Postnatal development and activation of L-type Ca$^{2+}$ currents in locus ceruleus neurons: implications for a role for Ca$^{2+}$ in central chemosensitivity

Ann N. Imber and Robert W. Putnam

Department of Neuroscience, Cell Biology and Physiology, Wright State University Boonshoft School of Medicine, Dayton, Ohio

Submitted 21 December 2011; accepted in final form 6 March 2012

Imber AN, Putnam RW. Postnatal development and activation of L-type Ca$^{2+}$ currents in locus ceruleus neurons: implications for a role for Ca$^{2+}$ in central chemosensitivity. J Appl Physiol 112: 1715–1726, 2012. First published March 8, 2012; doi:10.1152/japplphysiol.01585.2011.—Little is known about the role of Ca$^{2+}$ in central chemosensory signaling. We use electrophysiology to examine the chemosensitive responses of tetrodotoxin (TTX)-insensitive oscillations and spikes in neurons of the locus ceruleus (LC), a chemosensitive region involved in respiratory control. We show that both TTX-insensitive spikes and oscillations in LC neurons are sensitive to L-type Ca$^{2+}$ channel inhibition and are activated by increased CO$_2$/H$^+$ concentrations. Spikes appear to arise from L-type Ca$^{2+}$ channels on the soma whereas oscillations arise from L-type Ca$^{2+}$ channels that are distal to the soma. In HEPES-buffered solution (nominal absence of CO$_2$/HCO$_3^-$), acidification does not activate either oscillations or spikes. When CO$_2$ is increased while extracellular pH is held constant by elevated HCO$_3^-$, both oscillation and spike frequency increase. Furthermore, plots of both oscillation and spike frequency vs. intracellular [HCO$_3^-$]$_i$ show a strong linear correlation. Increased frequency of TTX-insensitive spikes is associated with increases in intracellular Ca$^{2+}$ concentrations. Finally, both the appearance and frequency of TTX-insensitive spikes and oscillations increase over postnatal ages day 3–16. Our data suggest that 1) L-type Ca$^{2+}$ currents in LC neurons arise from channel populations that reside in different regions of the neuron, 2) these L-type Ca$^{2+}$ currents undergo significant postnatal development, and 3) the activity of these L-type Ca$^{2+}$ currents is activated by increased CO$_2$ through a HCO$_3^-$-dependent mechanism. Thus the activity of L-type Ca$^{2+}$ channels is likely to play a role in the chemosensitive response of LC neurons and may underlie significant changes in LC neuron chemosensitivity during neonatal development.

calcium; electrophysiology; hypercapnia; ventilation; bicarbonate; calcium oscillations; tetrodotoxin

CENTRAL RESPIRATORY CONTROL has been shown to involve multiple locations within the brain stem. These areas contain neurons whose firing rates are altered in response to changes in CO$_2$/H$^+$, referred to as chemosensitive neurons (13, 26, 34). One area identified as being involved in central chemoreception is the locus ceruleus (LC) (5, 10, 15). Most research on the chemosensitivity of LC neurons and other chemosensitive areas of the brain stem have focused on the role of pH-sensitive ion channels, especially K$^+$ channels, as the basis for neuronal chemosensitive signaling (32, 33). Little is known, however, about the potential role of Ca$^{2+}$ ions in central chemosensitive signaling.

The cellular basis for the firing rate response to hypercapnia of chemosensitive neurons is not fully known. It is believed to be due to CO$_2$-induced changes of pH inhibiting acid-sensitive channels (34). Thus studies of CO$_2$-sensitive cellular mechanisms by necessity include H$^+$-sensitive mechanisms, and both intra (pH$_i$) and extra (pH$_e$)-cellular pH changes have been considered as possible chemosensitive stimuli (14, 28, 33). For example, numerous acid-sensitive K$^+$ channel targets for hypercapnia-induced acidification have been demonstrated in LC neurons, including inwardly rectifying K$^+$ channels, TASK channels, an A current, and a delayed-rectifying K$^+$ channel (16, 21, 32). It has been proposed that the magnitude of the firing rate increase in response to hypercapnia is the result of acid-induced inhibition of these multiple K$^+$ channels, which would decrease the outward K$^+$ conductance, leading to depolarization and increased firing rate in response to CO$_2$ (16).

Evidence in LC neurons suggests that Ca$^{2+}$ may also play a role in chemosensitive signaling. When the fast Na$^+$-channel blocker tetrodotoxin (TTX) is applied to block Na$^+$ action potentials in LC neurons, either TTX-insensitive action potentials (spikes) (45), smaller rhythmic membrane potential (V$_{m}$) oscillations (9, 18, 23), or both can be observed. TTX-insensitive oscillations are inhibited by cobalt, cadmium, high Mg$^{2+}$ (11.5 mM), or the L-type Ca$^{2+}$-channel inhibitor nifedipine, suggesting they arise from Ca$^{2+}$ channels (9, 15, 30, 45). TTX-insensitive spikes are not as frequently reported, but are also inhibited by Ca$^{2+}$-free solutions (45). These findings strongly suggest the presence of Ca$^{2+}$ channels in LC neurons.

The possible significance of Ca$^{2+}$ channels in the neuronal chemosensitive response is profound (4, 12, 24). Multiple studies have documented the importance of extracellular Ca$^{2+}$ to intracellular signaling in cultured H$^+$-sensitive PC12 pheochromocytoma cells (39–41). The injection of an intracellular Ca$^{2+}$ chelating agent into chemosensitive areas of the ventral medullary surface decreased the adaptive ventilatory response to hypercapnia in rats (20). In peripheral chemoreceptors, increases in [HCO$_3^-$], associated with hypercapnia result in the phosphorylation and activation of membrane Ca$^{2+}$ channels, thereby resulting in an increase in intracellular Ca$^{2+}$ levels (Ca$^{2+}_{i}$) and enhanced exocytosis of neurotransmitters (42). Similarly in LC neurons, TTX-insensitive oscillations can be activated by high CO$_2$/HCO$_3^-$ in the absence of a change in pH$_e$ (15). These studies strongly suggest a role for Ca$^{2+}$ in the chemosensitive response of LC neurons.

