Hypoxic excitation in neurons cultured from the rostral ventrolateral medulla of the neonatal rat

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Mazza, Emilio, J r., Norman H. Edelman, and Judith A. Neubauer. Hypoxic excitation in neurons cultured from the rostral ventrolateral medulla of the neonatal rat. J Appl Physiol 88: 2319–2329, 2000.—Neurons within cardiorespiratory regions of the rostral ventrolateral medulla (RVLM) have been shown to be excited by local hypoxia. To determine the electrophysiological properties of these excitatory responses to hypoxia, we developed a primary dissociated cell culture system to examine the intrinsic response of RVLM neurons to hypoxia. Neonatal rat neurons plated on medullary astrocyte monolayers were studied using the whole cell perforated patch-clamp technique. Sodium cyanide (NaCN, 0.5–10 mM) was used, and membrane potential (V_m), firing frequency, and input resistance were examined. In 11 of 19 neurons, NaCN produced a V_m depolarization, an increase in firing frequency, and a decrease in input resistance, suggesting the opening of a cation channel. The hypoxic depolarization had a linear dose response and was dependent on baseline V_m, with a greater response at more hyperpolarized V_m. In 8 of 19 neurons, NaCN produced a V_m hyperpolarization, decrease in firing frequency, and variable changes in input resistance. The V_m hyperpolarization exhibited an all-or-none dose response and was independent of baseline V_m. These differential responses to NaCN were retained after synaptic blockade with low Ca²⁺-high Mg²⁺ or TTX. Thus hypoxic excitation 1) is maintained in cell culture, 2) is an intrinsic response, and 3) is likely due to the increase in a cation current. These hypoxia-excited neurons are likely candidates to function as central oxygen sensors.

respiratory; sympathetic; sodium cyanide; whole cell perforated patch clamp

However, the populations of neurons that are involved in such regulation have been shown to have different responses to central hypoxia, leading to increases or decreases in respiratory or sympathetic output (51).

The respiratory response to hypoxia represents the integration of stimulation of the arterial chemoreceptors and the depressant effects of central hypoxia on neuronal excitability (37). The predominant effect of mild to moderate hypoxia on the central respiratory network is primarily to promote respiratory depression, although very severe hypoxia promotes high amplitude respiratory output in the form of gasping (6, 20, 31, 51). In anesthetized, peripherally chemodenervated cats, Wasicko et al. (51) demonstrated that progressive brain hypoxia produced a depression of both phrenic nerve and inspiratory synchronous output while concomitantly causing a moderate increase in tonic sympathetic nerve activity. At more severe levels of hypoxia, gasping occurred in both the phrenic and inspiratory synchronous sympathetic output, whereas tonic sympathetic nerve output was maintained.

Recent evidence suggests that the stimulatory effect of hypoxia on both central respiratory and sympathetic output may be due to direct excitation of rostral ventrolateral medullary (RVLM) neurons of the pre-Bötzinger and C1 regions, respectively (35, 39, 46–49). Local microinjection of either DL-homocysteic acid or sodium cyanide (NaCN) into the pre-Bötzinger complex of the RVLM produced a gasp-like output in the phrenic neurogram (46, 47). Similarly, local microinjection of NaCN into the C1 region of the RVLM produced an increase in sympathetic nerve output and an increase in arterial blood pressure (48). Furthermore, iontophoretic application of either L-glutamate or NaCN into this region was found to excite the putative RVLM sympathoexcitatory neurons in a dose-dependent reversible manner in vivo (49). The mechanisms by which neurons of these two regions are excited by hypoxia are unresolved. Whether these neurons are disinhibited by neurons more vulnerable to hypoxia or are intrinsically excited by local hypoxia via cellular responses similar
to those of other chemosensitive tissues, e.g., a change in cationic currents (19), is yet to be determined. Thus the aim of the present study was to develop a primary dissociated cell culture system to examine the intrinsic response of RVLM neurons to hypoxia induced with NaCN. Specifically, we tested the hypothesis that the excitatory response to NaCN is maintained in cell culture and is intrinsic to a unique subpopulation of neurons due to a change in cationic conductance.

METHODS

The electrophysiological response to NaCN-induced hypoxia was examined using the whole cell perforated patch-clamp technique on dissociated RVLM neurons cultured on medullary astrocyte monolayers.

