Highly H⁺-sensitive neurons in the caudal ventrolateral medulla of the rat

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The ventral surface of the caudal ventrolateral medulla (cVLM) has been shown to generate intense respiratory responses after surface acid–base stimulation. With respect to their chemosensitive characteristics, cVLM neurons have been less studied than other rostral-most regions of the brainstem. The purpose of these experiments was to determine the bioelectric responses of cVLM neurons to acidic stimuli and to determine their chemosensitive properties. Using extracellular and microiontophoretic techniques, we recorded electrical activities from 117 neurons in an area close to the ventral surface of the cVLM in anaesthetised rats. All neurons were tested for their sensitivity to H⁺. The fluorescent probe BCECF was used to measure extracellular pH changes produced by the microiontophoretic injection of H⁺ in brainstem slices. This procedure provided an estimation of the local changes in pH produced by microiontophoretic H⁺ application in the anaesthetised rat. Neurons coupled to the respiratory cycle, R (n = 51), were not responsive to direct stimulation with H⁺. Sixty-six neurons that did respond to H⁺ stimulation were uncoupled from respiration, and identified as NR neurons. These neurons presented distinct ranges of H⁺ sensitivity. The neuronal sensitivity to H⁺ was mainly assessed by the slope of the stimulus–response curve, where the steeper the slope, the higher the H⁺ sensitivity. On this basis, NR neurons were classed as being either weakly or highly sensitive to H⁺. NR neurons with a high H⁺ sensitivity (n = 12) showed an average value of 34.17 ± 7.44 spikes s⁻¹ (100 nC)⁻¹ (mean ± S.D.) for maximal slope and an EC₅₀ of 126.76 ± 33 nC. Suprathreshold H⁺ stimulation of highly sensitive NR neurons elicited bursting pattern responses coupled to the respiratory cycle. The bursting responses, which were synchronised with the inspiratory phase and the early expiratory phase of the respiratory cycle, lasted for several seconds before returning to the steady state firing pattern characteristic of the pre-stimulus condition. These NR neurons, which possess the capacity to detect distinct H⁺ concentrations in the extracellular microenvironment, are excellent candidates to serve in a chemoreceptor capacity in the caudal medulla.

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The ventral surface of the caudal ventrolateral area of the medulla oblongata (cVLM), which is Loeschcke’s area, has classically been shown to participate in central chemoreception. The rapid respiratory response to metabolic or respiratory acidosis is drastically abolished when the ventrolateral medulla is cooled, lesioned or chemically blocked in anaesthetised animals with peripheral chemoreceptor denervation (Schläfke & Loeschcke, 1967; Berndt et al. 1972; Loeschcke, 1982; Forster et al. 1998). The cVLM is not unique in its function as a central chemoreceptor, but rather it is generally accepted that central chemoreception is widely distributed in various areas of the brain (Nattie, 2000). In most previous studies, the caudal-most part of the ventrolateral medulla has not received the same sort of attention as the more frequently explored rostral region. Besides the physiological function of the cVLM in chemoreception, its homologue in humans, the arcuate nucleus, is generally thought to be involved in the regulation of cardiorespiratory function and to be implicated in the development of sudden infant death syndrome (Filiano, 1994; Gilson et al. 1994; Matturri et al. 2000).

Different techniques have been used to test central chemosensitivity, which complicates the comparison of data obtained from different experimental models. Chemical and electrical stimulation of the exposed ventral surface of the medulla in in vivo experiments on cats and rats induce drastic changes in breathing pattern as well as in neuronal responses (Loeschcke et al. 1970; Schläfke et al. 1970). Experimental changes in the concentration of carbon dioxide (CO₂) and hydrogen ions (H⁺), when applied systemically or upon many brainstem areas, are able to non-specifically stimulate neurons in different brain areas,
masking the \textit{in vivo} physiological response of single central chemoreceptors (St John & Wang, 1977; Middendorf & Loeschcke, 1978). \textit{In vitro} studies have also demonstrated the effects of acid–base stimuli on neuronal activity in the ventrolateral medulla (Richerson, 1995; Putnam, 2001; Wiemann & Bingmann, 2001; Filosa et al. 2002). To date, it appears that CO$_2$/pH chemosensitivity is shared by numerous neurons within the lower brainstem (Ballantyne & Scheid, 2000). Wherever central chemoreceptors are located, they must show a higher sensitivity to changes in proton concentration ([H$^+$]) than other brainstem neurons as well as have an anatomical and/or functional relation to the respiratory network (Wang et al. 1998).

Since functional and anatomical data (Fukuda et al. 1978; Issa & Remmers, 1992; Paul et al. 1995) point to the cVLM as a possible origin of respiratory responses to acid–base stimulation, we performed experiments to identify neurons in this region that exhibited chemoreceptor characteristics. We also chose to use microiontophoretic injection of H$^+$ in anaesthetised rats because it allowed us to more precisely stimulate neurons suspected of being chemoreceptive in nature, while the respiratory system was intact and the animal was breathing spontaneously.

\textbf{METHODS}

\textbf{Animal preparation}

Experiments were performed on 50 Albino Wistar rats of either sex, ranging in weight from 250 to 450 g. All protocols were carried out in accordance with the European Community Guide (86/609) for the Care and Use of Laboratory Animals. Briefly, the animals were anaesthetised with an intraperitoneal (I.P.) injection of chloral hydrate (300 mg kg$^{-1}$), following which the femoral artery and vein were cannulated for measurement of arterial pressure and additional administration of anaesthetic. For subsequent maintenance of anaesthesia, doses of 55 mg kg$^{-1}$ were administered I.V. when animals demonstrated corneal or flexion withdrawal reflexes. Using sterile procedures, the animal under study was placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) and the dorsal surface of the medulla was exposed at the level of the first intervertebral space. Rectal temperature was maintained at 37–38°C using heating pads. Before being placed in position, the mouthpiece and ear bars were covered with pramocaine (Tronothane 1%, Abbot Laboratories, Sligo, Ireland), a long-lasting viscous local anaesthetic. Animals breathed spontaneously and their respiratory cycles were monitored by means of a thermistor (Yellow Spring, CO, USA) located in the nasal air stream.