Despite the apparent role of these Ca$^{2+}$-based currents in chemoreception, variations in preparations among different studies have prevented a clear, direct assessment of the role of observed oscillations and spikes in the chemosensitive response of LC neurons. Much of the previous work on TTX-insensitive oscillations and spikes in LC neurons has focused on their general characteristics and their relationship to cell-cell signaling (18, 45). In several studies, not all LC neurons
show oscillations or spikes in the presence of TTX (9, 30). In others, oscillations appear to be due to Na\(^+\) channels and can be completely inhibited by TTX (18, 29). When TTX-insensitive oscillations or spikes were not observed, Ba\(^+\) or Ba\(^2+\) and tetraethylammonium (TEA) were used to induce both oscillation and spike activity (2, 18). Thus further work needs to be done to characterize the nature of and the conditions that promote the appearance of Ca\(^2+\) currents and the role of those Ca\(^2+\) currents in the chemosensitive response of LC neurons.

Interestingly, recent evidence suggests that the firing rate response of LC neurons to hypercapnia changes during early neonatal development, decreasing markedly in LC neurons from rats older than postnatal day 10 (P10) (16). Immunohistochemical studies in mice and rats have noted marked quantities of Ca\(^2+\)-sensitive proteins including large conductance Ca\(^2+\)-activated K\(^+\) channels (6, 37). We hypothesize that Ca\(^2+\) may be the main factor in this developmental transition. Consistent with this, we suggest that either TTX-insensitive spikes or oscillations arise from Ca\(^2+\)-sensitive channels on the cell membrane of chemosensitive LC neurons. We hypothesize that these Ca\(^2+\) channels are activated by a HCO\(_3^-\)-dependent mechanism, and not by changes in pH/pHa. Activation of these channels is expected to elevate Ca\(^2+\), potentially affecting intracellular mechanisms. We speculate that this elevated Ca\(^2+\) serves to activate Ca\(^2+\)-dependent K\(^+\) channels and thereby reduce the firing rate response of LC neurons to hypercapnia. This could be the basis for the decreased firing rate response of LC neurons during development if Ca\(^2+\) oscillations/spikes show a corresponding increased activation after age P10.

The purpose of the current study was therefore to examine the presence of natively occurring oscillations and spikes in LC neurons from neonatal rats ages P3–P16 to: 1) determine whether observed oscillations and spikes are Ca\(^2+\)-based using both TTX and nifedipine; 2) study the role of changes in pH and HCO\(_3^-\) in hypercapnia-induced activation of oscillations/spikes; 3) show that activation of these Ca\(^2+\) currents by hypercapnia results in increased intracellular Ca\(^2+\) levels; and 5) determine whether these Ca\(^2+\) currents exhibit marked increases during early postnatal development in LC neurons.

A preliminary account of some of this work was published previously (17).

METHODS

Slice preparation. Mixed sex neonatal Sprague-Dawley rats postnatal age P3–P16 were anesthetized using a CO\(_2\) overdose or hypothermia and rapidly decapitated. Removal of the brain stem and subsequent coronal brain slicing using a vibratome (PelcoVibratome 1000) were carried out in ice-cold (4–6°C) artificial cerebrospinal fluid (aCSF) solution as previously described (15, 35). Slices containing the LC region were then incubated in room temperature aCSF equilibrated with 95% O\(_2\)/5% CO\(_2\) until use 1–4 h after slicing. During experiments, slices were superfused continuously by gravity flow (~4 ml/min) using solutions held at 35°C. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Wright State University and were in agreement with standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by American Association for Accreditation of Laboratory Animal Care and is covered by National Institutes of Health Assurance (no. A3632–01).

Solutions. Unless otherwise specified, all brain slices were immersed in aCSF solution. This solution consisted of (in mM): 124 NaCl, 1.3 MgSO\(_4\), 5 KCl, 1.24 KH\(_2\)PO\(_4\), 10 glucose, 2.4 CaCl\(_2\), 26 NaHCO\(_3\), and was equilibrated with 95% O\(_2\)/5% CO\(_2\) (pH 7.45 at 35°C). Hypercapnic acidotic solutions were identical except for being equilibrated with 85% O\(_2\)/15% CO\(_2\), pH 6.9, or in one experiment 90% O\(_2\)/10% CO\(_2\), pH 7.10. This percentage of CO\(_2\) was chosen to maximize cellular effects of hypercapnic acidosis (28, 35). Isydoric hypercapnic solutions were identical to hypercapnic acidotic solutions except that NaHCO\(_3\) was raised to 77 mM replacing NaCl isosmotically, bringing the pH back to 7.45. During experiments, slices containing LC neurons were superfused with aCSF containing 1 μM TTX to study the TTX-insensitive current. In solutions without CO\(_2\)/NaHCO\(_3\), the NaHCO\(_3\) in aCSF was replaced isosmotically with HEPES buffer and the solution was equilibrated with 100% O\(_2\). HCl and NaOH was used to pH the HEPES aCSF solution to 7.45 and 6.9, resembling the normal aCSF and hypercapnic acidotic solutions, respectively. The whole cell pipette filling solution consisted of (in mM): 130 K-glucurate, 0.4 EGTA, 1 MgCl\(_2\), 0.3 GTP, 2 ATP, and 10 HEPES, plus either 50 μM pyramine or 250 μM Fura-2. The pipette filling solution pH was buffered to ~7.35 using KOH. The filling solution was designed with low EGTA and no added Ca\(^2+\) to minimize washout of the chemosensitive response (15).

All of our external solutions were equilibrated with the standard high levels of O\(_2\) (95%-100%). It has been pointed out that this is hyperoxic to in vivo levels of O\(_2\) within the brain and often results in hyperexcitability of neurons (11). Although we do not know what impact this may have on the response of Ca\(^2+\) channels to hyperoxia, we chose to use this level of O\(_2\) to compare our results to earlier studies and because no generally agreed upon lower level of O\(_2\) is currently available.