Medullary astrocyte cultures. To minimize synaptic interaction, RVLM neurons were plated at low density (60 cells/mm²) on astrocyte monolayers, shown by McCarthy and DeVellis (30) to increase neuronal survival. Specifically, medullary astrocyte monolayers were used because neurons grown on astrocytes taken from their native brain regions show increased survivability (40). In a sterile isolation hood, 8–10 neonatal Sprague-Dawley rats (4–5 days old) were decapitated, and their brains were removed and placed in a petri dish containing sterile cold basal salt solution (BSS) buffered with PIPES (4°C, pH 7.0). With the use of a stereoscopic dissecting microscope (×40), the medullas were dissected in sterile cold PIPES-BSS and the pia mater was carefully removed. The medullas were moved to another petri dish containing fresh, sterile, cold PIPES-BSS and minced into 1- to 2-mm² pieces. The minced tissue was moved to a sterile plastic centrifuge tube containing 2.5 ml of 0.1% trypsin solution (Sigma Chemical) and then incubated for 30 min in a water bath at 37°C. The plastic centrifuge tube containing the minced tissue was removed from the water bath and centrifuged briefly for 30 s. The supernatant was removed, and the tissue was resuspended in DNase (0.32 mg/ml; Boehringer Mannheim) and DMEM supplemented with 10% fetal bovine serum, 2% L-glutamine, and 2% penicillin-streptomycin (GIBCO Life Science Technologies) to a final volume of 2 ml. The tissue was mechanically dispersed via gentle trituration (9–10 times) through a flame-narrowed glass Pasteur pipette. The dispersed cells were then filtered through a 50-µm nylon mesh (Small Parts) and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in fresh complete DMEM (DMEM-CM) as described above. The dissociated cells were then plated in 75-cm² culture flasks (Falcon, Becton-Dickinson) by pipetting 1 ml of the solution containing the dissociated cells into each culture flask and bringing the total volume up to 10 ml with fresh DMEM-CM. The flasks were then incubated with 1 ml of DMEM-CM per well at 37°C in a 5% CO₂ environment for 2 h. To obtain nearly pure medullary astrocyte cultures, the flasks were shaken on a rotary shaker for 6–8 h at 200 rpm, 46–72 h after cultures were plated onto the culture flasks. This procedure removed loosely adherent cells such as neurons and oligodendrocytes. The medium was suctioned off and immediately replaced with fresh DMEM-CM. This procedure was done twice a week until confluent astrocyte monolayers were obtained (7–10 days).

The day before astrocytes were harvested, circular coverglasses (18 mm in diameter; Fisher Scientific) were autoclaved and placed into sterile 12-well Falcon cell culture trays (Becton-Dickinson) in a sterile isolation hood. Glass coverglasses were submerged in fresh poly-L-lysine (1 mg/ml; Sigma) for 5 min. The poly-L-lysine was removed, and the coverglasses were washed twice in sterile distilled water. The culture trays containing the poly-L-lysine-coated coverglasses were then exposed to ultraviolet (UV) light for 2 h in the sterile isolation hood. After exposure to UV light, the culture trays were dosed and allowed to air dry for at least 24 h before being used for astrocyte plating.

Pure confluent medullary astrocyte cultures no more than 2 wk old were used for harvesting. The medium was first suctioned from the flasks and replaced with 1 ml of trypsin (0.25%) and 4 ml of 0.02% EDTA in Ca²⁺- and Mg²⁺-free PBS (37°C, pH 7.2). The flasks were then shaken on a rotary shaker at 200 rpm for 2–5 min. This allowed for the astrocytes to come off the flask and redissociate. The redissociated astrocytes were then removed from the flasks and placed in a plastic centrifuge tube and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the astrocytes were resuspended in DMEM-CM and filtered through a 50-µm nylon mesh. The astrocyte number was counted, and viability was checked using a hemacytometer with trypan blue exclusion (0.08%; GIBCO). The cells were then plated onto round poly-L-lysine-coated cover glass (18 mm in diameter) in 12-well culture trays at a density of 35 cell/mm². The cultures were incubated with 1 ml of DMEM-CM/well at 37°C in a 5% CO₂ incubator. The cultures were fed twice weekly by suctioning off 50–75% of the medium and replacing it with approximately the same volume of fresh DMEM-CM. Confluent monolayers within the culture wells were obtained 7–10 days later and were used for neuronal cell culture.

Dissociated medullary neuronal cultures. Neuronal cultures were prepared according to the method adapted from Kay and Wong (24). Under a sterile environment, four neonatal Sprague-Dawley rats (0–1 days old) were decapitated and the craniums were cut sagittally and removed. The brains, including the cortices, cerebellum, and brain stem, were removed and placed in a petri dish containing PIPES-BSS at 4°C. The medulla, from the obex to the pontomedullary border (~2.0 mm), was isolated using a stereoscopic dissecting microscope (×40). The medulla was then transversely sectioned (800 µm), and the RVLM containing the C1 and pre-Boëtziinger regions was dissected out as shown in Fig. 1. The sections of the RVLM were moved to another petri dish containing fresh cold PIPES-BSS and minced into 1- to 2-mm² pieces. The minced tissue was moved to a sterile plastic centrifuge tube containing 2.5 ml of 0.1% trypsin solution (Sigma Chemical) and incubated for 20 min in a water bath at 37°C. The plastic centrifuge tube containing the minced tissue was removed from the water bath and centrifuged briefly for 30 s. The supernatant was removed, and the minced tissue was resuspended in DNase (0.32 mg/ml) and DMEM-CM. The tissue was mechanically dispersed via gentle trituration (9–10 times) through a flame-narrowed glass Pasteur pipette. The dispersed cells were then transferred through a 50-µm nylon mesh (Small Parts) and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in fresh complete DMEM (DMEM-CM) as described above. The dissociated cells were then plated in 75-cm² culture flasks (Falcon, Becton-Dickinson) by pipetting 1 ml of the solution containing the dissociated cells into each culture flask and bringing the total volume up to 10 ml with fresh DMEM-CM. The flasks were then incubated in a 5% CO₂ environment at 37°C. To obtain nearly pure medullary astrocyte cultures, the flasks were shaken on a rotary shaker for 6–8 h at 200 rpm, 46–72 h after cultures were plated onto the culture flasks. This procedure removed loosely adherent cells such as neurons and oligodendrocytes. The medium was suctioned off and immediately replaced with fresh DMEM-CM. This procedure was done twice a week until confluent astrocyte monolayers were obtained (7–10 days).
µg/ml), and brain-derived neurotrophic factor (10 ng/ml) incubated at 37°C in a 5% CO2 environment. The cultures were fed twice weekly by suctioning off 50–75% of the medium and replacing it with approximately the same volume of fresh DMEM-N2 plus brain-derived neurotrophic factor.

