. 2E (mean data)], 2 mM Cs\(^{+}\) did not reduce outward currents, which corroborates previous findings from mammalian neurons where Cs\(^{+}\) applied at low millimolar concentrations effectively blocked \( I_h \), but did not reduce outward K\(^{+}\) currents (53). In contrast, Fig. 2D (bottom; representative recording) and Fig. 2F (mean data) shows that ZD-7288 reduced outward currents; thus resulting in greater reduction of outward currents compared with Cs\(^{+}\) (Fig. 2H; \( P = 0.0003; T_\text{ref} = 7.267; \) two-tailed unpaired \( t \)-test). Similar to the observation of Do and Bean (10), this result suggests that ZD-7288 may directly inhibit or indirectly lead to the inhibition of K\(^{+}\) channels responsible for the measured changes in outward currents. However, we cannot exclude the possibility that ZD-7288 specifically blocked \( I_h \) and, as a result, improved the space-clamp conditions in the slice preparation, which led to artifactual reductions in outward current. This possibility, however, seems less likely, because we did not observe a similar decrease in outward current after application of Cs\(^{+}\). We, therefore, decided to use 2 mM Cs\(^{+}\) to block \( I_h \) in subsequent current-clamp experiments.

Fig. 1. Characterization of the hyperpolarization-activated current \( (I_h) \). A (representative \( I_h \) recording) and B [mean steady-state current (\( I_{\text{steady-state}} \))-voltage data; \( n = 11 \)] show that locus coeruleus (LC) neurons from bullfrogs contain a slowly activating, noninactivating, inwardly rectifying current that slowly activates upon a 3-s step from −52 to −142 mV in voltage clamp. The solid circle in A indicates the time point where the \( I_{\text{steady-state}} \) was measured for each voltage, as plotted in B. C: two families of current recordings elicited from a more positive (−72 mV) and more negative (−112 mV) holding potential (\( V_{\text{hold}} \)) to determine the reversal potential \( (E_h) \) of \( I_h \). \( E_h \) can be estimated by extrapolating the intersection of the instantaneous component of the current evoked from each \( V_{\text{hold}} \). The time point of the instantaneous component used for estimation of \( E_h \) is indicated for currents elicited from −72 mV (○), and for currents elicited from −112 mV (□) \( V_{\text{hold}} \). D: the mean \( E_h \) (−40.7 ± 3.2 mV) as determined by the described method. Values are means ± SE.
**I_h** Activation Modulates Temperature Sensitivity

Although we have shown that cooling reduces the hyperpolarization-activated depolarizing "sag" in LC neurons (47), we wanted to directly measure the effects of cooling on the conductance and voltage dependence of I_h activation. Additionally, we intended to study how I_h activation state can influence spontaneous firing and cold sensitivity; therefore, we also assessed the sensitivity of the conductance and voltage-dependent activation to cAMP to validate cAMP as an agonist of I_h (46). When neurons were cooled to 20°C, a 3-s prepulse that sufficiently activated I_h, neurons were then stepped to the test potential of −92 mV to elicit tail currents. After the measurements at 20°C, the neurons were cooled to 10°C. Consistent with the notion that cAMP alters the open-closed probability without influencing single-channel conductance (54), Fig. 3B shows that presence of cAMP does not influence G_h max at either 20 or 10°C (two-way ANOVA; *P* = 0.7511; *F*(1,42) = 0.1021). In contrast, cooling reduced G_h max regardless of cAMP concentration (Holm-Sidak’s multiple-comparisons test; *P* < 0.0001 for control and cAMP; *T*(42) = 4.898 (control); *T*(42) = 4.654).

cAMP typically leads to I_h activation by shifting the voltage dependence of activation to more depolarized potentials (3). A

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**Figure 2.** I_h (I\textsubscript{steady-state}-instantaneous current) is sensitive to 2 mM Cs\textsuperscript{+} and ZD-7288, but ZD-7288 reduces outward currents (I\textsubscript{outward}). A: representative family of I_h traces [V\textsubscript{hold} = −72 mV; −72 to −132 mV; change (Δ) = −10 mV] before and after application of 2 mM Cs\textsuperscript{+} (n = 7; top) and 50 μM ZD7288 (n = 5; bottom). B and C: summary current-voltage (I−V) data of the steady-state I_h recorded in control artificial cerebral spinal fluid (aCSF) (solid circle, Cs\textsuperscript{+} experiments; solid square, ZD-7288 experiments), 2 mM Cs\textsuperscript{+} (shaded circle) or 50 μM ZD7288 (shaded square), and isolation of the Cs\textsuperscript{+} T (open circle) or ZD-7288-sensitive current (open square). D: representative family of I\textsubscript{outward} (K\textsubscript{s}) traces (V\textsubscript{hold} = −92 mV; −92 to +28 mV) before and after application of 2 mM Cs\textsuperscript{+} (n = 4; top) and 50 μM ZD7288 (n = 4; bottom). E and F: summary I−V data of the peak I\textsubscript{outward} at each voltage at the time point marked by the solid circle in D. E: the near superimposed I−V relationship recorded in control aCSF (solid circle) and 2 mM Cs\textsuperscript{+} (shaded circle) demonstrates that there is no 2 mM Cs\textsuperscript{+}-sensitive I\textsubscript{outward} (open circle). F: in contrast, I\textsubscript{outward} elicited in ZD-7288 (shaded square) are reduced compared with currents evoked in aCSF (solid circle), leaving a large ZD-7288-sensitive current (open square). G: summary of mean percent inhibition of the maximum I_h (I\textsubscript{h max}) 2 mM Cs\textsuperscript{+} achieves slightly greater block of I_h compared with ZD-7288 (*P* = 0.0069; two-tailed unpaired *t*-test). H: summary of mean percent inhibition of the maximum I\textsubscript{outward} (I\textsubscript{outward max}) 2 mM Cs\textsuperscript{+} does not inhibit I\textsubscript{outward}, whereas ZD-7288 reduces the I\textsubscript{outward max} by −50% (*P* = 0.0003; two-tailed unpaired *t*-test). Values are means ± SE. *P* < 0.05. **H**