\textbf{Microiontophoretic procedure}

The four-barrelled micropipettes used for iontophoresis were filled with the following solutions: acetic acid (1 M, pH 2.66), sodium chloride (NaCl) (1 M, pH 7.4), and Direct Blue 15 (Sigma, Inc.; 2% in sodium acetate 0.5 M). Passing positive current pulses through the barrel containing the acetic acid resulted in the ejection of hydrogen ions. One of two NaCl-filled barrels was used for automatic balancing of the current injection. The specific effect of H$^+$ application on neurons was checked by passing a non-specific cationic (Na$^+$) current through the second of the NaCl-filled micropipette barrels. Applying negative current pulses of 2–7 $\mu$A (50% duty cycle) over a 10–15 min period through the barrel containing the Direct Blue 15 permitted labelling of the recording sites for subsequent histological localisation. A Neurophore BH2 (Harvard Apparatus, Edenbridge, Kent, UK) device was used to control the duration and intensity of the current pulses. Stimulation pulses varied in intensity and time, ranging from 14 to 150 nA and from 1 to 50 s. Doses were measured in units of charge (coulombs, C), according to the equation: $Q = I \times t$, ($I$ = current; $Q$ = charge; $t$ = time; 1 nC = 1 nA s).

\textbf{Acidifying procedure}

The free acid form of BCECF (2',7'-bis-(2-carboxyethyl)-5- and 6-carboxyfluorescein) (Molecular Probes Europe, Leiden, Netherlands) was used as a fluorescent probe to measure the acidification of extracellular fluid produced by the current injection of H$^+$. The free acid form of BCECF was chosen because of its low membrane permeance and because we were interested in measuring pH changes in the extracellular fluid. BCECF, often used in the acetoxyethyl (i.e. membrane permeant) form, is most commonly calibrated by using nigericin and potassium to expose cells to known pHs (Thomas et al. 1979). In contrast, calibration of BCECF in the extracellular fluid is uncommon (Chatton & Spring, 1994; Vanderheyden et al. 2000; Street et al. 2001). We performed an \textit{in vitro} calibration of BCECF as a form of estimating the actual pH of the extracellular space at the recording sites in the brainstem of the anaesthetised rats. A two-step procedure was used to do this. The first step determined changes in fluorescence of the BCECF acid probe in artificial cerebrospinal fluid (ACSF) buffered to different pHs (range 4–9). The second step quantified the changes in fluorescence produced by current injection of H$^+$ into brainstem slices.

\textbf{Step 1.} Several rectangular capillary tubes made of borosilicate glass (0.1 mm path length, 2 mm width, 50 mm total length) (Vitro Dynamics, Inc., Rockaway, NJ, USA), were filled with 50 $\mu$M BCECF-free acid in ACSF solution (135 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 10 mM glucose and 0.1 mM Heps) buffered at different pH values. The tubes were placed on the optical stage of an inverted microscope (Zeiss, Axiovert 35) equipped with a fluorescence imaging system. A cooled (−35°C) CCD camera (MCD220, SpectraSource, Agura, CA, USA) (12-bit resolution) was positioned in the other optical port of the microscope for image capture.

The fluorescence emitted by BCECF at an excitation wavelength of 450 nm is not dependent on pH as this is the wavelength of the isobestic point; however it is dependent on pH at 490 nm excitation and for this reason it is possible to use a ratiometric procedure for calibration (Rink et al. 1982; Gryniewicz et al. 1985; Bright et al. 1987). This enables one to obtain a saturation curve for BCECF and hydrogen ions with minimal influence of the fluorophore concentration, solution volume, illumination intensity, photobleaching, inner filtering or scattering. In this way, each capillary tube was excited with light at both 490 nm and 450 nm, and fluorescence emitted at 520 nm was captured by the CCD camera and saved for subsequent analysis. For statistical purposes, several excitation were performed on small areas in distinct locations of every tube using the diaphragm in the fluorescence light path of the microscope.

SpectraSource image analysis software allows for the fluorescence of a defined area of the images to be precisely measured. We selected an area and measured fluorescence values in that area over a series of images. The mean and S.D. of the 490/450 nm
ratio values were used to construct a calibration curve. The deconvolution function for the calibration points provided a pK of 7.38 for the dye. After calibration, pH values could be calculated according to the equation:

$$\text{pH} = \text{pK} + \log \left( \frac{R_{\text{max}} - R}{R - R_{\text{min}}} \times \frac{F_{450_{\text{max}}}}{F_{450_{\text{min}}}} \right)$$

where $R$ is the ratio of fluorescence obtained using the 490 nm and 450 nm excitation wavelengths. Using this procedure, each ratiometric value of fluorescence and thus pH value could be matched to one of 256 grey levels (Fig. 1A).

**Step 2.** Medullary slices were used for measuring changes in pH-dependent fluorescence produced by microiontophoretic H+ injections onto living nerve tissue. Deeply anaesthetised rats (ethyl-ether) were decapitated and the medulla block removed and fixed to a plate in cooled ACSF solution bubbled with a mixture of 5% CO₂ and 95% O₂. Slices of 300 μm thickness were cut with a vibrotome (Lacer, UK). After incubation, slices were positioned in a chamber adapted for continuous solution superfusion (1–2 ml min⁻¹) and the chamber placed on the optical stage of the inverted microscope. The tip of a micropipette containing acetic acid solution (1 M, pH 2.66) was positioned close to the inferior surface of the slice. By passing positive current through the micropipette, changes in pH could be monitored as a decrease in fluorescence when the tissue was excited with light of wavelength 490 nm. The optical focus of the microscope was moved under fluorescent illumination to optimise visualisation of the change in fluorescence produced by the injection of H⁺ into the slice.