Measurement of intracellular pH/Ca\(^2+\). The pH-sensitive fluorescent dye pyrane (50 μM) or the Ca\(^2+\)-sensitive dye Fura-2 (250 μM) was added to the pipette filling solution and loaded into LC neurons using whole cell patch pipettes as previously described (35). Pyramine-loaded neurons were excited alternately at 450 and 415 nm by light from a 75-W xenon arc lamp for pH-sensitive and -insensitive recordings, respectively, using a Sutter Lambda 10–2 filter wheel. Fura-2-loaded neurons were alternately excited at 340 and 380 nm. Emitted fluorescence at 515 (pyramine) or 505 nm (Fura-2) was directed to the Nikon multi-image port module and then to a GenII Sys Image intensifier and a CCD camera. Subsequent fluorescence images were acquired using a Gateway 2000 E-3100 computer and collected/processed using the software MetaFluor 4.6r. Image acquisition could be achieved within ~2 s and was repeated every 60 s for pyramine and every 30 s for Fura-2. Light was blocked between acquisitions to reduce photo bleaching. For pyramine, the 450/415 fluorescence ratio (R\(_{450}\)) was determined and the following calibration curve was used to convert to intracellular pH (pH\(_I\)): pH\(_I\) = 7.5651 + log([N\(_A\) − 0.1459]/(2.0798 − N\(_A\))), where the experimental R\(_{450}\) values were divided by a calibration R\(_{450}\) value at pH 7.4 to yield N\(_A\) (35). The Fura-2 fluorescence was not calibrated, and arbitrary fluorescence units were used instead to monitor increases or decreases in R\(_A\) and thus increases or decreases in Ca\(^2+\).

Electrophysiological recordings. Whole cell recordings were used throughout this study. Pipettes were made from thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm) pulled to a tip resistance of ~5 MΩ as previously described (15, 27, 35). LC neurons were visualized using an upright microscope (Nikon, eclipse microscope 80i) with a ×60 water-immersion objective and subsequently patched via formation of a gigohm seal with the cell membrane. Membrane potential (Vm) was measured in current clamp mode and current injected via an Axopatch 200B amplifier. Firing rate (FR) was measured using a slope/height window discriminator (FHC model 90-24) by 10.220.32.246 on April 18, 2017 http://jap.physiology.org/ Downloaded from

J Appl Physiol • doi:10.1152/japplphysiol.01585.2011 • www.jappl.org
resting $V_m$ of $-45$ to $-60$ mV and a spontaneous firing rate of $<4$ Hz. The reversibility of all electrophysiological responses to altered superfusate solutions was verified by a return to baseline values upon change of the solution back to normal aCSF. Patched recordings of TTX-insensitive currents using the above techniques lasted $>45$ min without any evidence of washout of the response (15), and electrophysiological responses to most solution changes were observed in $<2$ min. Multiple hypercapnic pulses caused the same activation of the chemosensitive Ca$^{2+}$ current under investigation without a decrease in response.

Drugs. TTX, carbenoxolone, pyranine, nifedipine, and Fura-2 were purchased from Sigma-Aldrich (St Louis, MO). Nifedipine was prepared as a stock solution of 50 mM in EtOH prior to use. TTX, pyranine, and Fura-2 stocks were prepared in dH$_2$O.

Data analysis and statistics. Where applicable, analysis for changes in frequency ($\Delta$frequency) was calculated by the following: $\Delta$frequency = [(hypercapnic average frequency − control average frequency)/control average frequency] $\times 100\%$. All values are expressed as means ± SE. Significant differences between two means were determined by Student’s $t$-tests or paired $t$-tests. Comparisons of more than two means were assessed using ANOVA with multiple paired comparisons. In all cases, means were considered significantly different if $P \leq 0.05$.

RESULTS

Ca$^{2+}$ oscillations and spikes in LC neurons. When TTX is applied to block Na$^+$ action potentials in a whole cell-patched LC neuron, a rhythmic, TTX-insensitive current, can be observed as either a small amplitude oscillation or larger amplitude spikes. Figure 1A shows the typical appearance for both TTX-insensitive oscillations and spikes from neonatal LC neurons in 5% CO$_2$-aCSF plus TTX. Oscillations observed in 30 neurons from 12 slices from neonatal rats aged P8–P13 occur at a frequency of 0.3–1.0 Hz and are $\sim 10$ mV in amplitude. Rapid depolarizing TTX-insensitive spikes with amplitudes between 20 and 40 mV often synchronize with the depolarizing rise of oscillations. In agreement with previous studies (15), our data demonstrate that the addition of the L-type Ca$^{2+}$ current blocker nifedipine completely and reversibly eliminates both TTX-insensitive oscillations and spikes ($n = 5$, neurons from 5 slices; Fig. 1, B and C). This suggests that both oscillations and spikes arise from the activity of L-type Ca$^{2+}$ channels.

Despite the similar pharmacological characteristics of oscillations and spikes, they show differing voltage responses under whole cell patch conditions. Figure 2A shows the sensitivity of spikes to changes in $V_m$ due to injected current through a whole cell patch on the LC soma. These results were repeated in 23 neurons from 14 slices from neonates aged P5–P14. Typically, TTX-insensitive spikes appear upon depolarizations to approximately $-35$ mV or greater. Depolarizations from the average resting membrane potential of approximately $-45$ mV to $-35$ mV can be observed upon exposure to TTX. When additional depolarizing current is applied, large increases in spike frequency can be observed, whereas hyperpolarizing current can completely eliminate the appearance of spikes, indicating that TTX-insensitive spikes are voltage sensitive (Fig. 2A). In contrast, TTX-insensitive oscillations appear to be largely insensitive to changes of $V_m$ in the LC neuron soma. Figure 2B shows a step-wise hyperpolarization of the LC soma resting $V_m$ to approximately $-70$ mV with no observed change in either the amplitude or frequency of oscillations.