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normalized conductance-voltage relationship in Fig. 3C estimates the fraction of activated $I_h$ at a given $V_m$. To determine the $V_{0.5}$, we fit a Boltzmann function to the normalized conductance curve, as shown in Fig. 3C. $V_{0.5}$ values indicate that, in control pipette solution at 20°C, $I_h$ is half activated ($V_{0.5}$) at $-89.1 \pm 3.0$ mV, and increasing cAMP depolarizes the $V_{0.5}$ to $-74.9 \pm 2.4$ mV [Fig. 3D; one-way ANOVA; $P < 0.0001$; $F_{(3,42)} = 21.27$]. Cooling to 10°C hyperpolarized the $V_{0.5}$ to $-107.1 \pm 2.4$ mV (from $-89.1 \pm 3.0$ mV at 20°C). In the presence of cAMP at 10°C, $V_{0.5}$ was half maximal at $-88.32 \pm 3.89$ mV, compared with $-74.9 \pm 2.4$ mV at 20°C. Consistent with our hypothesis and previous observation that cooling eliminates the depolarizing voltage “sag” (47), we confirm that cooling inhibits $I_h$ by reducing the maximal conductance and hyperpolarizing voltage-dependent activation, with or without cAMP present. In addition, cAMP shifted the $V_{0.5}$ by approximately $+15$ mV, indicating that cAMP activates $I_h$ in IC neurons of bullfrogs. These results suggested that $I_h$ would be more active in neurons dialyzed with cAMP at 20°C, but similarly inhibited at 10°C.

$I_h$ Is Not Active at Rest, but Influences Firing Frequency and $V_m$ When Activated

These next experiments used whole cell current-clamp recordings to characterize and compare spontaneous firing in control pipette solution and in the presence of the $I_h$ agonist, cAMP. Summary data are presented in Fig. 4A. Mean firing...
rates were greater in cAMP-containing neurons (Fig. 4A; P < 0.0001; T_{27.27} = 4.752; unpaired, two-tailed t-test with Welch’s correction). Most LC neurons fired action potentials spontaneously at 20°C with a mean frequency of 0.72 ± 0.12 Hz (n = 23; Fig. 4B). Consistent with enhanced activity of a hyperpolarization-activated depolarizing current (6), Fig. 4B shows that cAMP eliminated most of the action potential undershoot (afterhyperpolarization) in neurons dialyzed with cAMP. Furthermore, the R_{in} that the headstage encounters during a negative current injection allows qualitative insight into whether addition of cAMP increased firing through net channel opening or closing. If cAMP acted predominantly through I_{h} activation, we would expect to observe decreases in R_{in} compared with control. R_{in} was measured in some of the neurons in each group and was reduced in the presence of cAMP [control (n = 20): 530.1 ± 34.1 MΩ vs. cAMP (n = 12): 383.4 ± 26.1 MΩ; P = 0.0051; T_{30} = 3.02; two-tailed t-test]. Other action potential and membrane properties are included in Table 1, and the waveforms are presented in Fig. 4, bottom.

The previous experiments suggest that I_{h} may increase firing frequency when activated. To remove ambiguity associated with population studies and account for the fact that cAMP is not a specific agonist of I_{h}, we isolated the contribution of I_{h} to spontaneous firing and V_{m} in control and elevated cAMP using 2 mM Cs+ at 20°C. If I_{h} contributed to V_{m} and spontaneous firing, we expected that blocking I_{h} with Cs+ would reduce

Table 1. Membrane and action potential properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Control</th>
<th>cAMP</th>
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</thead>
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<tr>
<td>n</td>
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<td>24</td>
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<tr>
<td>Membrane potential, mV</td>
<td>−54.43 ± 0.93</td>
<td>−53.61 ± 1.12</td>
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<tr>
<td>V_{threshold}, mV</td>
<td>−44.95 ± 1.14</td>
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<tr>
<td>Depolarization rate, mV/ms</td>
<td>120.70 ± 5.84</td>
<td>80.04 ± 8.08*</td>
</tr>
<tr>
<td>Repolarization rate, mV/ms</td>
<td>−91.66 ± 4.64</td>
<td>−60.54 ± 5.98*</td>
</tr>
<tr>
<td>Half-width, ms</td>
<td>0.77 ± 0.03</td>
<td>1.11 ± 0.09*</td>
</tr>
<tr>
<td>Afterhyperpolarization, mV</td>
<td>−13.59 ± 0.83</td>
<td>−8.42 ± 1.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. V_{threshold}, threshold voltage. *P < 0.001. †P < 0.0001.
firing frequency and hyperpolarize the membrane. Figure 5A shows an example of an LC neuron before and after the application of 2 mM Cs⁺. Exposing LC neurons containing control pipette solution to 2 mM Cs⁺ had no net effect on firing rate and Vᵐ; however, as demonstrated in Fig. 5B, this response was variable (Fig. 5B; n = 8; P = 0.40; T₇ = 0.7969; two-tailed paired t-test). Cs⁺ also did not alter Vᵐ (Fig. 5C; n = 8; P = 0.4938; T₇ = 0.7218; two-tailed paired t-test).

