Multiple targets of chemosensitive signaling in locus coeruleus neurons: role of K\textsuperscript{+} and Ca\textsuperscript{2+} channels

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Filosa, Jessica A., and Robert W. Putnam. Multiple targets of chemosensitive signaling in locus coeruleus neurons: role of K\textsuperscript{+} and Ca\textsuperscript{2+} channels. Am J Physiol Cell Physiol 284: C145–C155, 2003. First published September 18, 2002; 10.1152/ajpcell.00346.2002.—We studied chemosensitive signaling in locus coeruleus (LC) neurons using both perforated and whole cell patch techniques. Upon inhibition of fast Na\textsuperscript{+} spikes by tetrodotoxin (TTX), hypercapnic acidosis [HA; 15% CO\textsubscript{2}, extracellular pH (pH\textsubscript{e}) 6.8] induced small, slow spikes. These spikes were inhibited by Co\textsuperscript{2+} or nifedipine and were attributed to activation of L-type Ca\textsuperscript{2+} channels. Upon inhibition of both Na\textsuperscript{+} and Ca\textsuperscript{2+} spikes, HA resulted in a membrane depolarization of 3.52 ± 0.61 mV (n = 17) that was reduced by tetraethylammonium (TEA) (1.49 ± 0.70 mV, n = 7; P < 0.05) and absent (−0.97 ± 0.73 mV, n = 7; P < 0.001) upon exposure to isohydric hypercapnia (IH; 15% CO\textsubscript{2}, 77 mM HCO\textsubscript{3}, pH\textsubscript{e} 7.45). Either HA or IH, but not 50 mM Na-propionate, activated Ca\textsuperscript{2+} channels. Inhibition of L-type Ca\textsuperscript{2+} channels by nifedipine reduced HA-induced increased firing rate and eliminated IH-induced increased firing rate. We conclude that chemosensitive signals (e.g., HA or IH) have multiple targets in LC neurons, including TEA-sensitive K\textsuperscript{+} channels and TWIK-related acid-sensitive K\textsuperscript{+} (TASK) channels. Furthermore, HA and IH activate L-type Ca\textsuperscript{2+} channels, and this activation is part of chemosensitive signaling in LC neurons.

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The current model of cellular chemosensitivity, based on chemosensitive cells located both in the brain stem and the carotid bodies, is that a chemosensitive signal such as high CO\textsubscript{2}/H\textsuperscript{+} results in a neuronal membrane depolarization, presumably through inhibition of a K\textsuperscript{+} channel, resulting in an increased spike frequency (17, 35, 36, 42, 63, 64). A number of findings suggest that this model may be simplistic. When using hypercapnic stimuli without a change of extracellular pH (pH\textsubscript{e}), an increase in spike frequency without an apparent membrane depolarization has been observed in both LC (26) and medullary raphe neurons (62). Furthermore, an increase in membrane input resistance, which should be associated with K\textsuperscript{+} channel closure in response to chemosensitive signals, is often very small (18, 42), suggesting that multiple channels may be affected, with some closing and others opening. Indeed, Richerson (43) suggested the possibility of several chemosensitive channels being affected by chemosensitive signals in concert.

Another complicating feature of the current model is that the search for the chemosensitive channel has resulted in several possible candidates. For example, in medullary raphe neurons, Richerson (42) and colleagues have shown that the ionic mechanisms that lead to an increased spike frequency could be mediated through modulation of transient outward currents, Ca\textsuperscript{2+} currents, and Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents. Recently, they have identified a novel intracellular pH (pH\textsubscript{i})-sensitive cation current (59). In dorsal medullary neurons, a decrease in an unspecified outward K\textsuperscript{+} conductance has been claimed to be the chemosensitive target (19). In LC neurons, Pineda and Aghajanian (39) showed a decrease in the outward conductance of inwardly rectifying K\textsuperscript{+} (K\textsubscript{ir}) channels in response to HA, whereas Wellner-Kienitz et al. (64) showed a decreased outward K\textsuperscript{+} conductance of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (K\textsubscript{Ca}) in response to HA in fetal medullary neurons. In peripheral chemosensitive cells, changes of pH\textsubscript{e} and pH\textsubscript{i} resulted in inhibition of both K\textsubscript{Ca} channels, as well as of the TWIK-related acid-sensitive K\textsuperscript{+} (TASK) channel (7, 8, 10). Finally, during hypercapnia, Ca\textsuperscript{2+} channels are activated in peripheral chemosensi-
tive cells (9, 47, 48, 67) and in invertebrate central chemosensitive neurons (24). Therefore, possible channel targets of chemosensitive neurons include $K_r$, $K_{Ca}$, TASK, and $Ca^{2+}$ channels. Depending on the complement of channels expressed in any particular chemosensitive neuron, its response to a given chemosensitive stimulus could involve effects of multiple channels, and this response could differ from other chemosensitive neurons that express a different complement of channels.