To quantify the fluorescence changes produced by different injections of H⁺, we initially chose an area with lowest fluorescence in an image produced by the smallest injection of H⁺. The same area at the same coordinates was used to measure subsequent changes of fluorescence induced by H⁺ injections of different amplitudes. In this way, the measurement error due to the radial gradient of fluorescence produced by H⁺ injections was minimised by restricting the measured area to the core of the injection in the central region of the acidified zone. Fluorescence values at different pHs were obtained by the ratiometric procedure using excitation at the 490 nm and 450 nm wavelengths as well as carrying out normalisation and subtraction of the background fluorescence signal. A neutral density filter was used to attenuate the effects of photobleaching. Fluorescence images were digitised and stored on the hard disk of a computer for further analysis.

The acidified tissue area grew exponentially with the amount of charge injected. The average diameter of the acidified area was 10 μm (range: 5–50 μm). Finally, the values of the 490/450 nm ratios were substituted into the equation of the calibration curve obtained in step 1 and the pH value thus calculated. Tissue pH decayed depending on the rate of charge injection. The relationship between ΔH⁺ charge and ΔpH was $-1.04 \times 10^{-3}$ pH units nC⁻¹. The range of estimated pH changes during *in vivo* experiments was 7.4–6.8 as calculated from the calibration of slices (Fig. 1).

**Recordings**

Extracellular unit recordings were carried out with one of the barrels containing NaCl for which the tip impedances ranged from 2 to 10 MΩ. Neuronal activity was recorded through a high-input impedance amplifier (NEC-11, Biomedical Engineering Corp., New York, USA), filtered, and monitored on a storage oscilloscope. Criteria for electrical isolation of single neuronal cell activity were the following: the amplitude of the electrical signal had to be at least three times larger than the basal noise, the signal had to have a constant waveform compatible with the extracellular appearance of action potentials, and the signal could be stably monitored for more than 15 min. Neurons tested for H⁺ sensitivity had to elicit at least four consistent responses to a predetermined level of H⁺ charge injection in order to be retained for later analysis. The area explored extended from 0.5 mm medial to 3 mm lateral and from −4.0 mm to −5.5 mm with respect to the interaural line in the rostro-caudal axis, and from 2.5 mm dorsal down to the ventral surface in the horizontal plane (Paxinos & Watson, 1997). The electrocardiogram, arterial pressure, respiratory activity and neuronal activity were digitised by means of an A/D input impedance amplifier (NEC-11, Biomedical Engineering Corp., New York, USA), filtered, and monitored on a storage oscilloscope.

**Figure 1. Calibration of the acidification procedure in the extracellular space of nervous tissue**

A, calibration curve for BCECF under *in vitro* conditions. Ratios of the fluorescence emitted by BCECF at 490 nm and 450 nm excitation wavelengths are plotted against distinct pH buffered solutions. $K_C$: dissociation constant. B, BCECF calibration in brainstem slices. The relationship between the charge of the stimuli (top x-axis) and the change in the tissue pH is represented by filled squares and its regression by the continuous line. The relationship between time (bottom x-axis) and pH is represented by filled circles and its regression by the dashed line. Inset shows three images of a brainstem slice and the size effects of three different H⁺ stimulations. White arrows show the areas where pH decreased. Calibration bars for images: 100 μm. Vertical calibration bar: grey scale for changes in fluorescence induced by H⁺ stimulation.
converter (Neurodata Inc., Pasadena, CA, USA) with sampling rates of between 11 and 88 kHz. All records were stored on videotape and converted to ASCII files when needed for analytical purposes.

Data analysis
Data from neurons tested for H⁺ sensitivity were stored in an ASCII file and analysed off-line with a custom-made software program written in the C language. The software was able to distinguish a spike by its waveform on the basis of the rise-time from 10% to 90% of the peak amplitude and on the half-width duration of the spike. A Windows discriminator function was enabled by selecting a value of signal amplitude with reference to the S.D. and basal noise. The firing rate was analysed using a moving average of the values of 10 interspike intervals (ISIs). The value of the pre-stimulus firing rate was calculated as the mean of ISIs during 5 s periods of recordings. The maximal increase in firing rate from each response was calculated as the maximal value from the moving average. The measurement of the regularity of the pre-stimulus firing rate and the subsequent discriminant analysis was based on the ISI mean value for 150 s. Digitised data were exported to a conventional spreadsheet (Microcal Origin 5.0) for mathematical calculations and statistical analysis. A minimum of five data points, each one representing the mean value of at least three responses to the same level of stimulation, were chosen for the construction of a dose–response curve. Mathematical fitting of data was carried out by a 500 iterations procedure for linear and dose–response functions implemented in the spreadsheet. Plots of both H⁺ charge and estimated pH stimulus against firing rate were used to classify the neuronal responses. Thresholds for each fitting were determined as the pH value when the increasing frequency of action potentials reached the half value between the foot of the steepest slope and the pre-stimulus frequency in the sigmoidal fit. EC₅₀ values were obtained after non-linear fitting (Boltzmann function) of the neuronal responses to H⁺ stimulation. The slope values of the neuronal responses were calculated by linear fitting of data. Statistical significance was determined using a two-sample t test assuming unequal variances, with values of P < 0.05 considered as significant. All values are expressed as the mean ± S.D.