In contrast to neurons containing control pipette solution, Fig. 5D illustrates that all neurons containing elevated cAMP underwent reductions in firing (Fig. 5E; P = 0.015; T₅ = 3.611; two-tailed paired t-test) and slight membrane hyperpolarization (Fig. 5F; P = 0.03; T₅ = 2.983; two-tailed paired t-test) in response to Cs⁺ application. We conclude that Iₜ does not make little to no contribution to the spontaneous firing and Vᵐ under control conditions. Consistent with our voltage-clamp experiments, cAMP activates Iₜ to enhance firing, presumably through small depolarization of Vᵐ.

Enhancing Iₜ Reverses Cold Activation

The next series of experiments was conducted to determine whether elevated cAMP/Iₜ influences cold activation. Consistent with previous reports (47), LC neurons from bullfrogs increase firing frequency during cooling (Fig. 6A). In 23/24 neurons containing control ICF, firing frequency increased from 0.56 ± 0.08 Hz to 2.19 ± 0.30 Hz (Q₁₀ = 0.37 ± 0.06; mean and Q₁₀ represent all neurons cooled) (Fig. 6B; P < 0.0001; T₄₆ = 4.978; two-way ANOVA with Holm-Sidak’s multiple-comparisons test) upon transition from 20 to 10°C. To test the hypothesis that Iₜ activation would reduce the magnitude of the cold-activated response, neurons containing 100 μM cAMP were exposed to the cooling ramp. In direct contrast with neurons containing control pipette solution, 20/24 (83%) neurons with elevated cAMP decreased firing rates from 1.82 ± 0.33 to 0.72 ± 0.15 Hz during cooling from 20 to 10°C (Q₁₀ = 3.81 ± 0.87; mean and Q₁₀ represent all neurons cooled containing cAMP) (Fig. 6, C and D; P < 0.01; T₄₆ = 3.357; two-way ANOVA with Holm-Sidak’s multiple-comparisons test). Thus cAMP qualitatively changes the response to cooling compared with control [P < 0.0001; two-way ANOVA; F(1.46) = 34.73]. Cooling also resulted in a smaller membrane depolarization in neurons dialyzed with cAMP (Fig. 6E; control: n = 23; 6.50 ± 0.69 mV vs. cAMP: n = 20; 1.55 ± 0.65 mV; P < 0.0001; T₄₁ = 5.266; unpaired, two-tailed t-test). Cooling increased steady-state Rₘ, but Rₘ was not different in neurons containing elevated cAMP (n = 7) compared with control (n = 8) (control: 892 ± 149 vs. 100 μM; cAMP: 825 ± 30 MΩ; P = 0.6739; T₇.₆₇₂ = 0.4374; unpaired, two-tailed t-test with Welch’s correction), suggesting that Iₜ was equally inhibited by cooling whether or not neurons were dialyzed with cAMP. Overall, these results demonstrate that elevated cAMP, potentially through increasing active Iₜ before cooling, leads to reversal of the typical cold response in LC neurons.

