Acidosis-Stimulated Neurons of the Medullary Raphe Are Serotonergic

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INTRODUCTION

Despite wide variations in daily activity and metabolic requirements, the arterial blood concentration of carbon dioxide is maintained remarkably constant through precise feedback control of ventilation. The neurons responsible for sensing CO2, the central chemoreceptors, have not yet been identified at the cellular level. These neurons were once thought to be located exclusively within the ventrolateral medulla (Mitchell et al. 1963; Schlafke 1981), but recent evidence indicates that they are more widely distributed (Nattie 1999). Several brain stem nuclei contain chemosensitive neurons that are now candidates for this role (Dean et al. 1990; Kawai et al. 1996; Neubauer et al. 1991; Pineda and Aghajanian 1997; Richerson 1998), including the medullary raphe (Richerson 1995; Wang et al. 1998).

When studied in vitro, there are two types of intrinsically chemosensitive neurons in the rat medullary raphe, one stimulated and the other inhibited by a decrease in pH induced either by an increase in CO2 or a decrease in [NaHCO3] (Richerson 1995; Wang et al. 1998). Both types of neurons have a degree of chemosensitivity that is consistent with their response to CO2 playing a functional role under physiological conditions. For example, acidosis-stimulated neurons respond to a decrease in extracellular pH from 7.4 to 7.2 with a mean threefold increase in firing rate (Wang et al. 1998). These two types of chemosensitive neurons have been proposed to be central respiratory chemoreceptors, acting in opposite ways to modulate ventilation and other CO2/pH-sensitive brain functions (Richerson 1995). A role for the raphe and serotonin in chemoreception is supported by observations in vivo that some raphe neurons in cats increase their firing rate in response to inhalation of CO2 (Veasey et al. 1995), that focal microinjection of acetazolamide into the rat medullary raphe stimulates ventilation (Bernard et al. 1996), and that a chemical lesion of serotonergic neurons with 5,7-dihydroxytryptamine causes hypoventilation and blunting of the response to inhaled CO2 in rats (Mueller et al. 1984).

The medullary raphe nuclei include the raphe pallidus, raphe magnus, and raphe obscurus. The neurons present within these nuclei are heterogeneous; however, the principal cell type is serotonergic, comprising ~25% of raphe neurons (Mason 1997). Within some raphe neurons, the neuropeptides TRH and substance P are found co-localized with serotonin (Dean et al. 1993; Iverfeldt et al. 1989). When studied in vivo or in brain slices, serotonergic neurons of the raphe have characteristic and relatively homogeneous electrophysiological properties. These properties include a highly regular firing pattern (Aghajanian and Vandermaelen 1982a; Jacobs and Azmitia 1992; Jacobs and Fornal 1991; Mason 1997; McCall and Clement 1989; Vandermaelen and Aghajanian 1983) and wide action...
potentials (Aghajanian and Vandermaelen 1982a; Bayliss et al. 1997a) a following by an afterhyperpolarization (AHP) that reverses at a voltage 25 mV more positive than the predicted Nernst potential for K⁺ (Penington and Kelly 1993). Serotonergic neurons also display prominent A current (Aghajanian 1985; Segal 1985) and respond to a depolarizing current pulse with spike frequency adaptation followed by a prolonged AHP (Aghajanian and Vandermaelen 1982b; Bayliss et al. 1997b).

A primary dissociated cell culture system has been developed to permit detailed study of the intrinsic properties of raphe neurons (Wang et al. 1998). This approach offers advantages for electrophysiological studies, including stability of recordings and control of the extracellular milieu. It is also possible to record from neurons that have grown for prolonged periods, during which they develop a mature response to CO₂ (Wang and Richerson 1999). The use of cell culture to study chemosensitivity is possible because the response to acidosis of medullary raphe neurons is similar in culture (Wang et al. 1998) and in brain slices (Richerson 1995), including a similar time course in postnatal development of chemosensitivity (Wang and Richerson 1999). When studied in this culture system, the subset of raphe neurons that are stimulated by acidosis are relatively homogeneous. They tend to have a similar morphology with a larger, multipolar soma (Wang et al. 1998). Their firing patterns are also similar, with a low variability in their interspike interval (Wang et al. 1998), a measure of regularity of firing at a short time scale that has been associated with serotonergic neurons in vivo (Mason 1997). Their homogeneity suggests that acidosis-stimulated neurons might comprise a specific neuronal phenotype, and their location and firing pattern suggest that they might be serotonergic. Here, this possibility was tested by first recording from rat ventromedial medullary (VMM) neurons grown in long-term primary cell culture and then using immunohistochemistry to identify their neurotransmitter content. The basic electrophysiological properties of raphe neurons in culture were also studied to determine how closely they reflect their counterparts in situ, which is important for determining whether the results obtained using this approach are relevant to serotonergic neurons in vivo.