Electrophysiology. Recordings were made from neurons cultured from the RVLM >4 days old. The nystatin-perforated patch-clamp technique in the whole cell configuration, adapted from Horn and Marty (22), was used for all recordings. Nystatin is a pore-forming antimycotic that allows electrical coupling between pipette and cell interior but prevents the dialysis of macromolecules (ATP, proteins) that occurs during standard whole cell recordings. Thus stable recordings of spontaneous activity can be obtained for prolonged periods (1–2 h). The nystatin pores are freely permeable to monovalent cations (Na+, K+) and have been used to measure Cl− currents (26).

Pipettes were pulled from thin-walled borosilicate glass capillary tubes (1.5 mm outer diameter; World Precision Instruments) on a Flaming-Brown horizontal (P-87, Sutter Instruments) or vertical (P-83, Narishige Instruments) micro-pipette puller. Pipettes started with a tip diameter of 1.5–2 µm and were firepolished on a microforge (Narishige Instruments) to a final tip size of 1–1 µm and a resistance of 2–4 MΩ when filled with a standard internal pipette solution (containing in mM: 105 K₂SO₄, 15 KCl, 0.8 MgCl₂, and 10 HEPES, pH 7.2). Nystatin (1,000,000 units; Sigma) was freshly dissolved in DMSO at a stock concentration of 60 mg/ml and added to the internal pipette solution at a final concentration of 240 µg/ml. Solvation of the nystatin in the internal pipette solution was facilitated by ultrasonification for 20 s. To facilitate formation of the patch, pipette tips were first backfilled using negative pressure with plain internal pipette solution (100 µm from tip) and followed by the nystatin internal pipette solution. After formation of a gigaseal with gentle suction, the cells were allowed to equilibrate for 15–30 min until cell membrane potential (V_m) and input resistance were stabilized.

Coverslips were mounted in a Plexiglas recording chamber (volume of 1 ml), placed on an inverted Zeiss microscope with phase and Hoffman optics, and superfused with artificial cerebrospinal fluid (CSF; in mM: 125 NaCl, 3.5 KCl, 1 CaCl₂, 1 MgCl₂, 24 NaHCO₃, 0.6 NaH₂PO₄, and 15 glucose, bubbled with 95% O₂-5% CO₂ to pH 7.4) at a rate of 2 ml/min using a peristaltic pump. In experiments designed to reduce synaptic transmission, the MgCl₂ and CaCl₂ concentrations of the artificial CSF were changed to 5 and 0.5 mM, respectively (low Ca²⁺-high Mg²⁺ artificial CSF), or 0.5 µM TTX was added to the artificial CSF to block fast Na+ channel activity involved in action potential production. All experiments were performed at room temperature (23–25°C).

Hypoxia was produced by transiently exposing the cells to 10% NaCN (0.5–10 mM) dissolved in artificial CSF and equilibrated with 95% O₂-5% CO₂ into the perfusion line, resulting in a transient pulse. Phenol red (GIBCO) was added to NaCN solution as a way of determining when NaCN reached the recording chamber. The dose of NaCN that produced a response was used for the duration of the experiment. Bolus injection of the vehicle (0.3 ml; containing artificial CSF and phenol red) was used as a control.

Signals were amplified and filtered using an Axopatch 1D patch-clamp amplifier and CV-4 head stage (Axon Instruments) operating in the current-clamp mode. Firing frequency was measured on-line using a window discriminator (CWE) and a spike counter (CWE). Scaled output for current clamp (V_m) was recorded on videotape for permanent storage. These data were analyzed off-line after playback through a computerized, multichannel, digital data-acquisition package (CODAS, Dataq on a DTK-486DX computer) with a sampling rate of 2,000 samples·s⁻¹·channel⁻¹. Digital CODAS files were permanently stored on digital backup tape (3M) and ZIP drive (omega). Constant-amplitude current pulses injected via the recording pipette were produced with the CLAMPEX program of the pCLAMP data-analysis package (Axon Instruments). Action potential properties were examined with Axograph 3.5 (Axon Instruments). Data were also recorded in hard-copy form on a four-channel oscilloscope thermal chart recorder (Gould).

Neuronal recordings were analyzed off-line to examine changes in V_m, action potential firing frequency, input resistance, and action potential properties during NaCN administration. V_m was measured every 5 s and averaged over 60 s of baseline, peak, or nadir of the response, and 60 s of recovery. Both instantaneous firing frequency and average firing frequency (10-s bins) were examined during the experiment. Input resistance was determined by measuring the V_m responses to constant-amplitude current pulses (−10 pA; 1-s pulse at 0.2 Hz) given via the recording pipette during the experiment and consisted of two components. Peak input resistance (R_p) was defined as the maximum V_m response to the current pulse and occurred within the first 500 ms of the pulse. Steady-state input resistance (R_s) was defined as the point at which the V_m response to the injected current pulse reached a constant level and was measured in the last 500 ms.
of the current pulse. Latency of the initiation of the response, time to peak or nadir of the response, and the time constant \( t^{-c} \) of recovery were examined. The \( t^{-c} \) of recovery was defined as the amount of time necessary for the \( V_m \) to return to 37% of baseline.