Histological procedures
At the end of the experiments, animals were deeply anaesthetised (sodium pentobarbital, 300 mg kg⁻¹ i.v.) and transcardially perfused with saline solution for 5 min followed by 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4). The brainstem was removed, postfixed overnight and stored in a cryo-protecting solution until freezing. Coronal brain slices (40 μm thick) were obtained with a freezing microtome. Sections were mounted on gelatin-coated slides, air-dried, counterstained with the Nissl method, dehydrated, cleared with xylene and coverslipped with DPX (Panreac, Barcelona, Spain). Histological localisation of the recording sites was obtained in 95% of the experiments. The localisation of sites was made by an individual experimenter who was blinded to the H⁺ analysis results.

RESULTS
As a first approach, neurons (n = 117) in the caudal region of the medulla, in an extension overlapping Loescheke’s area, were classified by their bioelectric activities as: (a) R (n = 51), spontaneously active and related to the respiratory cycle or (b) NR (n = 66), spontaneously active but not related to the respiratory cycle. Both classes of neurons were recorded from and tested for H⁺ sensitivity. After delivery of stimuli

Figure 2. Respiratory-coupled neuron (R) unresponsive to H⁺ stimulation
R neuron activity and respiratory cycles with increasing H⁺ stimulations. A, upper panel, extracellular recording of a cVLM neuron with respiratory-related activity; middle panel, average firing frequency; and lower panel, respiratory cycle recording from an anaesthetised rat. B and C, upper panels, average firing frequency; lower panels, respiratory cycle recording from an anaesthetised rat. Application time of stimulus (for 100, 350, and 600 nC total charge) is depicted by the bar. There was no increase in firing rate after stimulation. Calibration bar shows time base. The arrow indicates expiration phase.
through the micropipette containing the acid solution, the values of maximal increases in firing rates were measured and statistically compared with pre-stimulus values. The characteristics to be studied to determine whether or not a neuron could be classified as sensitive to H⁺ stimulation were the following: (a) the threshold for activation by H⁺ exposure, (b) the characteristics of the dose-dependent response, and (c) the failure of a cationic current to produce the same response induced by H⁺ injection at the same stimulus intensity.

On the whole, R neurons were located further from the ventral surface of the medulla than NR neurons, at a depth of 2.5–3.0 mm from the dorsal surface, in an area corresponding to the caudal ventral respiratory group (Ezure et al. 1988). These cells were unresponsive to H⁺ stimulation at doses ranging from 50 to 700 nC (Fig. 2). There was no statistical significance ($P<0.05$) between maximum frequency of firing in pre-stimulus and post-stimulus values for all R neurons tested. Therefore, this group was classified as insensitive to H⁺ stimulation, at

![Figure 3](image-url)

**Figure 3.** Response of a low H⁺-sensitive non-respiratory (NR) cVLM neuron to H⁺ stimulation

A, NR neuron activity and respiratory cycles with increasing H⁺ stimulations. Upper panel, respiratory cycle recording from an anaesthetised rat, middle panel, extracellular recording of a cVLM neuron, lower panel, average firing frequency. The arrow indicates expiration phase. Application time of stimulus (108, 150 and 224 nC) is depicted by the bar. Calibration bar shows time base. These neurons showed low-level responsiveness to proton stimulation.

![Figure 4](image-url)

**Figure 4.** Response of a highly H⁺-sensitive cVLM neuron to acid stimulation

A, lack of response of a highly H⁺-sensitive NR neuron to non-specific cationic (Na⁺) stimulation. Upper panel, extracellular recording of a cVLM neuron; lower panel, average firing frequency. B, selective response to H⁺ stimulation of a NR neuron. Upper panel, extracellular recording of a cVLM neuron; lower panel, average firing frequency. C and D, average firing frequency with increasing H⁺ stimulations. E, respiratory cycle recording from the anaesthetised rat. The arrow indicates expiration phase. Application time of stimulus (for 56, 60 and 84 nC total charge) is depicted by the bar. Note that the duration of the response was proportional to the intensity and duration of the stimulation. Calibration bar shows time base.
least in the range used in this work. Of the 51 R neurons from which recordings were made, 35 were classified as expiratory and 16 as inspiratory, depending on the respiratory phase to which they were coupled.

NR neurons showed positive and different responses to H⁺ stimuli (Fig. 3). Fifty-four out of 66 neurons examined required stimulus intensities higher than 100 nC to weakly augment their firing rate, and higher than 200 nC to markedly increase their frequency of action potential firing (Fig. 3). The increase in firing rate showed a slope based on the group average of 6.55 ± 3.57 spikes s⁻¹ per 100 nC (or per 0.1 pH unit) and a threshold of 221.15 ± 48 nC (equivalent to 7.17 ± 0.05 estimated pH units) for the group exhibiting low H⁺ sensitivity. The mean EC₅₀ value for these neurons was 368 ± 84 nC (7.02 ± 0.09 estimated pH units), with a mean regression coefficient of 0.979. This group of neurons, although responsive to H⁺ stimulation, was classified as having a low sensitivity to H⁺ and low thresholds, low slope values and high EC₅₀ values (see Fig. 5).

Twelve neurons (18%) of the NR population in the cVLM from which recordings were made showed responses consistent with a fine discrimination of changes in [H⁺], and were therefore classified as being highly sensitive to changes in [H⁺]. A typical firing response of one of these neurons is shown in Fig. 4. This response was characterised by a progressive increase in firing rate following H⁺ stimulation. The microiontophoretic application of Na⁺ to the same neuron and at the same stimulus intensity revealed the selectiveness of the suprathreshold responses to H⁺ of this chemosensitive neuron (Fig. 4A and B). The increase in frequency of action potentials upon application of H⁺ reached a maximum (up to six times the pre-stimulus frequency) and then diminished towards the pre-stimulus firing rate (Fig. 4). Individual fitting of dose–response data for each NR cell is plotted in Fig. 5. A low threshold, a low EC₅₀, and a high slope as depicted in the upper left group characterised the stimulus–response fittings of highly H⁺-sensitive NR neurons. The threshold for this group of neurons was 28.84 ± 9.6 nC (7.37 ± 0.01 estimated pH units), the slope value was 34.17 ± 7.4 spikes s⁻¹ (100 nC)⁻¹ with a correlation for linear fit of 0.976. The average EC₅₀ for this group was 126.76 ± 33 nC (7.27 ± 0.04 estimated pH units) with a regression coefficient value of 0.978 (Fig. 5B).