A portion of this work was previously published in abstract form (Richerson and Wang 1998).

METHODS

Cells culture

Cultures of the VMM were prepared using methods described previously (Wang et al. 1998) except that cells were plated on CELLocate coverslips (Eppendorf, Westbury, NY) to aid in locating neurons after immunostaining. Briefly, cultures were prepared from neonatal (P0–P3) Sprague-Dawley rats using aseptic technique. A wedge of tissue from the ventromedial portion of the rostral one-half to two-thirds of the medulla was removed that contained the raphe pallidus, raphe magnus, raphe obscurus, and tissue immediately adjacent to these nuclei. Dissected tissue was digested for 30 min with papain, triturated, and plated on poly-l-ornithine- and laminin-coated CELLocate coverslips at a density of 0.5–1×10⁶ cells/ml. Cells were fed with either 10% fetal bovine serum (FBS) in modified Eagle’s medium (MEM) or 10% FBS/5% MEM/36% neurobasal medium with B27 supplement. Medium was conditioned for 1 day before use by glial cultures obtained from the VMM. In all cases, basic fibroblast growth factor (bFGF; 0.1–1 ng/ml) and fibroblast growth factor-5 (FGF-5; 1–10 ng/ml) were added to the culture medium to enhance survival (Lindholm et al. 1994). Cultures were maintained in an incubator at 37°C and 5% CO₂. Cells were first fed on day 4–7 with a half-volume change of neurobasal/B27 medium to which cytotoxic β-2-arabinofuranoside hydrochloride (Ara-C) (3 μM) was added to inhibit glial growth, and then fed with neurobasal/B27 medium approximately once per week. Penicillin (80 U/ml) and streptomycin (80 μg/ml) were added to the culture medium on the first day. FBS, bFGF, neurobasal medium, and B27 supplement were purchased from Gibco BRL Products (Gaithersburg, MD); MEM (No. 56419) was purchased from JRH Biosciences (Lenexa, KS). All salts and chemicals not otherwise listed were purchased from Sigma Chemical (St. Louis, MO).

Electrophysiological recordings

Neurons were transferred to a recording chamber, and continuously superfused at a rate of 3–4 ml/min with oxygenated Ringer solution containing (in mM) 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.3 NaH₂PO₄, 26 NaHCO₃, and 10 dextrose; pH 7.4 at 5% CO₂/95% O₂. As described previously (Richerson 1995; Wang et al. 1998), antagonists of ionotropic GABAergic and glutamatergic receptors were added to prevent synaptically mediated effects of CO₂, because it was of interest to study neurons with intrinsic chemosensitivity and nonchemosensitive neurons can be synthetically driven by neurons that are chemosensitive (Fukuda et al. 1980). Thus the bath solution contained 100 μM picrotoxin (PTX), 50 μM (+)-2-amino-5-phosphono pentanoic acid (AP-5), and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Experiments were performed at room temperature.

For those experiments in which the effects of changes in CO₂/pH were studied, the recording chamber was perfused with Ringer solution contained in one of two reservoirs. The Ringer solution in the control reservoir was bubbled with medical grade certified gas (Airgas Northeast; Cheshire, CT) with CO₂ of 5% and O₂ of 95%. The other reservoir contained the same Ringer solution bubbled with a mixture of CO₂ and O₂ whose ratio was controlled using a mass flowmeter/flow controller (Linde Gases; Somerset, NJ). Acid/base changes were induced by switching a valve to deliver Ringer solution to the chamber either from the reservoir that was bubbled with 5% CO₂/95% O₂ or from the other reservoir bubbled with either 9% CO₂/91% O₂ or 3% CO₂/97% O₂. This resulted in a change in extracellular pH from 7.4 to steady state values of ~7.13 or 7.61, respectively. Bath pH was continuously measured with a pH electrode (MI-414; Microelectrodes, Inc., Londonderry, NH) in the inflow to the recording chamber.