All data are expressed as means ± SE. Baseline data of neurons depressed by NaCN and neurons excited by NaCN were compared using a two-tailed Student’s unpaired \( t \)-test. Baseline data were compared with the peak or nadir of the response and tested for significance using a two-tailed Student’s paired \( t \)-test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

A total of 24 neurons met with our acceptance criteria and were studied. A recording was considered acceptable if \( V_m \) was less than \(-45 \text{ mV} \), the amplitude of the action potentials was greater than \(50 \text{ mV} \) and overshot \(0 \text{ mV} \), \( R_{pk} \) and \( R_{ss} \) were greater than \(500 \Omega \), and pipette seal resistance was greater than \(1 \text{ G} \Omega \). The neurons used in this study had a mean \( V_m \) of \(-61 ± 6 \text{ mV} \), action potentials with an average spike height of \(85 ± 14 \text{ mV} \) that all overshot \(0 \text{ mV} \), \( R_{pk} \) of \(945 ± 441 \text{ mV} \), \( R_{ss} \) of \(850 ± 422 \text{ mV} \), and a pipette seal resistance of \(2.6 ± 1.3 \text{ G} \Omega \). The majority of these neurons (20 of 24) were spontaneously active and had firing patterns that were either bursting or repetitive.

Baseline electrophysiological properties of cultured RVLM neurons. Neurons were challenged with bolus injections of NaCN, beginning with low doses (0.5–1 mM), until a change in \( V_m \) was observed. If no response was observed, the challenge was repeated with a higher dose (2–10 mM). In five neurons, the recording seal was lost before a dose was administered that evoked a change in \( V_m \). Results from these five neurons were not included in any further analysis.

The remaining 19 neurons were separated into two groups on the basis of their \( V_m \) and firing frequency response to NaCN-induced hypoxia: hypoxia excited or hypoxia depressed.

**Hypoxia-exited neurons** (\( n = 11 \)) were defined as neurons that demonstrated a reversible \( V_m \) depolarization and an increase in firing frequency in response to NaCN. Of the neurons that were excited by NaCN, 1 of 11 neurons fired with a bursting pattern, 4 of 11 were quiescent at baseline, and 6 of 11 had a repetitive firing pattern. Figure 2 illustrates a NaCN-induced excitation in a bursting (Fig. 2A), quiescent (Fig. 2B), and repetensively firing (Fig. 2C) neuron. NaCN produced a \( V_m \) depolarization in all three of these neurons, ranging from 4 to 11 mV, and a concomitant increase of two- to sevenfold in firing frequency. Group data showed that hypoxia-exited neurons depolarized by an average of \(7 \text{ mV} \) from a mean baseline of \(-62 ± 2 \text{ to } -55 ± 2 \text{ mV} \) \( (P < 0.001) \) and had an increase in firing frequency from \(2.9 ± 0.5 \text{ to } 6.8 ± 0.5 \text{ Hz} \) \( (P < 0.01) \). The mean latency to initiation of the excitatory response was \(30 ± 4 \text{ s} \) with a mean latency to peak response of \(85 ± 14 \text{ s} \). The \( V_m \) and frequency responses to bolus injections of NaCN were reversible and reproducible, returning to baseline values with a \( t^{-c} \) of recovery of \(204 ± 49 \text{ s} \).

**Hypoxia-depressed neurons** (\( n = 8 \)) were defined as neurons that demonstrated a reversible \( V_m \) hyperpolarization and a decrease in action potential firing frequency in response to NaCN. Of the neurons that were depressed by NaCN, seven of eight neurons had a repetitive firing pattern, whereas one of eight of these neurons had a bursting pattern. Figure 3 shows a representative RVLM neuron in which NaCN produced a \( V_m \) hyperpolarization from \(-56 \text{ to } -63 \text{ mV} \) and a decrease in firing frequency from \(1.5 \text{ to } 0.1 \text{ Hz} \). Group data showed that hypoxia-depressed neurons hyperpolarized by an average of \(4 \text{ mV} \) from a mean baseline of \(-61 ± 3 \text{ to } -65 ± 2 \text{ mV} \) \( (P < 0.001) \) and had a decrease in firing frequency from \(3.5 ± 0.5 \text{ to } 1.3 ± 0.3 \text{ Hz} \) \( (P < 0.01) \). The dynamics of the depressed hyperpolarization response did not differ significantly from the excited response; the mean latency to initiation of the hypoxic-depressant response was \(34 ± 9 \text{ s} \) with a mean latency to peak response of \(126 ± 32 \text{ s} \). Likewise, the hyperpolarization response peaked at \(35 ± 9 \text{ s} \) with a mean latency to peak response of \(126 ± 32 \text{ s} \).
Depolarization and reduction in firing frequency in response to a bolus injection of NaCN were reversible and reproducible, with a time constant of recovery of 280 ± 49 s. A bolus injection of an equal volume of vehicle (0.3 ml of artificial CSF) had no effect on \( V_m \) or firing frequency of neurons in either category of response. There was also no difference in the mean dose of NaCN necessary to elicit either of the two responses (hypoxia-excited neurons: 5.0 ± 1.0 mM, hypoxia-depressed neurons: 3.7 ± 0.8 mM; \( P = 0.4 \)).