**Fig. 5.** Iₜ does not contribute to spontaneous firing and membrane potential (Vᵐ), unless pharmacologically activated by cAMP. A: representative whole cell recording of an LC neuron before and after application of 2 mM Cs⁺, indicating that inhibition of Iₜ does not alter firing rate (FR) and Vᵐ in neurons recorded using control pipette solution. Summary data are shown of the firing frequency (B) and Vᵐ (C) of LC neurons at 20°C before and after inhibition of Iₜ by 2 mM Cs⁺ (n = 8) (P = 0.40; two-tailed paired t-test) and Vᵐ (P = 0.4938; two-tailed paired t-test). The data point indicated by the red hexagon was used to generate the representative recording for neurons recorded with control pipette solution. B: representative whole cell recording of an LC neuron before and after exposure to 2 mM Cs⁺ in the presence of cAMP. In contrast to neurons recorded using control pipette solution, when cAMP is added to the pipette solution, inhibition of Iₜ reduces FR and induces a slight hyperpolarization of the membrane. Summary data are shown of the firing frequency (D) and Vᵐ (E) of LC neurons at 20°C in the presence of 100 μM intracellular cAMP (cAMP) before and after inhibition of Iₜ by 2 mM Cs⁺. The data point indicated by the blue hexagon was used to generate the representative recording for neurons recorded with cAMP pipette solution. Decreases in firing frequency (P = 0.015; paired, two-tailed t-test) and membrane hyperpolarization (P = 0.03; paired, two-tailed t-test) upon inhibition of Iₜ indicate that activation of Iₜ by cAMP contributes to enhanced firing frequencies of LC neurons at 20°C. Values are means ± SE. *P < 0.05; **P < 0.01.
Like the experiments conducted at 20°C, we next used Cs⁺ to isolate the contribution of I₉ to cold-induced firing responses in control and in the presence of the I₉ agonist, cAMP. Since I₉ does not contribute to $V_m$ and firing frequency in control, unsurprisingly, inhibition of I₉ using Cs⁺ did not influence cold activation (0.75 ± 0.27 Hz increase during cooling in control vs. 1.12 ± 0.72 Hz in Cs⁺; $n = 6; P = 0.403; \bar{T}_5 = 0.912$; two-tailed paired $t$-test). However, in support of our hypothesis that I₉ could negatively regulate cold activation, Fig. 7A depicts an example integrated firing rate trace of a neuron containing 100 μM cAMP during two cooling ramps: one in control aCSF, followed by one in the presence of 2 mM Cs⁺. A repeated-measures two-way ANOVA revealed an interaction between temperature and group (presence/absence of Cs⁺) [$P = 0.0024; T_{(1,100)} = 16.14$], indicating that response to cooling is qualitatively different when comparing neurons dialyzed with cAMP with and without Cs⁺. Cooling neurons dialyzed with cAMP ($n = 6$) resulted in reduced firing rates (Fig. 7B; left; 20°C: 1.42 ± 0.34 Hz vs. 10°C: 0.63 ± 0.28 Hz; $P < 0.05; T_{10} = 3.078$; Holm-Sidak’s multiple-comparisons test). In direct contrast, in the presence of Cs⁺, all neurons that previously decreased firing rates during cooling when dialyzed with cAMP responded with increased firing rates during the second cooling ramp (Fig. 7B; right; 20°C: 0.27 ± 0.50 Hz vs. 10°C: 0.94 ± 0.33 Hz; $P < 0.05; T_{10} = 2.604$; Holm-Sidak’s multiple-comparisons test). Reduced firing frequencies induced by cooling that was converted to increased firing frequencies following inhibition of I₉ is summarized in Fig. 7C by

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Fig. 6. I₉ agonist, cAMP, reverses normal responses to cooling in LC neurons. A: bath temperature (top), integrated FR (middle), and action potentials (bottom) from an LC neuron undergoing a cooling ramp in the presence of control intracellular fluid (ICF). The gray dashed line represents the interspike $V_m$ at $-56$ mV. Action potentials are shown from the numbered time points indicated in the integrated FR trace to highlight frequency and $V_m$ changes induced by cooling. Notice that cooling from 20°C to 10°C induces increases in firing frequency that can be reversed upon return to 20°C. B: summary data of FR from neurons at 20°C and 10°C. Each point at 20°C is connected by a line to the FR of the same neuron at 10°C, showing that LC neurons with elevated cAMP undergo reversible decreases in firing and considerably smaller depolarization during cooling from 20°C to 10°C. C: bath temperature, integrated FR, and action potentials from an LC neuron that contains elevated (100 μM) cAMP. The gray dashed line represents the interspike $V_m$ at $-56$ mV. Unlike control ICF, neurons with elevated cAMP undergo reversible decreases in firing and considerably smaller depolarization during cooling from 20°C to 10°C. D: summary data of FR from neurons at 20°C and 10°C containing elevated cAMP. The data point indicated by the blue hexagon was used to generate the representative recording for neurons recorded with cAMP pipette solution. Each point at 20°C is connected by a line to the FR of the same neuron at 10°C, showing that LC neurons with elevated cAMP undergo decreases in firing during cooling ($n = 23; P < 0.0001$; two-way ANOVA with Holm-Sidak’s multiple-comparisons test). The data point indicated by the red hexagon was used to generate the representative recording for neurons recorded with cAMP pipette solution. Each point at 20°C is connected by a line to the FR of the same neuron at 10°C, showing that LC neurons with elevated cAMP undergo decreases in firing during cooling ($n = 20; P < 0.01$; two-way ANOVA with Holm-Sidak’s multiple-comparisons test). E: mean change in $V_m$ in LC neurons containing control and elevated cAMP ICF ($P = 0.0002$; unpaired, two-tailed $t$-test). Values are means ± SE. **$P < 0.01$. ****$P < 0.0001$. 

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highlighting the differences in the change in firing rate evoked by cooling ($P = 0.0040$; $T_5 = 5.041$; two-tailed paired $t$-test). The depolarization due to cooling in these neurons was also significantly greater in the presence of 2 mM Cs$^+$ (Fig. 7D; $P = 0.0007$; $T_5 = 7.395$; two-tailed paired $t$-test).

To eliminate the possibility that the increase in firing rate during the second cooling ramp in the presence of Cs$^+$ is the result of dialysis time and not the inhibition of $I_h$, four additional neurons containing elevated cAMP, underwent two cooling ramps (Fig. 7E). For each neuron, the first and second cooling ramp both led to decreases in firing rate. This control experiment indicates that restoration of cold activation during exposure to Cs$^+$ was, presumably, the result of $I_h$ inhibition and not an artifact due to time or multiple cold exposures. Additionally, neurons ($n = 6$) were also recorded from each slice with normal pipette solution as a positive control for
cold-activated responses. Comparing cold-activated responses of cAMP + Cs⁺ (i.e., the recovered cold-activated response) with control revealed that Ih inhibition was, evidently, sufficient to restore cold activation to near-control levels (Fig. 7F; \( n = 6; P = 0.102; T_{10} = 1.801 \); unpaired two-tailed t-test).