The fact that different channels possess distinct pH sensitivities also implies that the types of chemosensitive signals could affect which channels are targeted by various chemosensitive stimuli. For example, decreased pH$_i$ has been shown to inhibit $K_r$ and $K_{Ca}$ channels, whereas decreased pH$_i$ has been shown to inhibit TASK channels (37, 38, 41, 50, 52). Thus it is likely that depending on the stimulus present at any given time, multiple channels rather than a single channel could be targeted by any given chemosensitive stimulus. Furthermore, it is still to be determined whether inhibition of pH-sensitive K$^+$ channels per se is the only mechanism by which chemosensitive neurons increase neuronal discharge in the presence of an acid challenge. Evidence of $Ca^{2+}$ channels determining spontaneous firing rate in LC neurons (96) and of $Ca^{2+}$ channel activation during HA (26) would suggest a potential role for $Ca^{2+}$ channels, independent of K$^+$ channel inhibition, in the chemotransduction pathway as well. In fact, such a pathway of activation has been proposed to be the basis for hypercapnia-induced increased firing rate in snail neurons (23).

In LC neurons, the chemosensitive pathway is further complicated by the complex electrophysiological membrane properties of these neurons. In addition to spontaneous Na$^+$ spike activity, LC neurons from young animals (P8–26) (65), and to a much lesser extent adults (66), exhibit subthreshold rhythmic oscillations (SRO) in their membrane potential ($V_m$). These oscillations have been attributed to inward $Ca^{2+}$ currents (65, 66) or to a combination of $Ca^{2+}$ and Na$^+$ currents (4) on the dendrites. Although not much is known about the physiological significance of these oscillations, they appear to be due to electrical coupling arising at the dendrites (4, 32, 35). An important property of these SROs is that, as with Na$^+$ spikes, they are sensitive to chemosensitive stimuli such as HA (4). The chemosensitive nature of these oscillations provides an additional tool to study chemosensitivity in LC neurons.

In this study, we have begun an investigation of the possible channel targets of chemosensitive signaling in LC neurons. Of the potential pH-sensitive channels, these neurons are known to express $K_r$, $K_{Ca}$, and TASK channels (3, 5, 13, 34, 39, 52). We hypothesize that multiple channels, including various K$^+$ and $Ca^{2+}$ channels, are involved in the increased firing rate induced by chemosensitive signals in LC neurons. We show that during intracellular acidification, L-type $Ca^{2+}$ channels are activated. Our data also show that the membrane depolarization observed in response to HA is largely mediated by an extracellular pH-sensitive channel and partly by an intracellular pH- and tetraethylammonium (TEA)-sensitive channel. Finally, we show that activation of L-type $Ca^{2+}$ channels by various chemosensitive stimuli is correlated with the ability of those stimuli to increase firing rate and that $Ca^{2+}$ channel blockage reduces the firing rate response to hypercapnia. These data suggest that there are multiple targets for chemosensitive stimuli, including at least two types of K$^+$ channels, as well as L-type $Ca^{2+}$ channels, and that $Ca^{2+}$ channels play a role in chemosensitive signaling in LC neurons.

A preliminary report of part of this work has previously been published (27).

**MATERIALS AND METHODS**

**Slice preparation.** Pontine LC slices were prepared from neonatal Sprague-Dawley rats postnatal (P) age P1–9. We have previously shown that the electrophysiological response of LC neurons to HA is the same in slices from rats age P1 to adult (54). All procedures are in agreement with the Wright State University Institutional Animal Care and Use Committee guidelines and were approved by the committee (AAALAC no. A3632-01). Rats were anesthetized by hypothermia and rapidly decapitated (16). The brain stem was subsequently removed and placed onto a vibrator (PelcoVibrator 1000). Coronal brain stem slices (300 μm) were cut into artificial cerebrospinal fluid (aCSF) (for composition, see Solutions) at 4–6°C. Slices were then incubated at room temperature in aCSF equilibrated with 95% O$_2$-5% CO$_2$ (pH $\sim$7.45) until needed. At the time of the experiment, slices were placed in a perfusion chamber and held with a nylon grid. Brain slices were continuously superfused via a gravity-fed system at a rate of 3–5 ml/min. Solutions were maintained at 40°C in a reservoir in a water bath and bubbled with the equilibrating gas. Solution flowed from the reservoir through stainless steel tubing (outer diameter 1.6 mm), a glass bubble trap, and finally through a thermoelectric Peltier assembly (29) to maintain temperature in the perfusion chamber at 35 ± 2°C.

**Solutions.** The aCSF was composed of (in mM) 5 KCl, 124 NaCl, 1.3 MgSO$_4$, 26 NaHCO$_3$, 1.24 KH$_2$PO$_4$, 10 glucose, and 2.4 CaCl$_2$ and was equilibrated with 95% O$_2$-5% CO$_2$, pH $\sim$7.45 (at 35°C). The composition of the HA solution was the same as the aCSF solution with the exception that it was bubbled with 85% O$_2$-15% CO$_2$, pH $\sim$6.8. This resulted in a bulk solution pH of 6.9 for HA in the perfusion chamber (26). We do not know the actual change of pH$_i$ that is seen by the neurons we studied (usually one or two cell layers into the slice) due to the change in bulk pH, to 6.9. Regardless, this represents a large stimulus. We and others have previously shown (39, 54) that smaller stimuli (pHi 7.7–7.15) increase LC neuron firing rate by 40–50%. We have chosen to use the larger stimulus in this study for ease of comparison with our previous work (26). For the IH solution, NaHCO$_3$ was increased to 77 mM (replacing NaCl isosmotically) and equilibrated with 85% O$_2$-15% CO$_2$, pH $\sim$7.45. The propionate solution consisted of aCSF solution with 50 mM NaCl being isosmotically replaced with 50 mM Na$^+$-propionate, pH adjusted to $\sim$7.45 with NaOH. The osmolality of all solutions was measured and adjusted to 300 mosmol/kg H$_2$O with the major salt of the solution.