These observations were repeated in 14 LC neurons from 10 slices from neonates aged P7–P16. As oscillations still demonstrate sensitivity to the L-type Ca$^{2+}$ channel inhibitor nifedipine (Fig. 1B), it is possible that the oscillations arise from channels located at some distance from the soma. In these distal regions, $V_m$ changes observed in the soma would be largely attenuated. Thus TTX-insensitive spikes and oscillations may arise from channels with similar electrophysiological and pharmacological characteristics, but respond differently to changes of $V_m$ due to being located in different regions of an LC neuron. Consistent with this hypothesis, the addition of 100 $\mu$M carbenoxolone was shown to inhibit TTX-insensitive oscillations, but not spikes, in 3 LC neurons from 3 slices aged...
This suggests that TTX-insensitive oscillations are dependent upon communication via gap junctions whereas spikes are not (Fig. 2C). The conclusion that channels causing the rhythmic, TTX-insensitive oscillations are located some distance from the LC soma is also supported by intracellular Ca\(^{2+}\)/H11001 studies. Figure 3, A and B, shows the measurement of intracellular Ca\(^{2+}\)/H11001 in the soma of two different LC neurons in the presence of TTX. In Fig. 3A, the neuron demonstrated TTX-insensitive spikes but not oscillations. Simultaneous electrophysiological and imaging studies of this neuron showed that increases in spike frequency correlated with a concurrent increase in somal Ca\(^{2+}\) concentrations (increase in Rfl; Fig. 3A). When hyperpolarizing current was injected to inhibit the TTX-insensitive spikes, intracellular Ca\(^{2+}\) rapidly returned to baseline levels. In Fig. 3B, the neuron was hyperpolarized to remove the presence of spikes, and oscillations were transiently stimulated by the addition of 15% CO\(_2\) (hypercapnic acidotic solution) (n = 6 from 6 slices in rats aged P5–P7; see Fig. 6). Increased oscillation frequency (typically from 0 to 0.5 Hz) was not associated with a change in the soma Ca\(^{2+}\) level (a typical resting Rfl value might be between 0.79 and 0.82, with no change between 5% and 15% CO\(_2\) as in Fig. 3B). This is consistent with TTX-insensitive spikes arising from the activation of Ca\(^{2+}\) channels located in the soma, whereas oscillations arise from Ca\(^{2+}\) channels located distal to the soma. The magnitude of the increase in somal Ca\(^{2+}\) concentrations appears to be a function of TTX-insensitive spike frequency with smaller changes of somal intracellular Ca\(^{2+}\) (<0.1 Rfl) being associated with relatively small increases in spike frequency (n = 10 neurons from 7 slices in rats aged P7–P13), whereas larger changes of intracellular Ca\(^{2+}\) (>0.1 Rfl) were associated with larger increases in spike frequency (n = 7 neurons from 4 slices in rats aged P8–P13; Fig. 3C). These data indicate the presence of L-type Ca\(^{2+}\) channels in the somal membrane.
capable of increasing somal Ca$^{2+}$ in LC neurons in a fashion that is dependent on spike frequency.

Effects of CO$_2$ and pH on Ca$^{2+}$ spikes and oscillations. To test the CO$_2$ sensitivity of the L-type Ca$^{2+}$ currents in the largely chemosensitive neurons from the LC, we exposed neurons from neonatal animals between P10 and P14 to HEPES-buffered aCSF at pH 7.45 (nominal absence of CO$_2$/HCO$_3^-$). In the absence of CO$_2$/HCO$_3^-$, the TTX-insensitive oscillations that were observed were very small, varied in appearance, and were sometimes completely absent ($n = 7$ from 4 slices from rats aged P11–P13; Fig. 4A). TTX-insensitive spikes were also absent in the HEPES-buffered solutions, but could be induced by membrane depolarizations to above −25 mV (Fig. 4A, inset). Restoring normocapnic, HCO$_3^-$-buffered aCSF to the same neuron restored the normal appearance of both TTX-insensitive oscillations and spikes. Notice the appearance of spikes without the addition of depolarizing current. C: returning to HEPES-buffered aCSF solution once again results in the inhibition of both spikes and oscillations.

Hypercapnic acidosis reversibly increased spike (Fig. 5A) and oscillation (Fig. 5B) frequency, as previously observed (15, 29). Because hypercapnic acidosis appears to activate L-type Ca$^{2+}$ channels, we tested whether it is a change of CO$_2$, HCO$_3^-$, or pH that is the basis for activation. For these studies we used isohydric hypercapnic solutions, in which the HCO$_3^-$ concentration is increased to maintain extracellular pH ($pH_e$) at 7.45 when equilibrated with 15% CO$_2$. To monitor $pH_e$ changes, we loaded the neuron with the pH-sensitive dye pyranine through the whole cell patch. Figure 6A shows the normal decrease in $pH_e$ due to exposure to hypercapnic acidosis (~0.15 pH unit), and the smaller decrease in $pH_e$ due to exposure to isohydric hypercapnia (~0.05 pH unit). Despite the marked larger changes in both $pH_e$ and $pH_i$ in hypercapnic acidotic vs. isohydric hypercapnic solutions, both oscillations and spikes demonstrated a similar increase in frequency when exposed to 15% CO$_2$ (Fig. 6B). This is clearly shown in Fig. 6C, in which spike frequency increases in a dose-dependent
fashion with respect to changes of CO₂ but not with respect to changes of pHₐ or pHᵢ. Here, the change in spike frequency was higher from exposure to 15% CO₂ than 10% CO₂ (n/11005 12 neurons from 10 slices in rats aged P9–P14), whereas there was no difference between the increases caused by 15% CO₂ and isohydric hypercapnia (n = 6 neurons from 5 slices in rats aged P8–P14). These data suggest that changes in pH are not necessary for the CO₂-dependent activation of the L-type Ca²⁺ current in LC neurons.

L-type Ca²⁺ channels from peripheral chemoreceptors were activated in hypercapnia by a pathway that involves increased intracellular HCO₃⁻ ([HCO₃⁻]ᵢ) (42). Interestingly, isohydric hypercapnia resulted in a somewhat higher increase in spike frequency than hypercapnic acidosis (Fig. 6C). In 6 of the neurons studied, spike frequency was measured in both isohydric hypercapnia and hypercapnic acidosis. In this subset of LC neurons, the increase in spike frequency in response to isohydric hypercapnia was found to be significantly higher than the response to hypercapnic acidosis (P < 0.001). This correlates with higher calculated values of [HCO₃⁻]ᵢ in isohydric hypercapnia than in hypercapnic acidosis. We further studied whether increased [HCO₃⁻]ᵢ could be involved in the activation of L-type Ca²⁺ channels by CO₂ in LC neurons. On the basis of our measurements of pHᵢ and the level of CO₂, we calculated [HCO₃⁻]ᵢ using the Henderson-Hasselbalch equation. A plot of TTX-insensitive spike frequency vs. [HCO₃⁻]ᵢ (Fig. 7A) (n = 11 from 8 slices aged P8–P14). Figure 7B shows the same relationship for the frequency of TTX-insen-
sitive oscillations \((n = 34\) from 20 slices aged P3–P16). Both charts indicate a strong correlation between \([\text{HCO}_3^-]\) and the frequency of the L-type TTX-insensitive current, suggesting that the activation of L-type \(\text{Ca}^{2+}\) channels by elevated \(\text{CO}_2\) may be mediated by \([\text{HCO}_3^-]\).