To summarize, these results show that Ih is likely to be closed under normal conditions in LC neurons, which precludes its ability to influence spontaneous firing and cold sensitivity. We expected that inhibition of Ih by cooling could dampen cold sensitivity when Ih was initially contributing to firing rate and \( V_m \), but, to our surprise, neurons underwent a completely opposite response to cooling when Ih was initially activated.

**Ih Density Correlates with Responsiveness to cAMP**

Most neurons (20/24) exhibited cAMP sensitivity (as represented in Fig. 8A1), but four neurons, represented in Fig. 8A2, appeared insensitive to increased cAMP. Given that Ih inhibition reversed the effects of cAMP, we reasoned that neurons dialyzed with cAMP resembling the control condition may result from lower Ih density. In the presence of cAMP, six "cAMP-sensitive" neurons (i.e., reduced firing rate with cooling), and the four "cAMP-insensitive" neurons (i.e., neurons that, like in the control condition, increased firing with cooling), Ih density was measured. We found that cAMP-sensitive neurons had greater Ih densities compared with cAMP-insensitive neurons (Fig. 8B; \( P = 0.019; T_{8} = 2.915 \); unpaired two-tailed t-test). Interestingly, Ih density correlated with the Q₁₀ (i.e., fold change over 10°C) of firing frequency [Fig. 8C; \( r^2 = 0.6459; P = 0.0051; F_{(1,8)} = 14.59 \); linear regression]. Figure 8C illustrates that neurons with greater Ih densities have firing frequency Q₁₀ values > 1, indicating that neurons with greater Ih density were most affected by cAMP (i.e., they had larger decreases in firing rate with cooling). Conversely, when neurons with low Ih densities are exposed to cAMP, they retain neuronal response to cooling as if cAMP was not present and, as a result, had firing rate Q₁₀ values < 1. These findings complement Cs⁺ antagonist experiments and suggest that, when Ih is initially low at physiological \( V_m \) [via cAMP + Cs⁺ (Fig. 7), cAMP with low Ih density (Fig. 8), or closed due to voltage (Fig. 6)], neurons increase excitability when acutely cooled. Conversely, when Ih is activated at rest in neurons that contain a sufficient Ih density, they reduce firing rates upon acute cooling.

**cAMP Does Not Act Through Ih-Independent Mechanisms in LC Neurons**

In addition to activation of Ih, increases in cAMP are linked to activation of PKA. To assess whether these responses we observed were exclusively due to Ih activation or by additional PKA-dependent mechanisms, we performed experiments with the membrane-permeable PKA inhibitor, H-89. If a portion of the changes we observed with elevated cAMP occurred as a result of PKA or kinases inhibited by H-89 (28), we expected that application of H-89 would result in firing responses that resembled the...
control condition or cAMP in experiments after applying Cs+.
As demonstrated in Fig. 9, A (left) and B, spontaneous firing of
neurons at 20°C containing elevated cAMP, regardless of
PKA inhibition, was greater compared with control \( P < 0.001; F_{(2,12)} = 12.81; \) one-way ANOVA with Tukey’s post hoc test]. cAMP-dialyzed neurons with and without inhibition of PKA (Fig. 9, middle and right) at 20°C and 10°C.

Fig. 9. Protein kinase A (PKA)-dependent processes are not involved in cAMP sensitivity of LC neurons at 20°C and 10°C. A: 1-min traces of action potentials representing mean firing frequencies recorded from neurons containing control ICF (top), cAMP (middle), and cAMP in the presence of the PKA inhibitor H-89 (bottom) at 20°C and 10°C. B: summary data for 20°C under the three conditions \( n = 5 \) for all treatments. Increasing cAMP in the presence of PKA inhibition still results in elevated firing frequency compared with control. No differences were observed between neurons containing elevated cAMP and without inhibition of PKA \( n = 5; P < 0.001; \) one-way ANOVA with Tukey’s multiple-comparisons post hoc test]. C: as indicated by the change in FR during cooling, mean data \( n = 5 \) for all treatments) are shown, indicating that LC neurons still undergo decreases in firing during cooling in the presence and absence of PKA inhibition with cAMP, elevated, in contrast to control ICF. Additionally, no differences in FR reductions were observed between neurons containing elevated cAMP with and without inhibition of PKA \( n = 5; P < 0.001; \) one-way ANOVA with Tukey’s multiple-comparison post hoc test). Values are means ± SE. **\( P < 0.01. \)
control of amphibians. We hypothesized that $I_h$ would contribute to spontaneous firing, but act as a brake to dampen the magnitude of cold activation typical of LC neurons. Although $I_h$ had marginal basal activity, this hypothesis was supported because activation of $I_h$ by an endogenous agonist, cAMP, elevated firing frequency of neurons (at baseline temperature) but, surprisingly, led to decreased firing, contrary to normal increases, during cooling. The interaction between temperature and various neurophysiological mechanisms, including the ionic basis of the action potential (19), synaptic transmission (23), firing frequency (4), molecular thermosensing (9), and circuit behavior (52) has received attention. This study provides new insight by demonstrating that the direction of temperature-dependent firing can be manipulated by altering a single membrane conductance, specifically, $I_h$.