Highly sensitive NR neurons usually responded to H⁺ injection with a progressive increase in firing frequency; however a few highly sensitive NR neurons (5 out of 12) showed a bursting pattern response after stimulation. The

Figure 5. Relationship between focal pH change and maximal firing rate responses of NR neurons in cVLM
A, estimated pH and proton charge during stimulation are plotted against the frequency of action potentials displayed by NR neurons. Continuous traces, dose–response curves of low H⁺-sensitive neurons. Dashed traces, dose–response curves of highly H⁺-sensitive neuron. B and C, statistical differences in slopes and EC₅₀ of both groups, NR neurons of low and high H⁺ sensitivity (** P < 0.001).
bursting pattern consisted of periods of high frequency firing of action potentials alternating with silent periods. This bursting pattern activity was synchronised with the respiratory cycle for several seconds after stimulation and lasted from 3 to 16 s (Fig. 6). The duration of the bursts ranged from 179 to 480 ms (mean ± S.D., 256.4 ± 67.9 ms, n = 40), and the maximum intraburst frequency of action potentials was 80 spikes s⁻¹. At the end of the bursting response, the intraburst frequency of action potentials decayed smoothly and the interburst interval shortened, returning again to a stable discharge mode (Fig. 6A).

In general, neurons exhibiting high H⁺ sensitivity discharged at slightly higher rates than neurons belonging to the low H⁺ sensitivity group under pre-stimulus conditions. The mean frequency values were 7.75 ± 2.82 spikes s⁻¹ and 6.61 ± 2.85 spikes s⁻¹ for the high and the low H⁺ sensitivity groups, respectively. The discharge pattern of low sensitivity neurons was more irregular than that of high sensitivity neurons as evidenced by the coefficient of variation of the interspike interval (CV isi). The low H⁺ sensitivity neurons with a mean interspike interval of 199.8 ± 391 ms (mean ± S.D.) showed a CV isi of 1.96 ± 1.59. The mean value for the interspike interval of the high H⁺ sensitivity group of neurons was 140.7 ± 57.13 ms and the CV isi was 0.38 ± 0.21. A two-way analysis of variance showed that the CV isi for the low and high H⁺ sensitivity groups was significantly different (P < 0.001).

Within the high H⁺ sensitivity group, a subset of neurons exhibiting bursting responses after acidic stimulation showed a more regular pattern of discharge as evidenced by the lowest CV isi (0.18 ± 0.67). However, the high H⁺ sensitivity subset without a bursting discharge pattern showed a CV isi of 0.52 ± 0.16. This was less regular than that of the bursting subset but more regular than that of the low H⁺ sensitivity group. A linear discriminant analysis (Mason, 1997), taking the mean (X) interspike interval and the standard deviation (S.D.) of the interval of the discharge pattern of the two groups of neurons as independent variables, permitted us to distinguish between the low and high H⁺ sensitivity neurons with an error less

![Figure 6. Bursting response of a highly H⁺-sensitive NR neuron of the cVLM to H⁺ stimulation](image)

A, suprathreshold response to a local injection of 290 nC of H⁺ (bar). Note that the neuron started to increase its firing rate during the second half of the stimulation period and rapidly passed to bursting activity which was synchronous with the respiratory cycle. Upper panel, respiratory recording; middle panel, extracellular recording of the cVLM neuron; lower panel, frequency histograms of action potential firing (bin: 100 ms). The bursts of neuronal activity were synchronised with the respiratory cycles and lasted more than 12 s. The frequency histogram in the lower panel shows the intraburst frequency. B, the pre-stimulus condition; C, a parcel of the bursting response. The data in C correspond to the square marked as C in A. The arrow indicates expiration phase. Note that bursts were synchronised with the inspiratory phase and the early expiratory phase of the respiratory cycle. Calibration bar shows time base.
than 16% (Fig. 7). The discriminant function for the separation between the low and high H⁺ sensitivity groups of neurons was of the form:

\[ Y(\bar{X}, \text{s.d.}) = 58 - \bar{X} + 0.98 \text{s.d.} \]  

(1)

The continuous line in Fig. 7 represents a value for function (1) equal to zero and defines the optimal boundary between the two groups of neurons. In logarithmic plots, this line appears as a curve. Under our experimental conditions, any neuron with a high H⁺ sensitivity should exhibit a value of the discriminant function < 0, whereas function values > 0 should identify any neuron with a low H⁺ sensitivity.

The histological localisation from the recording neurons was assessed by visualising the iontophoretic injection of Direct Blue 15 that was made into the same sites as those used for making recordings of neuronal activity. The anatomical distribution of the recorded neurons in the cVLM is represented in Fig. 8. Respiratory neurons, considered to be insensitive to H⁺ in this work, were localised far from the ventral surface, in the proximity of the retroambiguus and ambiguus nuclei. NR neurons with a low H⁺ sensitivity were loosely distributed throughout the studied area. One subset of highly H⁺-sensitive NR neurons was located close to the ventral surface of the medulla, at the level of the hypoglossal rootlet and beside the olivary complex, in the area known as the parapyramidal region. The other subset extended dorsally to the pyramids, in an area corresponding to the extension of the B3 serotonergic cell group.