**Neonatal development of L-type \(\text{Ca}^{2+}\) channels in LC neurons.** Consistent with our hypothesis and the developmental changes noted by Gargaglioni et al. (16), we noticed variation in the frequency of \(\text{Ca}^{2+}\) oscillations among animals when studying the effects of \([\text{HCO}_3^-]\). This variation appeared to be related to the age of the rat from which a neuron was studied, so we examined the effects of \([\text{HCO}_3^-]\) on \(\text{Ca}^{2+}\) oscillation frequency as a function of age (Fig. 7B). For neurons from rats older than P6, the frequency of oscillations was positively correlated with \([\text{HCO}_3^-]\). Furthermore, the oscillation frequency increased as the neonatal animal aged. Fit values are as follows: P3–P5, \(-R^2 = 0.0574\) (NS) \((n = 7\) from 6 slices); P7–P9, frequency = 0.0173 \([\text{HCO}_3^-]\), \(-0.2398, R^2 = 0.69\) \((P < 0.001)\) \((n = 9\) from 6 slices); P10–P12, frequency = 0.0119 \([\text{HCO}_3^-]\), \(+0.438, R^2 = 0.52\) \((P < 0.001)\) \((n = 14\) from 7 slices); and >P13, frequency = 0.0105 \([\text{HCO}_3^-]\), \(+1.217, R^2 = 0.48\) \((P < 0.01)\) \((n = 4\) from 2 slices). These results suggest that L-type \(\text{Ca}^{2+}\) channels that are distal to the soma show considerable development during the early postnatal period.

To examine the effects of \([\text{HCO}_3^-]\) on spike frequency, it was necessary to control for voltage sensitivity. Neurons with spike frequencies from similar \(V_{\text{m}}\) values only were included in the data set in Fig. 7A. As with oscillations, spikes showed a strong positive correlation with \([\text{HCO}_3^-]\) \((n = 11\) from 8 slices aged P8–P14; fig. 7A). Due to the dependence of spike frequency on both \([\text{HCO}_3^-]\) and voltage, neurons were selected for this study based on the appearance of spikes between a control voltage of \(-32\) to \(-37\) mV. Neurons meeting these criteria were observed from rats aged P8–P14, and so developmental changes in spike frequency were not accurately represented. However, when spike frequencies were observed under normocapnic conditions as a control for \([\text{HCO}_3^-]\) in LC neurons, a strong dependence of spike frequency on age was seen (Fig. 8). Thus \(\text{Ca}^{2+}\) spikes and oscillations in LC neurons appear to be dependent upon \([\text{HCO}_3^-]\), and to undergo developmental increases from ages P3 to P16.

We studied in detail the age dependence of the appearance of \(\text{Ca}^{2+}\) oscillations and spikes in LC neurons in the presence of 5% \(\text{CO}_2\) and 15% \(\text{CO}_2\). A transition period existed (P4–P9) whereby the appearance of \(\text{Ca}^{2+}\) spikes and oscillations were highly variable and their amplitudes were smaller. Prior to age \(\sim\)P9 the \(\text{Ca}^{2+}\) currents observed in TTX were usually only seen after activation by hypercapnia \((n = 15\) in 8 slices; Fig. 9A). Upon return to normocapnia, the \(\text{Ca}^{2+}\) currents were no longer visible. During this transition period, TTX-insensitive oscillations activated by hypercapnic acidosis were often small in amplitude and frequency, and spikes were often absent without additional depolarizing current (Fig. 9B). After age \(\sim\)P10, TTX-insensitive oscillations and spikes were larger, and both oscillations and spikes occurred spontaneously without activation by hypercapnia. Our findings are summarized in Fig. 10. Of 35 neurons from 20 slices, 23 exhibited spikes. There were 23 neurons in this group younger than P9, 12 of which did not demonstrate spikes. The majority of neurons that did not show spikes \((n = 10\) were from rats younger than P7. Thus \(\text{Ca}^{2+}\) spikes are either largely absent or occur only in the presence of 15% \(\text{CO}_2\) in LC neurons from rats younger than P8–P9 (Fig. 10A). However, \(\text{Ca}^{2+}\) spikes were observed in most LC neurons from rats age P8–P9 even in the absence of hypercapnia. Spikes were also observed in all LC neurons from rats older than P10, both in 5% and 15% \(\text{CO}_2\) (Fig. 10A). A similar pattern of development was seen for \(\text{Ca}^{2+}\) oscillations (Fig. 10B). From 26 neurons aged younger than P9, 8 did not show oscillations, and the majority of these neurons were from animals younger than P7. In 57% \((n = 15)\) of neurons younger than P9, oscillations could be evoked by hypercapnia, whereas oscillations were observed in all 20 neurons from 11 slices aged P10 to P16. Once again, most LC neurons from rats younger than P10 showed no \(\text{Ca}^{2+}\) oscillations or only showed oscillations in the presence of 15% \(\text{CO}_2\), but both oscillations and spikes were omnipresent in LC neurons from rats older than \(\sim\)P10 (Fig. 10B). From this data set, there were 8 neurons from animals younger than P7 that showed neither oscillations nor spikes under any conditions. These data strongly suggest that \(\text{Ca}^{2+}\) channels develop markedly during the neonatal period in rat LC neurons.