Activation of $I_h$ Contributed to Enhanced Excitability at 20°C

We previously identified electrophysiological evidence of $I_h$ in LC neurons (47). This study confirms that $I_h$ in bullfrogs is similar to $I_h$ carried by HCN channels in excitable cells from other animals (3). We show that $I_h$ in LC neurons displays inward rectification, has a mean $E_h$ near −40 mV (Fig. 1), exhibits sensitivity to ZD-7288 and Cs⁺ at low millimolar concentration (Fig. 2), and undergoes a depolarizing shift in voltage-dependent activation with elevated cAMP (Fig. 3). These characteristics indicate that $I_h$ is presumably caused by HCN channel expression. Moreover, because cAMP induced an approximately +15-mV depolarizing shift in the $V_{0.5}$, our results suggest that $I_h$ is carried by channels similar to mammalian HCN isoforms 2 or 4, as opposed to the modestly cAMP-sensitive HCN 1 or 3 (3, 33, 57).

To elucidate the role of $I_h$ in regulating firing frequency, we exposed LC neurons to 2 mM Cs⁺. Because application of 2mM Cs⁺ had no net effect on firing rate and $V_m$ in LC neurons containing control pipette solution (Fig. 5, A–C), we conclude that $I_h$ plays at most, a small role under control conditions. These results corroborate a study indicating that $I_h$ plays little to no role in determining $V_m$ and sensitivity to light in intrinsically photosensitive retinal ganglion cells, most likely because $V_m$ lies positive to the base of the $I_h$ activation curve (55). Furthermore, $I_h$ plays a minimal role in determining $V_m$ and thermosensitivity of peripheral thermoreceptive neurons (37); however, $I_h$ seems to play a role in determining subthreshold responsiveness of cold thermoreceptors and may control oscillation frequency of sensory nerve endings (38). The control firing response to Cs⁺ that we observed were, admittedly, variable (Fig. 5B). Thus interneuron variation in $I_h$ density (Fig. 8), the neuromodulatory environment capable of altering $I_h$ activation at physiological $V_m$ (39), or the strength of an off target effect of Cs⁺ that we were not able to define may determine control firing.

In direct contrast to neurons containing control pipette solution, $I_h$ clearly contributes to spontaneous firing in neurons when activated by elevating cAMP (Fig. 5, D–F). As predicted by activating $I_h$ (a conductance with a $E_h$ positive to $V_m$), mean firing frequency was greater in the presence of cAMP (Fig. 4), and exposure to 2mM Cs⁺ reduced the firing rate and hyperpolarized the membrane in each neuron tested (Figs. 5, E and F). This result implies that 1) Cs⁺ is reasonably selective antagonist of $I_h$ in LC neurons, and 2) similar to other studies (17, 36), relatively small increases in $I_h$ at the base of its activation curve can influence the firing frequency, presumably through membrane depolarization (Figs. 5, D–F). Therefore, $I_h$ plays little to no role in setting firing frequency and $V_m$ under normal conditions, but exerts a uniform, excitatory influence when activated due to a depolarizing shift in voltage-dependent activation.

Elevated Resting $I_h$ Induced by cAMP Leads to Reduced Excitability During Acute Cooling

Under control conditions, nearly all LC neurons increase firing frequency during acute cooling. At present, we do not know the mechanisms that enable cold activation of LC neurons. Several thermosensitive ion channels or transporters could provide the substrate for cold activation (48), and previous work has demonstrated that the conductance densities of both thermosensitive and non-thermosensitive ion channels can shape firing responses during temperature changes (29, 56). To achieve greater spontaneous firing frequencies with cooling, as observed in the majority of LC neurons, cold must enhance depolarizing factors to outweigh inhibitory effects on action potential generation (19) associated with cold temperatures. This is illustrated in our summary diagram shown in Fig. 10, left, as an ~6.5-mV membrane depolarization (measured in this study; Fig. 6) induced by an unidentified $I_{cold}$. Previous data indicated that $I_{cold}$ is intrinsic to LC neurons, and preliminary results suggest that $I_{cold}$ is mediated by a background

![Diagram illustrating activation of $I_h$ at 20°C](http://ajpregu.physiology.org/)

*Fig. 10. Diagrams illustrating that activation of $I_h$ at 20°C increases firing frequency and offsets cold-activated responses. Top: increases in $I_h$ at 20°C enhance firing frequency. Pharmacological experiments in current clamp demonstrated that inhibition of $I_h$ at 20°C was sufficient to reduce firing frequency in the presence of cAMP, but not in control. Therefore, $I_h$ appears to play an excitatory role upon its activation at 20°C. Bottom left: during cooling, LC neurons increase firing frequency and depolarize by ~7 mV. $I_h$ does not likely contribute to this response because $V_m$ lies positive to the activation $I_h$. Since $I_h$ is closed due to voltage at rest, we include the contribution of $I_h$ as a 0 mV to the cooling response. In contrast to control, $I_h$ contributes to the firing frequency and $V_m$ in the presence of cAMP. Pharmacological experiments in current clamp showed that inhibition of $I_h$ at a greater starting value resulted in ~6-mV membrane hyperpolarization. Because cooling inhibits $I_h$, we estimate that inhibition of $I_h$ by cooling contributes 6 mV of hyperpolarization during the cooling stimulus that is not present under control circumstances. This hyperpolarizing influence emanating from inhibition of a greater starting $I_h$ appears to be sufficient to offset the depolarization associated with cooling and, therefore, reverse normal elevated firing frequency. $I_{cold}$, cold-induced depolarizing current.*
conduction that sets the $V_m$ (J. M. Santin and L. K. Hartzler, preliminary observation).