**Electrophysiological recordings.** Both perforated-patch and whole cell recordings were used throughout this study. Briefly, pipettes were made with thin-walled filament boro-
silicate glass (outer diameter 1.5 mm, inner diameter 1.12
mm) pulled to a tip resistance of ~5 MΩ. Perforated patch recordings were performed using a technique adapted from Rae et al. (40). The composition of the pipette filling solution for perforated patch recordings was (in mM) 130 CH₄O₃S, 130 KOH, 20 KCl, 5 HEPES, and 1 EGTA. While the tip of the pipette was filled with this solution, the rest of the pipette was backfilled with the same solution containing amphotericin B added to a final concentration of 240 μg/ml (40). The pipette filling solution for whole cell recordings was (in mM) 130 K⁺-gluconate, 10 KCl, 10 HEPES, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, and 2 ATP. Both perforated patch and whole cell solutions had their pH adjusted to ~7.35 with KOH. LC neurons were visualized (~720 magnification) with an upright microscope (Nikon Eclipse E600) using a ×40 water-immersion objective (N.A. 0.8, 3.0-mm working distance). Experiments were started at the point where a stable $V_m$ of ~−45 to −60 mV was established.

Electrophysiological recordings were conducted in current-clamp mode. The criteria for selecting a healthy LC neuron were that it had a resting $V_m$ ranging from ~−45 to −60 mV and spontaneous firing rate less than 4 Hz. Access resistance varied from neuron to neuron and between configurations, ranging from 10 to 60 MΩ for perforated patch recordings and from 8 to 16 MΩ for whole cell recordings. When LC neurons were exposed to tetrodotoxin (TTX), either with or without nifedipine or cobalt, sufficient hyperpolarizing current (10–30 pA) was injected to bring $V_m$ back to resting values. A similar current was employed when LC neurons were exposed to nifedipine alone (e.g., see Figs. 8 and 9). When LC neurons were exposed to TEA, a larger hyperpolarizing current (90–120 pA) was required to bring $V_m$ back to resting levels (e.g., see Fig. 4). Signals were amplified (BVC-700 Cornerstone by Dagan), filtered (1 kHz), and stored on tape for further analysis. Spikes and integrated firing rate were recorded with a multichannel slope/height window discriminator (P/N Model 700B, Bowdoinham, ME). $V_m$ and firing rate were analyzed using pCLAMP software version 8.0.2.

**Drugs.** Amphotericin B was prepared as a stock in DMSO (60 mg/ml) and diluted into the pipette filling solution to a final concentration of 240 μg/ml (40). All chemicals, including amphotericin B, cobalt chloride, TTX, TEA, nifedipine, and Na-propionate, were purchased from Sigma (St. Louis, MO).

**Data analysis.** Values are expressed as means ± SE. Changes in firing rate were tested for being significantly different from zero by using t-tests and were considered significant at $P < 0.05$. Values of percent change of firing rate were calculated by dividing the difference between each firing rate value during an acid challenge and the average firing rate before acidification (this ratio was multiplied by 100 to obtain percent change). All differences among three or more means were determined either by one-way ANOVA (see Fig. 6) or two-way ANOVA (see Fig. 10), with multiple paired comparisons done using Student-Newman-Keuls tests. All differences were considered to be statistically significant at $P < 0.05$.

**RESULTS**

**Properties of LC neurons.** LC neurons possess complex electrophysiological properties. In addition to regular Na⁺ action potentials (Fig. 1, $A1$ and $B1$), in the presence of the Na⁺ channel blocker TTX, TTX-insensitive oscillations and spikes can be observed (Fig. 1, $A3$ and $B3$). Furthermore, at hyperpolarizing $V_m$, so that the frequency of Na⁺ action potentials is reduced, SROs (Fig. 1, $B1$, arrowheads) are observed. For clarification, we will define action potentials observed in the absence of TTX as Na⁺ spikes and those observed in the presence of TTX as TTX-insensitive spikes (Fig. 1, $A3$, $B3$, arrow). The subthreshold $V_m$ oscillations observed in the absence of TTX will be referred to as SROs. The subthreshold $V_m$ oscillations observed in the presence of TTX, which are larger in amplitude than SROs, will be referred to as TTX-insensitive oscillations. The difference between Na⁺ spikes, SROs, TTX-insensitive oscillations, and TTX-insensitive spikes can be seen in Fig. 1, where the transition from one characteristic waveform to the other is observed upon addition of 1 μM TTX to the medium. Like Na⁺ spikes, SROs are sensitive to high CO₂/H⁺, their frequency increasing with HA (e.g., Fig. 2C), so they can also be used to assess chemosensitivity in LC neurons. Thus the criterion for selecting a chemosensitive neuron for this study was that exposure to high CO₂/H⁺ resulted in a significant and reversible increase in firing rate, observed either as an increase in Na⁺ or TTX-insensitive oscillations/spikes or as an increase in the frequency of SROs.