The amphotericin perforated-patch technique (Ra et al. 1991) was used for all current-clamp recordings of the firing rate response to changes in CO₂ because whole cell recordings result in loss of chemosensitivity (Dean and Reddy 1995; Richerson 1995). Perforated-patch recordings were also used for measuring action potential width, spike-frequency adaptation, the reversal of the postspike AHP, and the delay in action potential firing in response to a depolarizing pulse after a hyperpolarizing prepulse. Perforated patch electrodes (4–10 MΩ; borosilicate glass, Corning 7052) were filled with intracellular solution containing (in mM) 135 potassium methanesulfonate, 10 KCl, 5 N-2-hydroxy-ethylpiperazine-N’-N’,N’-N’-tetraacetic acid (EGTA; pH 7.2; osmolality 275 ± 10 mOsm). Amphotericin B was used as the ionophore as described previously (Ra et al. 1991; Richerson 1995).

All voltage-clamp recordings were made using whole cell recordings to minimize access resistance. The intracellular solution described in the preceding text was used for most of these recordings. However, in some voltage-clamp recordings of A current, EGTA was omitted and 50 μM CaCl₂ was added because a cation conductance that is activated by high [Ca²⁺]ᵢ was also studied in these neurons (Tiwari et al. 2000). The properties of A current were the same using...
either solution, so these two sets of data were combined. For all voltage-clamp data, baseline leak was subtracted.

The liquid junction potential was measured experimentally for each combination of electrode solution, bath solution, and electrode configuration used, and was \( \leq 1 \text{ mV} \) in each case. Fixed voltage offsets were nullled immediately prior to seal formation for each neuron. Neurons were considered healthy with resting potential \( \leq -45 \text{ mV} \), and action potential height \( \geq 60 \text{ mV} \). For current-clamp recordings of the response to acidosis, the membrane potential was amplified (Axopatch 1D, Axon Instruments, Foster City, CA), filtered (10 kHz low-pass), and acquired at 10 kilosamples/s with a computerized data acquisition system (AT-MIO-16F-5, National Instruments, Austin, TX) using custom-written software. For all other current-clamp recordings and for all voltage-clamp recordings, data were collected using a Digidata 1200 data-acquisition board with PClamp software (Axon Instruments).

Under the recording conditions used here, more than half the raphe neurons in culture fire spontaneously (Wang et al. 1998). For the majority of those that did not, constant depolarizing current injection was used to induce sustained firing during current-clamp recordings of the firing rate response to changes in CO\(_2\). A target firing rate between 0.5 and 2 Hz was used because rat serotonergic neurons fire within this range in vivo (Mason 1997). There was no relationship between the amount of current injected and the degree of sensitivity of neurons to changes in CO\(_2\) (data not shown). If a neuron required continuous hyperpolarization to decrease firing rate or stabilize membrane potential, that neuron was not used to study chemosensitivity. All recordings were made after at least 13 days in vitro [mean age = 32 ± 14 (SD) days in vitro, \( n = 80 \); range 13–70 days] since the response to acidosis does not develop until after that time (Wang and Richerson 1999).

Data analysis

As described previously (Wang et al. 1998), neurons were defined as chemosensitive if they demonstrated a response that was reversible and reproducible on two or more exposures to acid/base changes (i.e., four transitions between different pH levels); if the response was consistent in time course for each stimulus; if exposed to both acidosis and alkalosis, the response was opposite in sign (although not necessarily equal in magnitude); and if there was a statistically significant change in firing rate \( (P < 0.05, \text{Student's 2-tail t-test}) \) of \( \geq 20\% \) per 0.2 pH units for at least four transitions in CO\(_2\). These criteria were important for the current experiments as well as for previous studies where it was necessary to minimize false-positive responses (Wang and Richerson 1999; Wang et al. 1998).