We hypothesized that $I_h$ would function physiologically to blunt the magnitude of the cold response, because this depolarizing current is inhibited by cooling (Ref. 47; Fig. 3); however, a lack of $I_h$ activation at physiological $V_m$ precludes its ability to influence cold activation. In support of this notion, cold sensitivity was unaffected by 2 mM Cs$^+$ in neurons containing control solution in the patch pipette. Therefore, $I_h$ does not contribute to a change in $V_m$ during cooling and plays no role in regulating the normal cold-activated behavior of LC neurons. This is represented as 0-mV $\Delta V_m$ in the summary diagram (Fig. 10, left).

While $I_h$ did not play a role in determining cold activation under control circumstances, our hypothesis was supported when $I_h$ was initially activated by dialyzing neurons with elevated concentration of cAMP. Although we predicted a reduction in the magnitude of cold activation based on the fact that $I_h$ was now making a depolarizing contribution to firing rate and $V_m$ (Fig. 5), we unexpectedly found that activation of $I_h$ led to cold inhibition rather than cold activation. We arrived at this conclusion because neurons containing elevated cAMP, reduced firing frequency upon cooling (Fig. 6), and inhibition of $I_h$ was sufficient to revert cold-inhibited responses back to cold-activated responses that were similar to control neurons (Fig. 7). In support of this pharmacological experiment, neurons with low $I_h$ densities were insensitive to cAMP and exhibited cold-activated responses similar to the control neurons. By contrast, neurons with increasingly greater $I_h$ densities underwent more inhibition with cooling in the presence of cAMP (Fig. 8, B and C), suggesting that cAMP had a more substantial effect in neurons containing greater $I_h$ density. Because of causal (blocking the activated $I_h$ rescued cold activation) and correlative ($I_h$ density scaled proportionally with sensitivity to cAMP) evidence, our data indicate that augmenting the initial, baseline amount of $I_h$ is sufficient to convert cold activation to cold inhibition.

The most parsimonious interpretation of our data is that enhancing the initial amount of $I_h$ active at the physiological $V_m$ allows it to be inhibited by cooling, which opposes the $V_m$ depolarization caused by the currently unidentified, cold-depolarizing factors. An estimation of the effects of $I_h$ inhibition on the $V_m$ due to cooling was extrapolated from the membrane hyperpolarization caused by Cs$^+$ presented in Fig. 5F and included in the diagram in Fig. 10, right. We determined that inhibition of $I_h$ in the presence of cAMP resulted in an ~6-mV hyperpolarization (Fig. 5F). We, therefore, assume that inhibition of $I_h$ by cooling also contributes ~6 mV of hyperpolarization to the membrane during cooling, which was absent from control neurons. Thus greater initial $I_h$ at 20°C (Fig. 10, left) evokes a $V_m$ response to cooling that now includes a 6-mV hyperpolarization that offsets the ~6.5-mV depolarization by $I_{cold}$, resulting in a net depolarization of <1 mV instead of ~6.5 mV, as occurs in control neurons (Fig. 10, right). This prediction of the $V_m$ response to cooling in neurons dialyzed with cAMP based on findings from Cs$^+$ experiments that account for added $I_h$ inhibition ($\Delta V_m$ < 1 mV) is similar to the $V_m$ changes measured during cooling in the presence of cAMP ($\Delta V_m$ = 1.5 mV, Fig. 6). Although LC neurons dialyzed with cAMP still underwent modest membrane depolarization (1.5 mV), accompanied by reduced firing frequency during cooling, a similar response involving small-membrane depolarization with reduced firing frequency has been observed in *Aplysia* ganglion and rat hypothalamic neurons (5, 14). Additionally, since $I_h$ inhibition was sufficient to rescue cold activation in the presence of cAMP (Fig. 7F), our data do not suggest that cAMP influences thermosensitivity by manipulating $I_{cold}$. Stated simply, we conclude that increasing the contribution that $I_h$ makes to the $V_m$ before cooling enables its hyperpolarizing influence upon inhibition by cold temperature to offset normal cold-activated responses.