**DISCUSSION**

In this study, we have systematically characterized the appearance and development of chemosensitive TTX-insensitive current in LC neurons from neonatal rats. We found that 1) TTX-insensitive currents expressed as spikes and oscillations; 2) both spikes and oscillations were inhibited by the L-type \(\text{Ca}^{2+}\) channel inhibitor nifedipine; 3) spikes but not oscillations are capable of increasing somal \(\text{Ca}^{2+}\); 4) both spikes and oscillations were dependent on the presence of \(\text{CO}_2/\text{HCO}_3^-\); 5) \(\text{CO}_2\)-induce activation of spikes and oscillations appeared to be mediated by increased \([\text{HCO}_3^-]\); and 6) both oscillations and spikes showed a marked increase during neonatal development.

\(\text{Ca}^{2+}\) currents in LC neurons. A rhythmic, TTX-insensitive current in LC neurons, due to L-type \(\text{Ca}^{2+}\) channels, has been previously described (15, 29). In our work, we have shown that TTX-insensitive oscillations and spikes in LC neurons are both reversibly inhibited by the L-type \(\text{Ca}^{2+}\) channel inhibitor.
nifedipine, but respond differently to voltage changes in the soma of the LC neuron (see Figs. 1 and 2). Furthermore, our findings show that changes in TTX-insensitive spikes are associated with changes in the somal Ca$^{2+}$ levels, whereas changes in the TTX-insensitive oscillations are not (see Fig. 3). Thus the Ca$^{2+}$ channel-based oscillations and spikes may represent isolated populations of L-type Ca$^{2+}$ channels in the dendrites and soma, respectively. The distal population of Ca$^{2+}$ channels would not be affected by Vm changes in the soma, as we observed (Fig. 3). Alternatively, it has been suggested that TTX-insensitive oscillations reflect the strong synchronized firing patterns of LC neurons as seen through gap junctions (2, 23). In this theory, distal Ca$^{2+}$ channels actually reside in adjacent neurons and the oscillating currents travel through gap junctions. This theory is supported by the elimination of TTX-insensitive oscillations by the gap junction blocker carbenoxolone in LC neurons (3, Fig. 2C). However, in our current study, we show that TTX-insensitive spikes are not sensitive to carbenoxolone (Fig. 2C). Thus, immunohistochemical studies will be required to determine the actual distribution of L-type Ca$^{2+}$ channels in LC neurons, there can be little doubt that L-type Ca$^{2+}$ channels reside in or near the soma of LC neurons.

**Chemosensitivity of L-type Ca$^{2+}$ oscillations and spikes: mechanism of activation.** In agreement with previous studies, we have shown that both Ca$^{2+}$-based spikes and oscillations increase with CO$_2$ (15). This is unusual, because acidification is commonly expected to inhibit Ca$^{2+}$ channels (38, 43). In an attempt to determine the mechanism of hypercapnic activation, we exposed slices from P12 rats to HEPES-buffered aCSF with a nominal absence of CO$_2$/HCO$_3^-$. TTX-insensitive spikes could be seen, but only with depolarizing current bringing Vm to at least $-25$ mV, whereas oscillations were transient and reduced in amplitude (see Fig. 4). Both of these observations were unusual because the neurons were from older (>P10) rats. Normocapnic aCSF restored the normal appearance and chemosensitivity of the TTX-insensitive oscillations and spikes, and returning the patched neuron to HEPES-buffered aCSF again inhibited TTX-insensitive oscillations and spikes (see Fig. 4). These data suggest that CO$_2$/HCO$_3^-$ is necessary for the normal function of the L-type Ca$^{2+}$ channel oscillations and spikes in LC neurons. Moreover, CO$_2$/HCO$_3^-$ appears to shift the activation voltage for TTX-insensitive spikes to a more hyperpolarized Vm. These data may indicate a mechanism for L-type Ca$^{2+}$ current activation that is HCO$_3^-$ based (Fig. 11, pathway 1).

To examine a potential role for changes of pH in hypercapnic activation of the L-type Ca$^{2+}$ current in LC neurons, we used isohydric hypercapnic solutions. In isohydric hypercapnia the same increase in CO$_2$ is present as in hypercapnic acidotic solutions, but the extracellular pH is unchanged and intracellular acidification is roughly one-third or less than the intracellular acidification observed with hypercapnic acidosis (see Fig. 10).
spikes arising from the same type of Ca\(^{2+}\) channel and with hypercapnic activation being similar for oscillations and spikes. A mechanism for [HCO\(_3\)]\(_i\), activation of L-type Ca\(^{2+}\) spikes has been proposed for peripheral chemoreceptor glomus cells in the carotid body (42). In this mechanism, [HCO\(_3\)]\(_i\) activates L-type Ca\(^{2+}\) channels via a HCO\(_3\) -sensitive soluble adenylate cyclase (sAC). Increased intracellular HCO\(_3\) activates sAC, resulting in increased intracellular levels of cAMP, activation of protein kinase A, and activation of L-type Ca\(^{2+}\) channels due to their phosphorylation (Fig. 11, pathway 2). In peripheral chemoreceptive glomus cells, therefore, elevated [HCO\(_3\)]\(_i\) resulted in an increase in the magnitude of Ca\(^{2+}\) current as determined by whole cell voltage clamp recordings (42). It is possible that in LC neurons elevated [HCO\(_3\)]\(_i\) works through a similar pathway and causes an increase in the magnitude of the L-type Ca\(^{2+}\) current, but we did not directly study this pathway nor did we directly measure Ca\(^{2+}\) current. However, our findings suggest a shift in the threshold for voltage activation of L-type Ca\(^{2+}\) channels in LC neurons to more negative voltages (see Figs. 4 and 6). Thus, at any given voltage, increased [HCO\(_3\)]\(_i\)] should result in greater opening of L-type Ca\(^{2+}\) channels. Finally, we directly showed that hypercapnic activation of L-type Ca\(^{2+}\) channels resulted in a measurable increase in Ca\(^{2+}\)\(_i\) in the soma of LC neurons (see Figs. 3C and 11). Postnatal development of L-type Ca\(^{2+}\) oscillations and spikes, a role for Ca\(^{2+}\). Previous studies have shown that strong, rhythmic oscillations that regulate Na\(^+\) action potentials can be demonstrated in LC neurons from neonatal rats P1–P6 by blocking synaptic junctions with low Ca\(^{2+}\)/high Mg\(^{2+}\) (2, 29). These oscillations can be completely abolished by TTX. Moreover, these Na\(^+\)-based, TTX-sensitive oscillations in LC neurons were shown to increase in frequency over ages P2–P17 and were inhibited by carbenoxolone (9, 29). The Ca\(^{2+}\)-based oscillations under observation in this study are sensitive to both carbenoxolone and nifedipine and thus appear to rely solely on L-type Ca\(^{2+}\) channels and gap junctions (3, 15). Furthermore, Gargaglioni et al. (16) reported a shift in the chemosensitive response of LC neurons around age P10. Our findings suggest that the L-type Ca\(^{2+}\) oscillations in LC neurons undergo postnatal development over the age P3 to P16 such that prior to age ~P10, Ca\(^{2+}\) oscillations are typically absent without activation by hypercapnia (see Figs. 9 and 10). Thus these oscillations are distinct from Na\(^+\)-based oscillations and may also account for the shift in chemosensitivity suggested by Gargaglioni et al. (16). Over postnatal age P3–P16, Ca\(^{2+}\) oscillations increase in both amplitude and frequency (see Figs. 7B, 9, and 10). Prior to age ~P10, Ca\(^{2+}\) spikes are also typically absent without either exposure to hypercapnia or the addition of strong depolarizing current (see Figs. 9 and 10). After age ~P10, Ca\(^{2+}\) spikes appear without the addition of depolarizing current and show a steady increase in frequency under these conditions (see Fig. 8). Thus our data suggest the presence of an L-type Ca\(^{2+}\) current in the LC neuron soma that increases during early postnatal development and activates during exposure to CO\(_2\)/HCO\(_3\) to increase intrasomal Ca\(^{2+}\) levels. We do not know if this developmental change is due to changes in the properties of or changes in the level of expression of L-type Ca\(^{2+}\) channels during early neonatal development in LC neurons. Nevertheless, the development of these Ca\(^{2+}\) currents suggests that the role for Ca\(^{2+}\)