Differences in baseline electrophysiological properties could not predict whether a neuron would depolarize or hyperpolarize in response to NaCN. Table 1 summarizes the baseline data for \( V_m \), firing frequency, \( R_{pk} \), \( R_{ss} \), and action potential amplitude in all neurons examined as distinguished by their response to hypoxia. There was no significant difference between hypoxia-excited and hypoxia-depressed neurons in any of these parameters. Thus baseline electrophysiological properties did not predict a cell's response to NaCN-induced hypoxia.

The cumulative effect of repeated NaCN challenges did exhibit differences in baseline \( V_m \) and firing frequency over time between hypoxia-excited and hypoxia-depressed neurons. Figure 4 plots the mean and standard error of the baseline \( V_m \) and firing frequency for all 19 neurons under control artificial CSF conditions separated according to their differential response to hypoxia. Values were taken under control conditions before and after multiple NaCN challenges (4 ± 2 NaCN challenges in hypoxia-excited neurons and 3 ± 2 NaCN challenges in hypoxia-depressed neurons). At any given point in time, the variability of baseline \( V_m \) was small for both hypoxia-excited and hypoxia-depressed neurons (SE = 1.0 mV). However, there was a small but significant depolarization of \( V_m \) in hypoxia-excited neurons of 4.3 ± 1.2 mV over time (mean = 30 ± 4 min; \( P < 0.01 \)), whereas hypoxia-depressed neurons showed no significant change in \( V_m \) over time (mean = 27 ± 5 min; \( P = 0.66 \)). Baseline firing frequency was much more variable than \( V_m \) in both groups of neurons (SE = 1.8 Hz for hypoxia-excited and 1.5 Hz for hypoxia-depressed neurons), with a significant decline in firing frequency in hypoxia-depressed neurons, from 3.5 to 1.8 Hz, and a nonsignificant change in hypoxia-excited neurons, from 2.9 to 2.1 Hz.

Dependence of the response to NaCN on resting \( V_m \). To determine whether the response to NaCN was dependent on resting \( V_m \), the change in \( V_m \) was plotted against the baseline \( V_m \) before the NaCN challenge. As shown in Fig. 5A, the hypoxia-excited response was dependent on the initial baseline \( V_m \) with a greater depolarization occurring in response to hypoxia when the baseline \( V_m \) was more hyperpolarized. Extrapolating the line of best fit yields a reversal potential of approximately −53 mV (best-fit linear regression (r) = 0.75; \( P < 0.001 \)). In contrast, the hypoxia-depressed hyperpolarization response did not show a linear correlation with \( V_m \) (r = 0.17; \( P > 0.05 \); Fig. 5B). Thus the excited response to NaCN is dependent on resting \( V_m \), whereas the depressed response is not dependent on resting \( V_m \).

Dose-response relationship of the neuronal response to NaCN. The dose-response relationships of the change in \( V_m \) to increasing doses of NaCN at the same resting \( V_m \) are plotted in Fig. 6. Figure 6A illustrates a representative linear dose-response relationship in a hypoxia-excited neuron in which increasing doses of NaCN produced a linear increase in change in \( V_m \). This linear correlation between the change in \( V_m \) and the dose of NaCN in hypoxia-excited neurons is also observed when the group data are plotted at two different baseline potentials (Fig. 6B). In addition, the slope of this dose-response relationship is dependent on the baseline \( V_m \), with a steeper slope evident at more hyperpolarized baseline \( V_m \). For example, when baseline \( V_m \) was −60 mV, the slope of the dose-response relationship was 0.6 mV/mM (r = 0.67; \( P < 0.05 \)) compared with a slope of 1.9 mV/mM at a \( V_m \) of −65 mV. In contrast, hypoxia-depressed neurons exhibited more of an all-or-none response to NaCN over the range of doses used for this study. Figure 6C shows a representative dose-response relationship of a hypoxia-depressed neuron that exhibited this all-or-none \( V_m \) response to

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**Table 1. Baseline membrane potential, firing frequency, peak input resistance, steady-state input resistance, and action potential amplitude in both hypoxia-depressed and hypoxia-excited cultured rostral ventrolateral medullary neurons**

<table>
<thead>
<tr>
<th></th>
<th>Hypoxia Depressed</th>
<th>Hypoxia Excited</th>
<th>P</th>
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<tbody>
<tr>
<td>( V_m ), mV</td>
<td>−61 ± 3</td>
<td>−62 ± 2</td>
<td>0.70</td>
</tr>
<tr>
<td>FF, Hz</td>
<td>3.5 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>( R_{pk} ), MΩ</td>
<td>982 ± 170</td>
<td>1142 ± 163</td>
<td>0.36</td>
</tr>
<tr>
<td>( R_{ss} ), MΩ</td>
<td>957 ± 172</td>
<td>1123 ± 167</td>
<td>0.22</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>77 ± 6</td>
<td>87 ± 5</td>
<td>0.22</td>
</tr>
</tbody>
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Values are means ± SE; n (no. of neurons) shown in parentheses. \( V_m \), membrane potential; FF, firing frequency; \( R_{pk} \), peak input resistance; \( R_{ss} \), steady-state input resistance; AP, action potential. Baseline values for hypoxia-depressed and hypoxia-excited neurons were compared using an unpaired Student's t-test.
increasing doses of NaCN. When the group data were plotted and the linear regression line was derived for the hypoxia-depressed neurons (Fig. 6D), there was no correlation between change in \(V_m\) and dose of NaCN (\(r = 0.17\); \(P > 0.05\)). Therefore, although hypoxia-excited neurons show a linear dose-response relationship, with an increased sensitivity to NaCN at a more negative resting \(V_m\), this differs from hypoxia-depressed neurons, which show an all-or-none dose-response relationship to NaCN.