**Perforated patch vs. whole cell recordings.** Mean control values after establishing a seal for LC neurons when using the perforated patch configuration were $V_m$ of ~−46.0 ± 0.7 mV and firing rate of 0.98 ± 0.30 Hz ($n = 9$). Mean control values after establishing a seal for LC neurons when using the whole cell configuration were $V_m$ of ~−47.2 ± 0.4 mV and firing rate of 1.37 ± 0.27 Hz ($n = 13$). These values are comparable to previously reported values for LC neurons (2). One difference observed in the membrane properties of LC neurons when using these two configurations was the resting $V_m$. After establishing a whole cell patch, ~70% of the neurons stopped firing within 5–10 min. This was accompanied by a small membrane hyperpolarization. This phenomenon has been previously reported by Alreja and Aghajanian (2) and was attributed to rundown of cAMP. Even though hyperpolarization resulted in
cessation of Na⁺ spike activity, SROs were evident and had a frequency of 1.22 ± 0.10 Hz (n = 16). Thus the presence of an initial slow hyperpolarization was the main electrophysiological difference in LC neurons studied with whole cell techniques compared with perforated patch techniques.

Figure 2A shows the chemosensitive response of a LC neuron exposed to HA (15% CO₂) when using the perforated patch configuration. Under such conditions, LC neurons maintained their spontaneous firing activity and increased their firing rate (Fig. 2A; see also Ref. 26). This increased firing rate was reversed upon the removal of HA (Fig. 2A). LC neurons exposed to HA when using the whole cell configuration showed two different responses. Figure 2B shows a LC neuron where the spontaneous Na⁺ spike activity was lost in the absence of HA, but a reversible increase of the firing rate was evident in response to HA. In contrast, Fig. 2C shows a LC neuron where all Na⁺ spike activity was lost. However, the chemosensitive response in this neuron was still evident as a reversible increase in the frequency of SROs in response to HA with no evident Na⁺ spike discharge. Thus it appears that when using the whole cell configuration to study LC neurons, the chemosensitive response to HA can be observed either as an increase in spike frequency or an increase in SRO frequency.

When using whole cell recordings, we also noticed that reducing the EGTA and Ca²⁺ concentration in the pipette filling solution seemed to maintain normal membrane excitability because it increased the percentage of neurons that responded to high CO₂/H⁺ with an increase in Na⁺ spike activity but did not alter the SRO response (as shown in Fig. 2B) compared with neurons studied with pipettes filled with high EGTA and Ca²⁺ concentrations. Figure 2D shows the percentage of LC neurons that exhibited increased Na⁺ spike activity in response to high CO₂/H⁺ when using different concentrations of EGTA and Ca²⁺ in the recording pipette solution. When using our normal pipette solution of 10 mM EGTA/1 mM Ca²⁺ (46), only about 20% of LC neurons showed an increase in Na⁺ spikes with HA, and this percentage was similar when using 10 mM EGTA/0 mM Ca²⁺. The remainder of the neurons responded to HA with an increase of SROs only but showed no Na⁺ spike activity (e.g., Fig. 2C). However, nearly 70% of LC neurons responded to HA with increased Na⁺ spike frequency when the whole cell pipette was filled with a solution containing 0.4 mM EGTA/0 mM Ca²⁺ (Fig. 2D). These data suggest a potential role for Ca²⁺ in setting membrane excitability in LC neurons and further indicate that in contrast to previous observations (20, 42, 46), whole cell recordings can be used to study the chemosensitive response, at least in LC neurons. For the rest of our studies, when using the whole cell configuration we used 0.4 mM EGTA/0 mM Ca²⁺ in the pipette filling solution.

**Effects of HA on LC neurons in the presence of TTX.**

In an attempt to study the effects of HA on Vₘ in a previous study (26), we exposed LC neurons to HA in the presence of the fast Na⁺ channel blocker TTX. In the presence of 1 μM TTX, all Na⁺ spikes were inhibited. All neurons responded to HA with an increase in TTX-insensitive spikes (26). These spikes, observed in the presence of TTX, could be mediated either by a TTX-insensitive Na⁺ channel or by activation of Ca²⁺ channels. These spikes had a higher apparent threshold, a slower rise time, and were broader than the Na⁺ spikes (26), suggesting that they are Ca²⁺ spikes.

To test whether the TTX-insensitive spikes were due to Ca²⁺ channel activation, we exposed LC neurons to the nonspecific Ca²⁺ channel blocker Co²⁺. In the presence of 2 mM Co²⁺ (and 1 μM TTX), HA-induced spike and oscillatory activity was blocked (n = 12) (e.g., Fig. 3A) within 2–3 min of exposure, indicating that these spikes do indeed reflect the activity of Ca²⁺ channels.
Recent work on carotid body glomus cells has shown an augmentation of L-type Ca\(^{2+}\) currents during hypercapnia (56). To test whether the HA-induced Ca\(^{2+}\) spikes in LC neurons were mediated through L-type Ca\(^{2+}\) channel activation, we exposed these neurons to the L-type Ca\(^{2+}\) channel blocker nifedipine. As with Co\(^{2+}\), HA-induced spike activity (in the presence of 1 \(\mu\)M TTX) was completely blocked by 50 \(\mu\)M nifedipine \((n = 5)\) (e.g., Fig. 3B). Full blockage required at least 15 min of exposure to nifedipine. These data clearly indicate that HA activates L-type Ca\(^{2+}\) channels in LC neurons, most likely by an indirect pathway as previously observed in glomus cells (56).