---

**Fig. 11. Model of the chemosensitive K\(^+\)- and Ca\(^{2+}\)-sensitive pathways in LC neurons.** Numbered pathways are referred to in the text. Left represents a summary of the proposed role of H\(^+\)-sensitive K\(^+\) channels in the hypercapnic depolarization and increase in firing rate in chemosensitive LC neurons. Right depicts a possible pathway for hypercapnic activation of Ca\(^{2+}\) channels and potential roles for Ca\(^{2+}\) in chemosensitive signaling in LC neurons.

With a diminished acidification in both pH\(_o\) and pH\(_i\), it would be expected that the chemosensitive increase in frequency for both spikes and oscillations would be decreased if changes of pH played a role in their activation. This is especially true for Ca\(^{2+}\) spikes, because reduced acidification in LC neurons would result in a smaller hypercapnia-induced depolarization from H\(^+\)-inhibited K\(^+\) channels (15, 21). However, no change in the hypercapnic response of either oscillations or spikes was observed in isohydric hypercapnic solutions compared with hypercapnic acidotic solutions (Fig. 6B). Figure 6C summarizes the dose-dependent increase of Ca\(^{2+}\) spikes to CO\(_2\), demonstrating that while 15% CO\(_2\) clearly increases spike frequency over 10% CO\(_2\), there is no significant difference in spike frequency between the isohydric hypercapnic and hypercapnic acidotic solutions. This further supports a CO\(_2\)/HCO\(_3\)\(_i\)-based rather than a pH-based hypercapnic activation of L-type Ca\(^{2+}\) oscillations and spikes. In fact, a paired comparison of the spike frequency response to isohydric hypercapnia vs. hypercapnic acidosis showed that both spike frequency and [HCO\(_3\)]\(_i\) were significantly higher in isohydric hypercapnic vs. hypercapnic acidotic solutions, whereas Ca\(^{2+}\) was the same. This suggests that activation of L-type Ca\(^{2+}\) oscillations and spikes is due to increased [HCO\(_3\)]\(_i\). A relationship between [HCO\(_3\)]\(_i\) and Ca\(^{2+}\) spike and oscillation frequency is shown in Fig. 7. Interestingly, the slopes of the best-fit lines for age groups above P6 are very similar for both Ca\(^{2+}\) spikes and oscillations (~0.01–0.02 Hz/mm [HCO\(_3\)]\(_i\)). These data are consistent with oscillations and spikes arising from the same type of Ca\(^{2+}\) channel and with hypercapnic activation being similar for oscillations and spikes.
in the LC chemosensitive response may change as neonatal rats age and may account for shifts in the intrinsic chemosensitivity of LC neurons (16).

Membrane properties, gap junctions, and voltage-activated L-type Ca\(^{2+}\) channels: significance to TTX-insensitive oscillations and spikes. A discussion of changes in the membrane properties of LC neurons and their possible effects on oscillations and spikes is a complex topic. Our current data indicate that the amplitude of oscillations increases as the animal ages. This could be due to a change in the number of channels expressed that contribute to the rhythmic TTX-insensitive current or increases in the input resistance and changes in the gap junction coupling of neonatal LC neurons.

Studies have shown that action potentials, oscillations, and small fluctuations of the V\(_m\) are synchronized by gap junction coupling between LC neurons (2, 9, 18). Although injecting current into single LC neurons failed to induce action potentials in adjacent neurons, sustained (>100 ms) current injections could be passed between neonatal LC neurons (<10) when: 1) input resistance was increased using TEA; and 2) naturally occurring oscillations were inhibited using TTX and high MgCl\(_2\) (2, 9). Collectively, these findings led researchers to propose that oscillations were the result of the synchronized summation of single action potentials across multiple neurons coupled by low-resistance electrical pathways, such as via gap junctions (2, 18). In other words, the long electrotonic length and low resistance of the coupling allows multiple simultaneous fast depolarizations to combine as slower, smaller amplitude oscillations, while nonsynchronized depolarizations in a single neuron are filtered out. On the basis of this hypothesis, the TTX-insensitive oscillations that we observe could be the result of the activity of Ca\(^{2+}\) spikes across multiple gap-junction coupled neurons. This is in agreement with our current data, as both spikes and oscillations appear to have similar properties and development. During the transition age, for example, we can speculate that the depolarization induced by hypercapnia enables the activation of spikes within multiple neurons in the network, and thereby evokes oscillations in the patched neuron in addition to spikes. Thus increases in the amount of gap junction coupling would increase the amplitude of the oscillations observed in neonatal LC neurons. Frequency would be predominately dependent on the average resting V\(_m\) established by the network and the properties of the voltage-sensitive Ca\(^{2+}\) channels.