Intrinsic effect of NaCN on \(V_m\) and input resistance. The intrinsic effects of hypoxia on \(V_m\) and input resistance were examined during superfusion of the neuron with artificial CSF prepared with either low Ca\(^{2+}\)-high Mg\(^{2+}\) or TTX. Figure 7A demonstrates the changes in baseline \(V_m\) and the change in \(V_m\) during \(-10\)-pA current injections in a representative neuron excited by hypoxia under low Ca\(^{2+}\)-high Mg\(^{2+}\) conditions. In this example, a bolus injection of NaCN produced a \(V_m\) depolarization from \(-55\) to \(-49\) mV. The peak change in \(V_m\) (\(V_{pk}\)) in response to constant hyperpolarizing current pulses was reduced from a maximum of \(-70\) mV to a minimum of \(-57\) mV. The difference between \(V_m\) and \(V_{pk}\), which is proportional to the change in input resistance and is illustrated in Fig. 7, is decreased during the depolarization caused by NaCN (calculated \(R_{pk}\) decreased from \(1,540 \pm 30\) to \(775 \pm 78\) M\(\Omega\)). This decrease in input resistance during the depolarization response to hypoxia is consistent with the opening of a cation channel, i.e., an increase in an inward cationic conductance. A similar response to NaCN of this neuron was obtained in the presence of TTX (Fig. 7B). NaCN reduced \(V_{pk}\) in response to constant hyperpolarizing current pulse from a maximum of \(-74\) mV to a minimum of \(-53\) mV. This produced a reduction in the calculated \(R_{pk}\) from \(928 \pm 167\) to \(500 \pm 220\) M\(\Omega\). In all neurons in which input resistance was examined, the excitatory depolarization response to hypoxia was associated with a decrease in \(R_{pk}\) (\(n = 3\), consistent with the suggestion that hypoxia promotes an increase in an inward cation current.

The intrinsic mechanisms responsible for changes in \(V_m\) were also explored in hypoxia-depressed neurons under both low Ca\(^{2+}\)-high Mg\(^{2+}\) and TTX conditions. Figure 7C shows a hypoxia-depressed neuron in which NaCN produced a \(V_m\) hyperpolarization from \(-73\) to \(-76\) mV when superfused with low Ca\(^{2+}\)-high Mg\(^{2+}\). As shown in the comparison between \(V_m\) and \(V_{pk}\) in Fig. 7C, there is little change at the nadir of the response. Calculation of \(R_{pk}\) shows that there is a small decrease from \(995 \pm 28\) to \(925 \pm 20\) M\(\Omega\). In a different neuron, the depressant response to hypoxia in the presence of TTX is shown in Fig. 7D, demonstrating that NaCN produces a \(V_m\) hyperpolarization from \(-62\) to \(-65\) mV without much change in the difference between \(V_m\) and \(V_{pk}\). Calculation of \(R_{pk}\) shows a small increase from \(550 \pm 5\) to \(590 \pm 9\) M\(\Omega\). In all hypoxia-depressed neurons in which input resistance was examined, the changes in \(R_{pk}\) were variable from cell to cell (\(n = 6\)).

**DISCUSSION**

The results of this study demonstrate the usefulness of the in vitro-dissociated cell culture system for studying the effects of hypoxia on neurons of the RVLM; in
The NaCN-induced hypoxic excitation is retained in neurons dissociated and cultured from this region. In dissociated RVLM neurons, 60% of those studied were excited by NaCN-induced hypoxia in that NaCN caused a $V_m$ depolarization and an increase in firing frequency that were reversible and reproducible. In 40% of dissociated RVLM neurons studied, NaCN-induced hypoxia produced a depression of neuronal activity by causing a $V_m$ hyperpolarization and a decrease in firing frequency. The excitatory response to hypoxia was unique compared with the depressant response in that it was dependent on both resting $V_m$ and the dose of NaCN. Furthermore, the excitatory response to hypoxia was maintained when superfused in either low Ca$^{2+}$-high Mg$^{2+}$ or TTX, and depolarization was associated with a decrease in input resistance.

The baseline characteristics of neurons excited or depressed by NaCN were not significantly different. Thus neurons could not be distinguished, nor could their responses to NaCN be predicted, on the basis of their initial baseline intrinsic properties. However, there were several notable differences between hypoxia-excited and hypoxia-depressed neurons when the baseline $V_m$ and firing frequency were examined over the course of an experiment. Neurons excited by NaCN showed a small but significant $V_m$ depolarization of 4.3 mV over an average of 30 min without a significant change in baseline firing frequency. In contrast, neurons depressed by NaCN showed no significant change in $V_m$ but showed a significant decrease in firing frequency over the same 30-min time frame. The membrane depolarization seen in hypoxia-excited neurons over time may suggest a small cumulative effect of NaCN on the hypoxic excitatory mechanism specific to these neurons.

The lack of a significant change in firing frequency in hypoxia-excited neurons in contrast to the decrease in firing frequency seen in hypoxia-depressed neurons may be due to the small depolarization that minimizes the general effect of hypoxia, which is to reduce the opening probability of Na$^+$ channels. Cummins et al. (8, 9) have shown that NaCN and anoxia cause a negative shift in the Na$^+$ inactivation curve, thereby reducing excitability in both isolated hippocampal CA1 neurons and human cortical neurons. Thus the cumulative effect of NaCN over time that causes a membrane depolarization in hypoxia-excited neurons could act to counterbalance the effect of hypoxia on Na$^+$ channel activation and result in no net change in firing frequency. On the other hand, in hypoxia-depressed neurons, in which there is no significant change in $V_m$, the effect of NaCN on Na$^+$ channel kinetics would be unopposed in its ability to decrease firing frequency.