**Effects of TEA on the HA-induced \(V_m\) depolarization.**

In LC neurons, when Na\(^+\) spikes were blocked by TTX and Ca\(^{2+}\) channels were blocked by either Co\(^{2+}\) or nifedipine, HA induced a significant \((P < 0.001)\) membrane depolarization of 3.52 \(\pm\) 0.61 mV \((n = 17)\) (Figs. 3, A and B, and 6), suggesting that HA must affect channels other than Ca\(^{2+}\) channels. One possibility is that HA exposure inhibits K\(^+\) channels. Because, in other chemosensitive cells K\(_{ca}\) channels are inhibited by a fall of pH\(_i\) (64), we investigated this possibility with the use of TEA. In the presence of TTX and Co\(^{2+}\), exposure to 20 mM TEA resulted in a membrane depolarization (\(-4 mV\)) comparable to the one observed in the presence of HA (Fig. 4). At this concentration, TEA could inhibit K\(_{ca}\), K\(_{ir}\), and/or K\(_{v}\) channels, thus accounting for the membrane depolarization. We injected sufficient hyperpolarizing current to bring \(V_m\) back to its resting potential (Fig. 4). Under such conditions, HA exposure induced a significantly \((P < 0.05)\) smaller membrane depolarization of 1.49 \(\pm\) 0.70 mV \((n = 7)\) than that seen in the absence of TEA (Figs. 4 and 6). These data suggest that the HA-induced depolarization in LC neurons is in part mediated by inhibition of a TEA-sensitive K\(^+\) channel.

**The fact that the membrane depolarization was not completely blocked by TEA would suggest that TEA-insensitive channels might be targeted by HA as well.** Of the possible TEA-insensitive yet pH-sensitive K\(^+\) channels expressed in LC neurons, TASK channels are probably the most likely candidates. Recent studies have shown high expression of TASK-1 and TASK-3 channels in LC neurons (52, 57), and TASK channels are known to be highly sensitive to inhibition by extracellular acidification in the physiological pH range (22). To test whether TASK channels contribute to the HA-induced membrane depolarization, we exposed LC neurons to IH, which we have previously shown leads to a fall of pH\(_i\) but is not accompanied by any change of pH\(_o\) (26). Isohydric hypercapnia (IH), in the presence of TTX, resulted in an increase in Ca\(^{2+}\) spikes, with an apparent membrane hyperpolarization (probably due to activation of Ca\(^{2+}\)-activated K\(^+\) channels) (Fig. 5A). In the presence of TTX and either Co\(^{2+}\) or nifedipine, Ca\(^{2+}\) spikes were inhibited and under these conditions IH did not result in any change of \(V_m\) of LC neurons (\(\Delta V_m\) of \(-0.07 \pm 0.73 \text{ mV}, P > 0.1, n = 6\)) (Figs. 5B and 6).

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**Fig. 3. Effects of Ca\(^{2+}\) channel blockers on the HA-induced TTX-insensitive oscillations in LC neurons.** A: TTX-insensitive oscillations increased in frequency upon exposure to 2 mM Co\(^{2+}\) a nonspecific Ca\(^{2+}\) channel blocker. B: pre-exposure for 15 min to the L-type Ca\(^{2+}\) channel blocker nifedipine eliminated the TTX-insensitive oscillations, and they were not induced by exposure to HA. In both neurons, note that the effects of HA on \(V_m\) were reversed upon removal of HA.

**Fig. 4. Effects of the nonspecific K\(^+\) channel blocker tetraethylammonium (TEA) on the HA-induced membrane depolarization in the presence of 1 \(\mu\)M TTX and 2 mM Co\(^{2+}\).** In the presence of Na\(^+\) and Ca\(^{2+}\) channel blockers, HA induced a reversible membrane depolarization. The presence of 20 mM TEA resulted in a membrane depolarization which was compensated with sufficient depolarizing current (arrow) to return \(V_m\) to near its initial value. HA exposure in the presence of TEA resulted in a smaller, reversible membrane depolarization.

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**Fig. 5. Effects of isohydric hypercapnia (IH) in the presence and absence of the Ca\(^{2+}\) channel blocker nifedipine.** A: sample of a LC neuron exposed to IH in the presence of 1 \(\mu\)M TTX. Note the increased frequency of TTX-insensitive spikes and the apparent membrane hyperpolarization. B: sample of a LC neuron exposed to IH in the presence of 1 \(\mu\)M TTX and 50 \(\mu\)M nifedipine. Note that TTX-insensitive oscillations were completely blocked by the L-type Ca\(^{2+}\) channel blocker nifedipine.
between each pair of means is indicated by brackets. The level of significance of the difference between each pair of means is indicated by brackets.

Fig. 6. Effects of HA and IH on $V_m$ changes (in mV) in the presence of Na$^+$ and Ca$^{2+}$ channel blockers. In the absence of TEA, HA induced a significant membrane depolarization. In the presence of TEA, the HA-induced depolarization was significantly less. There was no membrane depolarization induced by exposure to IH. The height of each bar represents means ± SE. The number of neurons studied is given in each bar. The level of significance of the difference between each pair of means is indicated by brackets.

6). These results suggest that HA-induced depolarization is due in part to inhibition of TASK channels by decreased pH, as well as to inhibition of TEA-sensitive K$^+$ channels by decreased pH.