It has also been observed that in LC neurons from very young (<P6) or older (>24 days) neonatal rats in which oscillations are not seen, application of either Ba\(^{2+}\) or TEA can restore rhythmic oscillations (2, 18). Whereas Ba\(^{2+}\) increases the conductivity through voltage-activated Ca\(^{2+}\) channels, both drugs also increase the input resistance of neurons by blocking K\(^+\) channels. The combination of these two actions may facilitate the appearance of the synchronized depolarizations through distant, low-resistance gap junction pathways (23). However, the input resistance of individual LC neurons from neonatal rats younger than P15 was measured to be 67 M\(\Omega\) vs. values from adult rat LC neurons measured at 213 M\(\Omega\) using sharp-tip electrodes (9, 45). Because the amplitude of oscillations was found to decrease in rats over 24 days of age, this suggests that input resistance of individual neurons does not significantly contribute to the development of oscillation amplitude. Thus the loss of amplitude and synchronization of oscillations in LC neurons from animals >24 days old may support a loss of coupling between LC neurons as rats age (2, 9, 23).

A third possibility is that there is an increase in the number of Ca\(^{2+}\) channels expressed in LC neurons that contributes to the amplitude of spikes and, hence, the amplitude of oscillations. Although our current study does not quantitatively examine the amplitude of spikes or oscillations, our data suggest that there is an increase in the total Ca\(^{2+}\) current in LC neurons during the first three postnatal weeks. If so, Ca\(^{2+}\) could play an increasingly important role in chemosensitive signaling with development.

Significance. The presence of oscillations and spikes arising from L-type Ca\(^{2+}\) channels in LC neurons could be of significance in several ways. An amplification role has been proposed for the L-type Ca\(^{2+}\) oscillations, whereby an increase in the depolarizing current arising from Ca\(^{2+}\) channels (Fig. 11, pathway 3), and passing through gap junctions in individual neurons may serve to increase the number of neurons capable of responding to synaptic input (9). In the case of intrinsically chemosensitive neurons of the LC, this may mean synchronization of the gap-junction coupled network to the collective stimulations of single neurons that respond to CO\(_2\) and amplification of the chemosensitive response of the LC to hypercapnia.

A different role for Ca\(^{2+}\) oscillations has been proposed in the substantia nigra pars compacta in association with Parkinson’s disease (8). Here, an increase in rhythmic oscillations due to Cav1.3 Ca\(^{2+}\) channels was theorized to be associated with increased Ca\(^{2+}\) influx and eventual damage to the dopaminergic neurons, resulting in disease (Fig. 11, pathway 4) (8). A similar pathway may be at play in LC neurons in patients with post-traumatic stress disorder (PTSD). Evidence for abnormal LC neuron cell death was observed during postmortem exams of PTSD patients (7). Given the data for increased LC neuron Ca\(^{2+}\) channel activity during stress (i.e., increased TTX-insensitive oscillation frequency), it is possible that Ca\(^{2+}\) currents in LC neurons play a potentially damaging role similar to that seen in substantia nigra (4, 8, 19).

Our findings show that hypercapnia activates L-type Ca\(^{2+}\) channels and results in increased intracellular Ca\(^{2+}\). This raises the interesting and relatively unexplored possibility that Ca\(^{2+}\) plays a role in chemosensitivity (34). One potential role for Ca\(^{2+}\) would be that activation of L-type Ca\(^{2+}\) channels by hypercapnia augments CO\(_2\)-sensitive depolarization and increases the chemosensitive response (Fig. 11, pathway 3). Filosa and Putnam (15) showed that nifedipine resulted in a decrease in the firing rate response to hypercapnia, which is consistent with Ca\(^{2+}\)-channel activation enhancing the chemosensitive response of LC neurons. However, only LC neurons from animals younger than P9 were used in that study. In our current study, we show that an increase of the L-type Ca\(^{2+}\) current occurs during postnatal development (Figs. 8–10) and may result in the role of Ca\(^{2+}\) in chemosensitive signaling varying with postnatal age in LC neurons (see Figs. 8–10).

Beyond the potential effect of hypercapnia-induced Ca\(^{2+}\) current on V\(_m\), the increase in Ca\(^{2+}\) opens several possibilities for effects on chemosensitive signaling. For example, hypercapnia-induced increases in Ca\(^{2+}\) \(_{\text{c}}\) could activate Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{\text{Ca}}\)) in LC neurons (12, 16). Activation of K\(_{\text{Ca}}\) channels could then be acting as a “brake” to limit the
firing rate response of LC neurons to hypercapnia (Fig. 11, pathway 5). Aghajanian et al. (1) made a similar conclusion when the activity of KCa channels resulted in a negative feedback function on the spontaneous firing rate in LC neurons. Our findings indicate that the LC Ca2+ current develops during the initial postnatal period, suggesting that the firing rate response of LC neurons to hypercapnia may be reduced during neonatal development due to increased activation of KCa channels. This is consistent with the observations by Gargaglioni et al. (16) where a reduction of the chemosensitive response in LC neurons with neonatal development was reported. If the braking phenomenon does occur, it would suggest that LC neurons might play a reduced role in central chemosensitivity as neonatal development progresses. By extension, abnormalities with this braking pathway could lead to hypersensitivity of the respiratory response to hypercapnia, which has been found in pathological conditions such as panic disorder and sleep apnea (22, 25, 36, 44, 46). Thus it is likely that Ca2+ channels and intracellular Ca2+ concentration play important and perhaps varied roles in central chemosensitivity, at least in LC neurons, and more detailed studies of these roles is warranted.

ACKNOWLEDGMENTS
We acknowledge the advice and assistance of Dr. Keyong Li, and the preliminary work and technical expertise of Dr. L. Hartzler were greatly appreciated.

GRANTS
This work was supported by American Heart Association Predoctoral Fellowship (to A. N. Imber) and National Institutes of Health Grant R01 HL-56683 (to R. W. Putnam).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