A second distinguishing feature of hypoxic excitation is that the level of depolarization during exposure to NaCN is dependent on resting $V_m$, whereas the hyperpolarization response was not a function of resting $V_m$. These results suggest that the response to NaCN in the two subgroups of neurons described in this study rely on different mechanisms. Specifically, in neurons excited by NaCN as the resting $V_m$ is shifted to more positive values, the change in $V_m$ produced by NaCN is decreased, with an extrapolated reversal occurring at $-53$ mV. On the basis of this reversal potential and the changes in input resistance during NaCN excitation, it would seem unlikely that the response is due to the closure of a K$^+$ channel (28) or solely by the opening of a Ca$^{2+}$ or Na$^+$ channel (50). One likely candidate, however, is that of the mixed Na$^+-K^+$ cation current, or I$_{Na}$, which has its reversal potential in this region (41). This channel is highly modulated by changes in second messengers such as cAMP, cGMP, and intracellular Ca$^{2+}$ (21, 23) and has been suggested to play an important role in neonatal hypoglossal motoneurons (2). In addition, in rat thalamocortical neurons, hypoxia has been shown to cause a positive shift in the voltage dependence of I$_{Na}$ and to produce changes in its activation kinetics, causing a persistent increase in this current at resting $V_m$ (15). If present in hypoxia-excited neurons in this study, this current could potentially mediate the membrane depolarization and increase in firing frequency characteristic of the response to NaCN in these neurons.

In hypoxia-depressed neurons, the membrane hyperpolarization produced by NaCN was not dependent on resting $V_m$ and caused variable changes in input resistance...
distance. These characteristics make it difficult to speculate on cellular mechanisms responsible for the hyperpolarization response to NaCN. The opening of a K\(^{+}\) channel has been proposed to mediate the hyperpolarization response to hypoxia in other areas of the central nervous system (18, 25, 36, 38). The observation that the hyperpolarization response to NaCN was not dependent on resting \(V_m\) might suggest a change in the activity of the Na\(^+-\)K\(^+-\)ATPase. In hippocampal CA1 neurons, the Na\(^+-\)K\(^+-\)ATPase has been shown to mediate a transient, voltage-independent hyperpolarization that is evident in the posthypoxic period (17). Further studies will be necessary to determine the cellular mechanism mediating these differential responses to NaCN.

Another distinguishing feature of hypoxia-excited neurons compared with hypoxia-depressed neurons is the dose-response relationship with NaCN such that larger doses of NaCN produced larger levels of depolarization in a linear manner in hypoxia-excited neurons, whereas depressed neurons exhibited an all-or-none dose response. This suggests a specific sensing mechanism that can be activated in a graded manner in neurons excited by NaCN. In contrast, the all-or-none response of hypoxia-depressed neurons suggests that either the doses of NaCN used (1–10 mM) were out of the dose-response range for these neurons, and thus saturated the response, or that the depressant response to hypoxia is truly an all-or-none response and that a critical threshold is reached that activates the mechanism producing a maximum hyperpolarization in these neurons.

Advantages and limitations of the in vitro model. A specific aim of this study was to develop an in vitro-dissociated cell culture system to study the intrinsic effects of hypoxia on neurons of the RVLM. The advantage of this system is that it provides a means of examining individual, dissociated RVLM neurons in a low-density cell culture system, limiting the amount of synaptic interaction during hypoxia. In other primary neuronal cell culture systems, effective synaptic interactions do not occur until \(\geq\) 7 days in vitro. In addition, we used low Ca\(^{2+}\)-high Mg\(^{2+}\) and TTX recording solutions to chemically limit synaptic interactions. Thus, in the period in which we studied neurons (4–7 days in culture), the amount of synaptic interaction was limited.

One limitation to using primary cell culture systems is that the neuroanatomy is lost and thus specific cell types cannot be identified. A second limitation is that the neuronal phenotype present in vivo may change in vitro. We cannot know for certain that a RVLM neuron that is excited in culture would likewise be excited in vivo. However, for the purposes of this study, we tried to determine the basic characteristics that distinguish hypoxia-excited from hypoxia-depressed neurons. If the phenotype of neurons changes when they are grown in primary cell culture, such that they become hypoxia excited or hypoxia depressed, this may lead to insight into the cellular mechanisms that differentiate these two groups.

NaCN was used as the method for inducing chemical or histotoxic hypoxia. The major metabolic effect of NaCN is the binding of cytochrome aa\(_3\) of the mitochon-
Functional significance. Within the RVLM are functionally described areas important for respiratory and sympathetic control. The pre-Bötzinger complex is the hypothesized locus for central respiratory rhythmogenesis (45). It has been suggested that its role as a primary generator of respiratory rhythm is more important in the neonatal period. In addition, over the course of development, its role becomes less important as a primary generator of respiratory rhythm and becomes incorporated in a hybrid pacemaker-network model (4, 5, 16). The work of Solomon et al. (46) has shown that microinjection of DL-homocysteic acid, a glutamate agonist, into this region results in gasplike output in the phrenic neurogram. If NaCN is microinjected into this same region, a similar gasplike output is produced (47). Adjacent to the pre-Bötzinger complex is the C1 region that contains premotor sympathoexcitatory neurons that provide for tonic sympathetic output important to the maintenance of arterial blood pressure. The work of Sun and Reis (48) has shown that neurons within this region are sensitive to hypoxia in that systemic hypoxia or local application of NaCN to this region causes an increase in neuronal activity and an increase in sympathetic nerve output and arterial pressure. Thus there appears to be a specific sensitivity to hypoxia in these RVLM regions that modulate both respiration and sympathetic output. Our results do not imply that these are the only brain regions sensitive to hypoxia because many other brain regions have been proposed to contain neurons responsive to local hypoxia (10, 11). Thus hypoxic sensitivity may well be a distributed response with specificity linked to the function of its neural output.