Contribution of Ca$^{2+}$ channel activation to increased firing rate in LC neurons. Our data indicate that chemosensitive signals like HA (Fig. 3) and IH (Fig. 5A) activate L-type Ca$^{2+}$ channels in LC neurons. Here we address the possible role of this Ca$^{2+}$ channel activation in the chemosensitive response of LC neurons using two approaches: 1) comparing the effects of various chemosensitive stimuli on Ca$^{2+}$ channels and firing rate (Na$^+$ spikes), and 2) studying the effect of Ca$^{2+}$ channel blockers on the effect of HA and IH on firing rate.

In the first approach, we used three stimuli known to have different effects on the firing rate (Na$^+$ spikes) of LC neurons: HA, which causes a large increase in firing rate; IH, which causes an intermediate increase in firing rate; and propionate, which causes no increase in firing rate upon exposure but a small increase in firing rate upon removal (26). We quantified the percent change in Ca$^{2+}$ spike frequency in response to these three different acid challenges. Figure 7B shows the quantified data from the percent change in Ca$^{2+}$ spike frequency induced by HA (e.g., Fig. 3A) and IH (e.g., Fig. 5A) (1 mM TTX was added to the perfusate to see Ca$^{2+}$ spikes only). Both HA and IH resulted in a significant ($P < 0.001$ for both) increase in Ca$^{2+}$ spike activity of $1.03 ± 0.04$ Hz ($n = 6$) and $0.20 ± 0.04$ Hz ($n = 10$), respectively. In contrast, in the presence of 50 mM propionate, the frequency of Ca$^{2+}$ spikes was unchanged ($-0.03 ± 0.02$ Hz, $P > 0.05$, $n = 6$) (Fig. 7A and B). In fact, a small increase ($P < 0.025$) in Ca$^{2+}$ spike frequency of $0.24 ± 0.08$ Hz ($n = 6$) was observed upon removal of propionate (Fig. 10, A and B). Thus the effects of various acid challenges on Ca$^{2+}$ spike frequency are well correlated with the effects of these acid challenges on Na$^+$ spike frequency, suggesting a role for Ca$^{2+}$ channel activation in chemosensitive signaling.

In the second approach, we blocked Ca$^{2+}$ channels and measured the effects of HA and IH on firing rate. We observed the normal reversible increase in firing rate induced by HA in LC neurons (Figs. 8A, 1–3, and 9A, 1–3). In the presence of 50 μM nifedipine, there was a small increase in firing rate which was compensated with sufficient hyperpolarizing current to bring firing rate back to its basal rate (Figs. 8A4, arrow and 9A4, arrow). Exposure of LC neurons to HA in the presence of nifedipine resulted in a significantly ($P < 0.05$) smaller increase of Na$^+$ spike frequency (Fig. 8B, 5–7) of $1.07 ± 0.03$ Hz ($n = 4$) compared with the HA response of these neurons in the absence of nifedipine of $1.82 ± 0.12$ Hz ($n = 9$) (Fig. 10). IH caused an increase ($P < 0.001$) in firing rate of LC neurons of $0.94 ± 0.21$ Hz ($n = 12$) (Fig. 10) that was only partially reversible (26). This increased firing rate in response to IH was abolished ($0.26 ± 0.16$ Hz, $P > 0.05$, $n = 5$) in the presence of 50 μM nifedipine (Figs. 9B, 5–7, and 10). A two-way ANOVA comparing acid challenge and the presence or absence of nifedipine was performed. Both nifedipine ($P < 0.01$) and acid ($P < 0.001$) were
significant sources of variation. All pairwise comparisons were significant ($P < 0.05$). However, there was no significant ($P = 0.88$) interaction between nifedipine and acid challenge. While these data indicate that activation of L-type Ca$^{2+}$ channels contributes significantly to the increased firing rate of LC neurons in response to HA and IH, it is not a requirement for this response.

The results of the experiments using both approaches indicate that the activation of Ca$^{2+}$ channels by acid challenges plays a role in the chemosensitive response of LC neurons.

**DISCUSSION**

In this study, we present new findings on the possible mechanisms underlying the chemosensitive response of LC neurons in neonatal rats and offer data that suggest a more complicated model of cellular chemosensitive signaling in these neurons. First, we show that the chemosensitive properties of LC neurons are maintained when using the whole cell patch configuration. Second, our data suggest that multiple K$^+$ channels are inhibited during HA. Third, we present evidence for activation of L-type Ca$^{2+}$ channels by acid challenges and show that this activation may play a role in the chemosensitive response of LC neurons.

**Use of the whole cell configuration to study chemosensitivity.** Previous studies of chemosensitive neurons have found that the electrical response of these neurons to acid challenges is rapidly lost when using whole cell pipettes, suggesting the washout of some cytoplasmic factor necessary for chemosensitive signaling (20, 42, 46). In the first part of the current study, we show...
that the whole cell configuration can be used for the study of chemosensitivity in LC neurons. Our data suggest that in LC neurons, even under conditions where spontaneous activity is lost (no NA+ spike activity) due to slow, membrane hyperpolarization, the sustained presence of SROs, which respond to chemosensitive stimuli (4), can be used to study chemoreception. We have further found that by lowering EGTA-Ca2+ concentrations in the whole cell pipette, we increased the number of LC neurons that respond with increased NA+ spikes to chemosensitive stimuli. These findings with whole cell pipettes suggest that Ca2+ may play a role in setting the membrane excitability of LC neurons.