**DISCUSSION**

The present report has looked at the H⁺ stimulation of putative chemosensitive neurons in the cVLM of the medulla, an area whose surface has been shown to respond to acid–base stimulation. Using anaesthetised rats and H⁺ microiontophoresis techniques, we have identified a unique group of cVLM neurons with graded sensitivity to H⁺ that have not been described thus far. Within this group, some neurons demonstrated in-phase responses with respiration after H⁺ stimulation. Although the anatomical connections of these neurons with respiratory nuclei remain to be determined, both their functional relationship to the respiratory output and their high sensitivity to H⁺ strongly suggest their involvement in central chemoreception.
In order to be considered as candidates for central chemoreceptors, cells must meet a minimum number of criteria such as displaying different thresholds to common respiratory stimuli as changes in pH, P_{CO_2} or P_{O_2}, dose-dependent responses to increasing stimuli, anatomical connectivity with other brain structures related to respiratory regulation, and functional coupling of the chemoreceptor response to the respiratory output (Richerson, 1998; Wang et al. 1998; Wang & Richerson, 2000). Various technical requirements are needed in order to establish the presence of these characteristics. These include the ability to produce local acidification and to grade stimuli while recording neuronal activity, and the ability to identify anatomical or functional connections with the respiratory network. All of these were possible using our experimental approach.

In contrast to the scarcity of data from in vivo experiments using direct somal application of hydrogen ions, most data on central chemosensitivity at present proceed from in vitro experiments or from in vivo experiments using inhalation or infusion of CO₂. Using multibarrelled micropipettes, we were able to locally and gradually acidify nervous tissue in a physiological range and over a small area (5–50 μm in diameter) (Fig. 1) and thereafter to characterise the threshold and the dose–response curve of putative chemosensitive neurons. Marino & Lamb (1975) previously used extracellular recordings and microiontophoretic injection of H⁺ in deeper regions of the ventrolateral medulla of the cat. They recorded just 3 out of 74 putative chemosensitive NR neurons compared to our 12 highly H⁺-sensitive neurons out of 66 (18%). The number of highly H⁺-sensitive neurons in our data is in accordance with previous studies by Wang & Richerson (2000). This difference in the percentage of chemosensitive cells between our study and that of Marino & Lamb (1975) might be explained by the distance of the recording sites away from the ventral surface of the medulla in the latter study. Marino & Lamb (1975) also showed that the direct iontophoretic application of nontoxic amounts of hydrogen ions to the external environment surrounding the cell bodies of all respiratory neurons was incapable of reproducing the effects of CO₂. Accordingly, we were incapable of altering the firing rate of respiratory neurons by means of H⁺ iontophoresis over a wide range of stimulus amplitudes (Fig. 2). These findings indicate that neurons spontaneously firing in phase with the respiratory cycle do not have intrinsic chemoreceptive properties.

Perhaps, the most remarkable characteristic of the H⁺-sensitive neurons in our results has been their high sensitivity to H⁺ stimulation (34.17 spikes s⁻¹ (100 nC)⁻¹). This might be due to the direct injection of protons close to neurons from which recordings were made. Using the same technical approach in the cat, Marino & Lamb (1975) found a range of neuronal responses (5–80 spikes s⁻¹), which is in agreement with the data reported here (Fig. 5). Nattie et al. (1993) identified a group of neurons in the retrotrapezoid nucleus (RTN) of the cat that became respiratory-modulated in hypercapnia. However, in eucapnia, these neurons did not discharge in phase with the respiratory cycle. Their firing rate increased from 5.1 ± 2.5 to 52.9 ± 13.5 spikes s⁻¹ during hypercapnic stimulation with 7% CO₂. This hypercapnic stimulation is approximately equivalent to a change of 0.2 pH units, as shown by Wiemann et al. (1998) and Wang et al. (2002). In our results, H⁺ injections producing a decrease of 0.2 units of the estimated pH on high H⁺-sensitive neurons increased their mean firing rate in a similar range (Fig. 5). Although more rostrally located than neurons from which we recorded, the respiration-modulated neurons in the RTN might coincide functionally, according to their discharge responses, to our highly H⁺-sensitive neurons in the cVLM.

Other observations of medullary neurons responding to CO₂ or H⁺ have been reported in more rostral sites of the VLM (Podorski, 1976; Schlafke, 1981). More recently, other authors used CO₂, H⁺ or acetazolamide through diffusion probes in the VLM under in vivo and in vitro conditions to produce focal acidification over an area of diameter 300–500 μm, while simultaneously recording from remote neurons and/or stimulating the respiratory output but not the activity of those neurons directly (Coates et al. 1991, 1993; Issa & Remmers, 1992; Nattie & Li, 1996; Li & Nattie, 1997; Erlichman et al. 1998).