The mechanism by which the level of O2 is transduced is still unknown. In other O2-sensing systems, such as the carotid body and pulmonary artery, several mechanisms have been hypothesized. It has been proposed that O2 has a direct effect on an O2-sensitive K+ channel (28). Decreases in O2 tension lead to a closure of this channel, causing a Vm depolarization, followed by an influx of Ca2+ and subsequent release of neurotransmitter. A second hypothesis is that changes in O2 tension are sensed by the cytochrome aa3 of the electron transport chain (12, 33, 34, 52). Inhibition of electron transport by decreased O2 levels results in a decrease in ATP formation and mitochondrial depolarization, resulting in increases in cytosolic Ca2+ and subsequent release of neurotransmitter (13). Several heme proteins have been suggested to be important in the O2 chemosensitivity of these tissues. For example, it has been hypothesized that there is a heme moiety within the cell membrane in close proximity to a K+ channel that causes the closure of this channel when O2 levels decrease (27). Another proposed mechanism is that the heme protein NADPH oxidase mediates O2 chemosensitivity by regulating levels of O2 radicals within the cell, resulting in closure of K+ channels (7). Recently, the enzyme heme oxygenase has been shown to be important for oxygen chemosensitivity in the carotid body (44). Heme oxygenase mediates its response through the production of carbon monoxide, which

drial electron transport chain, thereby halting aerobic metabolism (14, 43). In addition, NaCN also binds to heme metalloenzymes, thereby affecting their activity. There is much debate as to whether chemical hypoxia induced with NaCN has the same effects as hypoxic hypoxia, or low O2 (1, 9). We did not compare the effects of chemical hypoxia with hypoxic hypoxia in our studies. However, Sun and Reis (48–50) made this comparison both in vivo and in vitro. They found in both RVLMM premotor sympathoexcitatory neurons (hypoxia excited) and respiratory-related neurons (hypoxia depressed) that chemical hypoxia and hypoxic hypoxia produced the same qualitative response. In addition, Buckler and Vaughan-Jones (3) showed similar effects of hypoxic hypoxia and NaCN on carotid body activity. Thus NaCN is a reasonable means of eliciting hypoxic responses in chemosensitive systems, allowing for the production of transient episodes of hypoxia that are both reversible and reproducible.

Fig. 7. Effect of hypoxia on input resistance in hypoxia-excited and hypoxia-depressed neurons. Solid lines indicate Vm, whereas dotted lines indicate membrane potential peak response (Vpk) to constant hyperpolarizing current pulse injection (−10 pA; 1-s pulse at 0.2 Hz). Difference between Vm and Vpk, indicated by hatched areas, is proportional to the change in peak input resistance. A: representative neuron excited by hypoxia under low Ca2+-high Mg2+ conditions in which NaCN produces a Vm depolarization (−55.1 mV to −49.1 mV) and a decrease in the difference between Vm and Vpk. B: when the superfusion solution is changed to TTX-artificial cerebral spinal fluid (CSF), excitatory response to hypoxia is maintained as is the decrease in the difference between Vm and Vpk. C: hypoxia-depressed neuron under low Ca2+-high Mg2+ conditions in which NaCN produced a Vm hyperpolarization from −73 mV to −76 mV with a small decrease in the difference between Vm and Vpk. D: different hypoxia-depressed neuron superfused in TTX-artificial CSF in which NaCN produces a Vm hyperpolarization from −62 mV to −65 mV and a small increase in the difference between Vm and Vpk.

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increases the level of cGMP, resulting in changes in such things as ion channel function. Preliminary studies have suggested that inhibition of heme oxygenase blocks the hypoxic excitatory response in cultured RVLM neurons (29). This finding suggests that heme oxygenase may be important to the O₂-sensing function of this region.

In conclusion, we have developed a model system in which the responses of primary dissociated RVLM neurons to NaCN-induced hypoxia were studied. The excitatory response to NaCN seen in an in vivo and in vitro slice is maintained in neuronal cell culture, and this response has a unique dose response and dependence on resting V_m that is intrinsic. In addition, we have begun to explore the ionic mechanism by which NaCN specifically excites these neurons. These data may suggest that RVLM neurons are involved in central O₂ chemotransduction important to the generation of gasping and the augmentation of sympathetic output during hypoxia.

We thank Theresa Hoang-Le for excellent technical assistance and Marcella Spioch for expert preparation of the manuscript.

This work was supported by National Heart, Lung, and Blood Institute Research Grants HL-58730 and HL-16022 and Training Grant HL-07467 and by a Predoctoral Fellowship Award from the American Heart Association-New Jersey affiliate.

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Received 27 December 1999; accepted in final form 2 April 2000.

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