Activation of Ca2+ channels by HA. A major finding of this study is that L-type Ca2+ channels are activated by acid challenges such as HA and IH. This was not expected because the effect of both extracellular and intracellular acidification on Ca2+ channels is generally believed to be channel inhibition (51, 60). L-type Ca2+ channels are modulated by a number of different factors, including membrane depolarization, protein kinases (PKA and PKC), and Ca2+ concentrations (1). L-type Ca2+ channels can be activated by membrane depolarization (11). In LC neurons, L-type Ca2+ channels were activated by IH in the absence of a measurable depolarization (Fig. 5), suggesting that the activation is not the result of membrane depolarization. It is possible, however, that L-type Ca2+ channels are expressed on the dendrites and that the Vm measured at the soma (as in this study) does not reflect the actual Vm at the site where these channels are expressed. L-type Ca2+ channels are expressed both in the soma as well as in the dendrites in other neurons (14, 28). Thus the possibility of L-type Ca2+ channel activation during acid challenge by depolarization in the dendritic processes cannot be ruled out.

It is also possible that L-type Ca2+ channels are activated by acid challenges in LC neurons by a depolarization-independent pathway, possibly involving a protein kinase. In another type of chemosensitive cell, the peripheral chemoreceptors or type-I glomus cells (30), L-type Ca2+ channels were shown to be activated in the presence of hypoxia (55, 61). Although hypoxia resulted in increased membrane excitability in these cells through both a membrane depolarization and activation of Ca2+ channels (61), it was shown that the mechanism of activation of Ca2+ channels was not directly linked to Vm changes but was attributed to a PKC-mediated pathway (56).

Is it clear that activation of L-type Ca2+ channels in LC neurons is not mediated solely by intracellular acidification because IH and propionate result in the same fall of pHi (26), but Ca2+ channels are activated by IH but not by propionate (Fig. 7). Further studies will be required to delineate the actual signaling pathway by which acid challenges activate L-type Ca2+ channels in LC neurons.

Effects of acidosis on Vm in LC neurons: evidence for activation of multiple K+ channels. When both L-type Ca2+ channels and NA+ spikes were inhibited, HA exposure resulted in a membrane depolarization. This depolarization is independent of L-type Ca2+ channel activation because it is observed even when Ca2+ channel blockers are present (Figs. 3, A and B, and 4). Its presence suggests that K+ channels are inhibited by HA. The depolarization may in fact be larger in the absence of Ca2+ channel blockers due to activation of inward Ca2+ currents, but it is hard to measure because of the presence of spontaneous NA+ spikes. It is thus possible that Ca2+ channel activation also contributes to the overall membrane depolarization during the chemosensitive response.

A number of different K+ channels have been shown to be sensitive to changes of either intracellular or extracellular pH. pHi sensitivity has been observed in members of the Kt (12, 39), the KCa (31, 49), and the Kv (6) channel family. pHi sensitivity has been observed in members of the TASK channel family (5, 22). The pH sensitivity of these channels in neurons is of particular physiological importance because inhibition of any of these channels could increase neuronal excitability. Many of the known pH-sensitive K+ channels are expressed in LC neurons (Ktr, KCa, and TASK) (34, 39, 57), making them potential targets in the chemosensitive pathway of these neurons. Our findings suggest that at least two K+ channels are inhibited by acid stimuli: a TEA-sensitive K+ channel (possibly Kt, KCa, and/or Kv) that is inhibited by intracellular acidification (such as with HA and IH); and TASK channels, inhibited by extracellular acidification (such as with HA but not with IH). Furthermore, the extent of K+ channel inhibition and the types of K+ channel(s) affected probably vary with the degree of intracellular acidification.

One factor to keep in mind when interpreting these data is that the contribution of K+ channel inhibition to membrane depolarization during an acid challenge...
was assessed in the presence of Ca\(^{2+}\) and Na\(^+\) channel blockers. This is important because channel blockage could change the membrane input resistance and thus the excitability of the neuron. Along these lines, it is also possible that blockage of both Ca\(^{2+}\) and Na\(^+\) channels alters the actual contribution of K\(^+\) channel inhibition to the chemosensitive response of LC neurons. The full identification of the specific channels affected by chemosensitive stimuli and their role in LC neuron responses must await voltage-clamp studies.

The general model for chemosensitivity assumes that a low neuronal pH inhibits a K\(^+\) channel resulting in a membrane depolarization, as is often observed (17, 35, 36, 42, 63, 64). Our data suggest a more complex relationship between pH changes and channel activation/inactivation, indicating multiple K\(^+\) channel targets and a major role for pH\(_{i}\) in setting resting V\(_m\) in the presence of a chemosensitive stimulus.

The role of Ca\(^{2+}\) channel activation in the chemosensitive response of LC neurons. In the present study, we found a clear relationship in LC neurons between the activation of L-type Ca\(^{2+}\) channels (Fig. 7B) and the increase in firing rate (26). We found that both HA and IH increased L-type Ca\(^{2+}\) channel activity and firing rate, but that propionate, which did not increase Ca\(^{2+}\) channel activity, also did not increase firing rate. This relationship is further strengthened by the fact that on removal of propionate, we see a small increase in both LC neuron firing rate (26) and Ca\(^{2+}\) channel activation (Fig. 7). This positive correlation between Ca\(^{2+}\) channel activation and increased firing rate in LC neurons suggests that activation of L-type Ca\(^{2+}\) channels plays a role in the increased firing rate of these neurons in response to acid challenges.