Data from isolated brainstem, slices or cell cultures of medullary neurons have provided information on the characteristics of the primary stimulus necessary to induce respiratory responses in brainstem preparations (For a review see Ballantyne & Scheid, 2000). In addition, some membrane properties and synaptic characteristics of neurons involved in responses to acid–base stimulation are also known. Voipio & Ballanty (1997), using H⁺-sensitive microelectrodes and controlling P_{CO_2} and HCO₃⁻, proposed that extracellular [H⁺] is the primary stimulatory factor in central chemosensitivity. The well known stimulatory effects of CO₂ on the respiratory network are not in contradiction with these data (Harada et al. 1985; Millhorn & Eldridge, 1986). Cellular mechanisms under-lying electrophysiological responses induced by alteration of the H⁺ concentration include Na⁺–H⁺ exchangers, ion channels and Na⁺–Cl⁻–HCO₃⁻ exchangers, the latter of which are not expressed or do not contribute significantly to acid extrusion from chemosensitive neurons (Wiemann & Bingmann, 2001). Inhibition of the type 3 Na⁺–H⁺ exchanger has resulted in intracellular acidification in the same range and time course as that produced by hypercapnia (Ritucci et al. 1998; Wiemann et al. 1999). Recently, Filosa et al. (2002) proposed that the primary
stimulus for chemosensitive responses is a change in the intracellular pH. The underlying mechanism might be related to the observation that neurons from various chemosensitive regions do not exhibit pH recovery in response to hypercapnic acidosis (Ritucci et al. 1997; Wiemann et al. 1998; Putnam, 2001). Moreover, Wiemann & Bingmann (2001) argued that the pHo or the pH gradient across the membrane of a chemosensitive neuron in the ventrolateral medulla oblongata was not responsible for (or was of minor importance in) the typical bursting increase of firing rate seen during hypercapnia. Conclusions from Filosa et al. (2002) are mainly based on the response of locus coeruleus neurons to a fall in pH induced by isothydric hypercapnia, with an absence of any change in pHo, and secondarily on the effect on firing rates induced by hypercapnic acidosis, isocapnic acidosis or Heps (N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid)-buffered acidification, all three of which resulted in the same change in pHo. Nevertheless, data from Filosa et al. (2002) also showed that the largest increases in firing rate occurred with the largest falls in pHo, that is, with hypercapnic acidosis and Heps acidification as the stimulating conditions. In fact, the authors of that paper proposed that changes in pHo, through inhibition of pHi regulatory systems leading to a sustained fall in pHo, play at least a modulatory role in chemosensitive signalling in locus coeruleus neurons. Therefore, whether pHo is a major part of the intracellular response of chemosensitive neurons or not, the chemosensitive response is enhanced in the presence of a fall in pHo.

In vitro preparations, although adequate in addressing cellular questions while showing recognisable respiratory output, still present some differences when compared to in vivo preparations. Major differences concern the respiratory rhythm, which is slower in vitro and displays shorter inspiratory bursts and a reverse response to hypercapnia compared to what is seen in vivo experiments (Ballantyne & Scheid, 2000). Most of those differences seem to be due to the effects of low temperature, deafferentiation and acid–base conditions in the in vitro preparations (Rekling & Feldman, 1998; St John, 1999). However, the overall responses displayed by CO2/H+-sensitive neurons in the ventrolateral medulla in slice cultures (Kawai et al. 1996) resemble those recorded extracellularly in our experiments. Taking into account the above, we chose the extracellular recording technique as an appropriate tool to observe neuronal activity while microiontophoresing H+ in situ as a means of identifying chemosensitive neurons in the cVLM.

In our report, a stimulus amplitude below 700 nC failed to elicit a H+-dependent response for all tested respiratory neurons located in the caudal medulla. Stimulus amplitudes higher than 700 nC were not used as these have been shown to produce neuronal damage (Marino & Lamb, 1975). Moreover, no reports have been published to date concerning the membrane potential response of rhythmic respiratory neurons to CO2. Therefore, the respiratory neurons recorded in this study were considered to be non-responsive to direct H+ stimulation, as distinct from responsive neurons at similar levels of the neuroaxis.

Our data show for the first time, and under in vivo conditions, bursting activity in neurons of the cVLM in response to changes in [H+] applied in close proximity to those neurons. The activity of these neurons was noted as changes from tonic firing to burst pattern activity that was synchronised with the spontaneous respiration of the animal. All H+-induced bursting neurons in our database belonged to the NR group. Similarly, data from Kawai et al. (1996) showed that the membrane potential of non-rhythmic inspiratory neurons, but not of rhythmic respiratory neurons, of the ventral respiratory group was sensitive to CO2. Interestingly, a change from a tonic to a bursting pattern in the discharge of brainstem neurons was observed by Okada et al. (1993) in an in vitro preparation of neonatal rats after acidification of the superfusing media. These authors recorded a few neurons in the medial and rostral parts of the ventral medulla, while we recorded our neurons in the caudalmost VLM. More recently, changes in the firing pattern of putative chemosensitive neurons have been described in in vitro studies. It seems that a slight intracellular acidification of about 0.05 pH units or a slight depolarisation (< 5 mV) irregularly increases neuronal firing rate, turning it into periodic bursting (Wiemann & Bingmann, 2001; Filosa et al. 2002). Given that we did not control the acid–base status of the rats, we cannot disregard the possibility of an effect of slight acidification on our findings; however the phenomenon of induced membrane depolarisation as an artefact using extracellular recordings is rare. Nevertheless, according to our data, all changes in the firing pattern from tonic to bursting were preceded by stable, continuous recordings and only after H+ delivery onto the neurons did any changes occur to produce a burst of action potentials. Therefore, the change in [H+]o was almost certain to be the trigger for the bursting response. Furthermore, the bursts were synchronised with the spontaneous respiratory rhythm.

The shift from steady to burst firing has been shown to be due to changes in membrane conductance for potassium and calcium in neurons of the periaqueductal grey (Sanchez & Ribas, 1991). In addition, the interburst period appeared to be governed by an after-hyperpolarisation that decreased with time, giving rise to a tonic discharge (Wiemann et al. 1999). The nature of the ion channels involved in the neuronal response to acid stimuli is not known at present. Inward rectifier potassium channels (IRK+) (Coulter et al. 1995), acid-sensing ionic channels (ASIC-H+) (Bevan & Yeats, 1991; Waldmann et al. 1997),
calcium-dependent potassium channels (Cook et al. 1984; Wellner-Kienitz et al. 1998) and a weakly rectifying ‘leak’ channel named the acid-sensitive potassium channel type 1 (TASK-1) have been related to cellular mechanisms in the chemosensitive response (Duprat et al. 1997; Bayliss et al. 2001). Thus, it appears that an ion channel-based mechanism could exist to explain the change in activity, from steady to burst response, seen in the NR neurons described here.