**Lack of $I_h$-Independent Actions of cAMP**

There are not currently any selective pharmacological activators of $I_h$/HCN channels. To fully address our hypothesis and study baseline firing and cooling responses with $I_h$ activated, we dialedyzed neurons with an endogenous agonist, cAMP. We recognized that cAMP is not a specific agonist of $I_h$ and that a portion of the changes we observed in the presence of cAMP could have been due to activation of other cAMP-dependent process (e.g., activation of PKA). Three lines of evidence indicate that differences in spontaneous firing and cold responses in the presence of cAMP were caused by action on $I_h$ and not $I_h$-independent processes. First, spontaneous firing rates and cold responses did not differ in the presence of the PKA inhibitor, H-89. If a component of the changes we observed in neurons dialyzed with cAMP was caused by PKA phosphorylation, we would have expected inhibition of PKA to cause spontaneous firing and cold responses to resemble the control condition [as occurred during application of Cs$^+$ (Figs. 5 and 7)]. Second, to rule out unaccountable factors independent of $I_h$, we compared the firing rates at 20°C in neurons dialyzed with control and cAMP pipette solutions with $I_h$ blocked using Cs$^+$. If other factors, outside of $I_h$, were influencing the firing frequency in neurons dialyzed with cAMP, we would have expected firing rates to differ when comparing these groups of neurons. We found that the firing rates did not differ between neurons containing control and cAMP pipette solutions when $I_h$ was blocked ($P = 0.4$). Finally, we showed that cold-inhibited responses in the presence of cAMP were converted back to cold-activated responses when we blocked $I_h$ during a second cold exposure. To ensure that cold activation was restored due to $I_h$ inhibition by Cs$^+$ application per se and not time-sensitive processes that could be activated by cAMP independently of $I_h$, we performed a time control experiment (Fig. 7F). In this series of experiments, neurons dialyzed with cAMP were cooled twice in aCSF. If time-dependent processes associated with prolonged dialysis and not inhibition of $I_h$ were responsible for rescuing cold activation in the presence of cAMP, we would have expected the second exposure of the time control to resemble the cold-activated responses of both control experiments and cAMP experiments performed in the presence of Cs$^+$. As shown in Fig. 7E, the second cold exposure in the time control experiment resulted in reduced firing frequency, nearly identical to the first cooling ramp. The time control experiment provides us with confidence that Cs$^+$ application and its probable action on the enhanced initial amount of $I_h$ per se is responsible for restoring cold-activated responses in neurons dialyzed with cAMP. Although these control experiments and analyses did not detect any $I_h$-independent effects of cAMP dialysis on baseline firing and cold
Insights into the Amphibian Respiratory Control System

LC neurons provide a CO2/pH-chemosensitive drive to breathe in the respiratory network of amphibians (34). Since cold activation is a property of chemosensitive neurons (47), we speculate that LC activity inversely proportional to temperature may stabilize respiratory parameters during changes in temperature. Our results demonstrating that initial activation of \( I_h \) was sufficient to reverse normal cold responses could be important for regulating breathing during changes in temperature. Although the physiological effectors of \( I_h \) activation are unidentified in LC neurons from bullfrogs, \( I_h \) is activated by several mechanisms that are likely to interact with chemosensitive LC neurons. For example, a soluble form of adenyl cyclase that senses bicarbonate and produces cAMP is expressed (35) and functional (22) in chemosensitive LC neurons of rats. Since LC neurons from frogs and rats exhibit similar responses to CO2/pH changes (46), a soluble adenyl cyclase-dependent process may elevate cAMP and, in turn, activate \( I_h \).

Additionally, nitric oxide (NO) signaling has been shown to activate \( I_h \) (58) and also facilitate respiratory discharge from the bullfrog brainstem (16, 18). We speculate that activation of \( I_h \) by NO signaling within the LC may contribute to portion of the excitatory action of NO in the respiratory network of amphibians. The significance of NO signaling on temperature sensitivity of respiratory output has not been addressed, but would be interesting to explore within the context of our findings. These are two physiologically conceivable mechanisms through which \( I_h \) could be activated in LC neurons to influence breathing. However, the involvement of cold activation of LC neurons and its modulation by the battery of factors that interact with \( I_h \) (reviewed in Ref. 3) remain unidentified and provide a mechanistic framework for future studies into anuran respiratory control.

General Implications for Considering Tissue Temperature in Electrophysiological Studies

Although the specific mechanisms that physiologically activate \( I_h \) in LC neurons of bullfrogs are currently unidentified, we would like to draw attention to the physiological significance associated with considering changes in tissue temperature that are highlighted by this study. These findings provide a unique, perhaps extreme, example demonstrating that the result of \( I_h \) activation on firing frequency is nonlinear across temperatures (Figs. 5 and 7). Specifically, an increase in firing rate induced by \( I_h \) activation that occurred in LC neurons at 20°C transformed firing rate changes in response to cooling from excitatory to inhibitory. Our diagram (Fig. 10), constructed by summing changes in the \( V_m \) measured under different conditions in this study, suggests that this nonlinear effect can be accounted for by enhancing the amount of \( I_h \) that contributes to the \( V_m \) under baseline conditions. Consequently, activation of \( I_h \) at baseline results in additional hyperpolarizing pull when cooled due to its inhibition that is not present when \( I_h \) is not initially activated under control circumstances. This finding illuminates an important point regarding the effects of \( I_h \) on firing rate and neuronal excitability at other temperatures. 

Perspectives and Significance

We demonstrated that enhancing \( I_h \) under baseline conditions was sufficient to stimulate firing, but led to a reversal of cold-activated firing responses during temperature changes. Given that HCN channels are regulated by various intra- and extracellular constituents, including cyclic nucleotides, ions, lipids, and interacting proteins (3), our results present the possibility that many factors could modulate temperature sensitivity of neuronal firing through actions on HCN channels. This implication is apparent for poikilothermic animals, like bullfrogs used in this study, whose entire nervous system is likely to undergo large changes in temperature, but might also have significance for mammalian nervous systems that also undergo changes in temperature during various physiological states. 
and pathological conditions. Results from the present study, therefore, underscore the importance of HCN channel modulation state as a critical regulator of neuronal activity with effects contingent on changing neuronal temperature.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J. M. S. and L. K. H. conception and design of research; J. M. S. performed experiments; J. M. S. analyzed data; J. M. S. interpreted results of experiments; J. M. S. prepared figures; J. M. S. drafted manuscript; J. M. S. and L. K. H. edited and revised manuscript; J. M. S. and L. K. H. approved final version of manuscript.

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