The involvement of L-type Ca\(^{2+}\) channels in the chemosensitive response of LC neurons was further indicated by the ability of Ca\(^{2+}\) channel blockers (Co\(^{2+}\) and nifedipine) to reduce the increased firing rate induced by HA (Figs. 8B and 10) and to eliminate the increased firing rate induced by IH (Figs. 9B and 10). We are aware of only one previous study showing that Ca\(^{2+}\) channel blockers can affect central chemosensory signaling. Wellner-Kienitz et al. (64) showed that Cd\(^{2+}\) (a putative L-type Ca\(^{2+}\) channel blocker), but not Ni\(^{2+}\) (a putative T-type Ca\(^{2+}\) channel blocker), inhibited the response to HA of medullary neurons in organotypic culture. These authors attributed this inhibition to an indirect effect of Ca\(^{2+}\) channel blockers on K\(_{Ca}\) channels (64). We believe that activation of Ca\(^{2+}\) channels may be directly involved in the chemosensitive response to HA and IH. First, Ca\(^{2+}\) channel activation has been implicated in chemosensory signaling in invertebrate neurons (23, 24). Second, HA-induced increased firing rate is only partially inhibited (Figs. 8B and 10), whereas IH-induced Ca\(^{2+}\) channel activation is completely inhibited (Figs. 3, 4, and 5) by Ca\(^{2+}\) channel blockers. Finally, propionate induces the same change of pH\(_{i}\) as IH but does not result in any Ca\(^{2+}\) channel activation (Fig. 7A) or in any increase in firing rate (26). Thus we propose that the activation of Ca\(^{2+}\) channels is a part of the activation of LC neurons by chemosensitive stimuli. Additional experiments are required to determine whether it is channel activation per se or the accumulation of intracellular Ca\(^{2+}\) that is important for LC neuron activation.

Chemosensitive signaling pathway in LC neurons. We propose a chemosensitive signaling pathway in LC neurons that is the sum of numerous activation processes involving multiple signals and targets. In this model, the activation of LC neurons by HA is the result of three activation steps: the inhibition of one or more TEA-sensitive K\(^+\) channels by intracellular acidification, the inhibition of TASK channels by extracellular acidification, and the activation of Ca\(^{2+}\) channels. When Ca\(^{2+}\) channels are inhibited, the other stimuli are sufficient to partially increase firing rate (Fig. 8B). In contrast, the activation of LC neurons by IH is the result of only two activation steps: the inhibition of one or more TEA-sensitive K\(^+\) channels by intracellular acidification and the activation of Ca\(^{2+}\) channels. TASK channels are not inhibited because there is no extracellular acidification. When Ca\(^{2+}\) channels are inhibited, IH only results in inhibition of TEA-sensitive K\(^+\) channels, which is presumed to be insufficient by itself to activate LC neurons (Fig. 9B). Finally, exposure of LC neurons to propionate only involves the inhibition of one or more TEA-sensitive K\(^+\) channels. TASK channels are not inhibited because there is no extracellular acidification and there is no Ca\(^{2+}\) channel activation (Fig. 7). Once again, this is insufficient to lead to an increase in firing rate of LC neurons (26). If medullary neurons are proposed not to express TASK channels, this model could also explain why Wellner-Kienitz et al. (64) observed complete blockage of HA activation of medullary neurons by Ca\(^{2+}\) channel blockers.

A similar model has been proposed by Richerson (43). His argument was that the response of a given chemosensitive neuron could be the sum of the effects of decreased pH on multiple targets. Such a system could lead to a large chemosensitive neuronal response to even a small change of pH\(_{i}\), by summing several small responses, and thereby explain the finding that ventilation is highly sensitive to even small changes of pH\(_{i}\) (25). Another possibility exists. It has been shown in at least one invertebrate preparation, the terrestrial snail, that chemosensitive neurons respond to hypercapnia by activating Ca\(^{2+}\) channels (23, 24). If this is a general feature of the chemosensitive response of invertebrate neurons, and K\(^+\) channels are not largely involved, the activation of Ca\(^{2+}\) channels by chemosensitive signals seen in LC neurons in the current study could represent a vestigial “invertebrate mechanism” with a “vertebrate mechanism,” involving K\(^+\) channel inhibition and Na\(^+\) channel activation, superimposed. This would imply a phylogenetic basis for the multiple effects of acid stimuli in chemosensitive neurons. Conversely, future studies may reveal marked similarities between chemosensory signaling in invertebrate and vertebrate neurons, suggesting a remarkable convergent evolution of chemosensory pathways, as previously suggested by Erlichman and Leiter (23). The
recent finding of HA-induced inhibition of K⁺ channels, as well as activation of Ca²⁺ channels, in chemosensitive neurons from snail (21) supports convergent evolution of the chemosensory pathways in snails and mammals.

Finally, we have studied LC neurons from neonatal rats (P1–9) exclusively. It is likely that the activity and expression of various ion channels changes with age, and it is known that the frequency and presence of SROs vary with development (65). Thus the chemosensory signaling pathway described in this study is most relevant for LC neurons from neonatal rats. Future studies in adult rats are needed to determine whether this pathway is maintained throughout development.

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