The mathematical differences describing the responses of NR neurons to H\(^+\) stimulation provide additional features for the classification of neurons on the basis of their chemosensitivity. The dose–response curves permitted differentiation to be made between chemosensitive neurons based on their threshold response and the steepness of the response, thus giving rise to NR neurons exhibiting low or high sensitivities to H\(^+\). The latter showed the lowest threshold and the steepest response to changes in pH (Fig. 5). This approach permitted basic criteria to be proposed for identifying neurons as central chemoreceptor candidates. Furthermore, the linear discriminant analysis of the discharge patterns provided a clear boundary between the two groups of neurons. The high H\(^+\) sensitivity group exhibited SD and CV\(_{\text{int}}\) values that were much lower than those of the low H\(^+\) sensitivity group, which is consistent with the more regular discharge rate of the high sensitivity group. This steady discharge pattern in the pre-stimulus condition is a characteristic of serotonergic neurons (Mason, 1997). However, the mean interspike interval of the chemosensitive neurons in this study was greater than the S.D. of the interspike interval by 58 ms or more, whereas the serotonergic neurons recorded by Mason (1997) showed a difference of more than 150 ms between the mean and the S.D. of the interspike interval. Therefore, from a strictly quantitative point of view, our H\(^+\)-sensitive neurons cannot be classified as serotonergic cells but can be classified as serotonergic-like neurons as proposed by Mason (1997). Nevertheless, this author used halothane-anaesthetised rat while we used chloral hydrate as an anaesthetic, which is known to not modify the firing rate by more than 20% as compared to the spontaneous rate of firing seen in unanaesthetised animals (Heym et al. 1984).

Despite the bursting activity and the intraburst frequency increase after H\(^+\) stimulation in the cVLM, there was no appreciable change in the breathing pattern (Fig. 6C). This should not be surprising given the probable minimal effect that one stimulated neuron would have in modifying the breathing pattern. Our results show that the post-stimulus bursting responses of NR neurons were synchronised with ventilation in an unusual manner. Burst action potential activity appeared in conjunction with inspiration and early expiration, suggesting a strong dependence of the activity on cyclic respiratory control. The bursting activity coupled to inspiration after H\(^+\) stimulation may provide support for anatomical connections to be present with innervating pathways associated with phrenic motoneurons and preganglionic neurons (Haxhiu et al. 1996; Hadziefendic & Haxhiu, 1999). The observed change in the discharge pattern of the bursting NR neurons also suggests that they were sending out tonic information when the extracellular pH levels were stable, and then shifted to an increased oscillatory behaviour after abrupt perturbation of [H\(^+\)], as one might expect of the proper response of a sensor engaged in maintaining homeostatic conditions.

The caudal ventrolateral medulla explored in this work has been shown to be connected to other nuclei that are well known for their relationship with cardiorespiratory control (Haxhiu et al. 1995; Pásaro et al. 2000). Other authors have related the parapyramidal area and the retrotrapezoid nucleus, the first of which corresponds to the area explored by us, to the respiratory output in response to hypoxia (Bodineau et al. 2000). Using immunoreactive techniques, Berquin et al. (2000) showed differences in the responses of neurons in the medulla oblongata to hypoxia and hypercapnia. Hypoxia elicited marked c-Fos labelling in the parapyramidal neurons located close to the ventral surface and in the nucleus of the solitary tract. Compared with hypoxia, hypercapnia evoked greater c-Fos labelling near the ventral surface of the paralolivar region, an area overlapping the site recorded in the present study (Sato et al. 1992). Recently, local acidification of the cVLM over the surface of Loeschcke’s area was shown to induce intense c-Fos immunoreactivity in the same brainstem area from which recordings were obtained in our work and in other respiratory-related nuclei (Douglas et al. 2001). These data highlight the fact that the area used in our study contains neurons susceptible to activation by changes in P\(_{\text{CO}_2}\)/[H\(^+\)].

We did not determine the anatomical connections of our candidate chemosensitive neurons to ventilatory group nuclei. However, one possible approach to looking for connections between chemosensitive neurons and the neurons controlling ventilation is to use the respiratory rhythm itself as an indicator of connectivity and to examine whether neurons showing this rhythm are also chemosensitive (Ballantyne & Scheid, 2000). In accordance with this approach, our data demonstrate the presence of chemosensitive neurons, apparently not related to respiration, that can be made to fire synchronously with respiration in the presence of an elevated hydrogen ion concentration in a manner that might account for such a functional connectivity to centres of respiration control. From a histological viewpoint, the localisation of our NR neuron candidates to central chemoreceptors in the proximity of the ventral surface of the cVLM fits well with an ideal location for such chemoreceptors, i.e. a region with a rich blood supply like that which surrounds the neighbouring area around the hypoglossal nerve root near
to the main bifurcation of the basilar artery (Bradley et al. 2002) and having ease of exchange of products with the CSF. This region corresponds to Loeschcke’s area (Fig. 8).

In conclusion, our results indicate that cVLM neurons with a resting activity synchronised with the respiratory cycle did not show any sensitivity to H⁺ stimulation. In contrast, a group of NR neurons nearer to the ventral surface and within the parapyramidal region of the caudal medulla, exhibited high sensitivity to H⁺ stimulation. The degree of cellular chemosensitivity and the phase-locked respiratory bursting responses to H⁺ stimulation found by us in the parapyramidal region of the caudal VLM have not been previously reported. These data show, for the first time in an in vivo model, the presence of neurons in the cVLM that can be characterised by their response to H⁺ stimulation, including an output related to the motor drive for breathing. These neurons represent excellent candidates for central chemoreception.

REFERENCES


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