To quantify the degree of chemosensitivity of neurons, several methods of analysis were used. First, for each neuron the mean steady-state firing rate was calculated during the last 60 s of each epoch at different levels of CO\(_2\). These values were then averaged for all epochs at a PCO\(_2\) of 5%, and likewise for all epochs at 3% and 9% PCO\(_2\), to get the mean steady-state firing rates for that neuron at each PCO\(_2\). Second, the mean steady-state firing rate calculated above was used to calculate the percentage change in firing rate for that neuron at each PCO\(_2\). Third, the chemosensitivity index (CI) described previously (Wang et al. 1998) was calculated for each neuron from the slope of the log firing rate versus extracellular pH relationship. The CI represents the firing rate of a neuron, as a percentage of control, in response to a decrease in extracellular pH of 0.2 units, where

\[
\text{Chemosensitivity index (CI)} = \frac{100 \times \frac{\ln(\text{FR}_9) - \ln(\text{FR}_5)}{\ln(P_{\text{PCO}_2})} - \frac{\ln(\text{FR}_5) - \ln(\text{FR}_3)}{\ln(P_{\text{PCO}_2})}}{\ln(0.032) - \ln(0.05)}
\]

\( \text{FR}_9 \) = mean steady-state firing rate (as % of control) at 9% CO\(_2\); \( \text{FR}_5 \) = mean steady-state firing rate (as % of control) at 5% CO\(_2\); \( \text{pH}_{\text{EC}} \) = mean extracellular pH at 9% CO\(_2\); and \( \text{pH}_{\text{EC}} \) = mean extracellular pH at 3% CO\(_2\). In those neurons only exposed to either 3 or 9% PCO\(_2\), the values at 5% PCO\(_2\) were used in the preceding equation in place of the missing values. Since this method of analysis would result in a large and misleading CI in neurons with low firing rates, the CI was modified here so that all epochs with steady-state firing rates of \( < 0.2 \text{ Hz} \) were assigned the value of 0.2 Hz. Neurons with mean steady-state firing rates \( < 0.2 \text{ Hz} \) under control conditions were not included in the analysis since such a low rate is not physiological for these neurons in the rat (Mason 1997).

For each neuron, the values of mean steady-state firing rate at each PCO\(_2\), percentage change in firing rate, and CI were then used to calculate the mean steady-state firing rates for all neurons within a group (e.g., all acidosis-stimulated neurons). The reason that three methods were used to quantify the responses was to provide more detail about the degree of chemosensitivity of these neurons and because it is not yet clear which method of analysis is most relevant to the effect of these neurons on their downstream targets. In neurons whose firing rate was relatively variable over the duration of recording, the CI is a more accurate measure of the percentage change in firing rate but can be artificially increased in neurons with low baseline firing rates. In contrast, the downstream effects of the raphe nuclei as a whole may be better expressed by the mean values of firing rate for all neurons within the raphe.

The regularity of firing was analyzed by measuring the standard deviation of the relative interspike interval (rISI) as described previously (Wang et al. 1998). Briefly, the rISI was the ratio of the ISI after that spike to the ISI before a spike to the ISI after that spike. For each recording, the rISI was calculated for \( \geq 10,000 \) spikes. A neuron that fired at a perfectly constant rate would have a rISI of 1 for each spike and a SD of the rISI of 0. This measure of regularity was adapted from that used by Mason (1997) for identifying serotonergic neurons in vivo. That method was modified here so that regularity that occurred over a short time scale could be measured in neurons whose firing rate changed significantly over a longer period. This was necessary, because changes in CO\(_2\) induced large changes in firing rate, even though the firing pattern from one spike to the next was still highly regular.

Action potential widths were calculated as the duration of time that the membrane potential exceeded action potential threshold. The threshold was determined as the membrane potential at which there was a rapid increase in Em\((t)\). A current (Connor and Stevens 1971) was quantified using a pulse protocol from a holding potential of \( -75 \text{ mV} \), a prepulse of 160 ms to \( -99 \text{ mV} \), and a test pulse of 150 ms to \( -15 \text{ mV} \). The amplitude of A current was then calculated as the difference between the peak outward current and the steady-state current at the end of the test pulse. Spike frequency adaptation was quantified by depolarizing neurons from resting potential with different levels of current for 4.5 s and calculating the initial firing rate from the first four spikes and the steady-state firing rate from the last four spikes during the pulse. The reversal potential of the postspike AHP was determined by biasing the resting membrane potential of neurons to different levels using tonic current injection and inducing action potentials with a brief depolarizing current pulse. The magnitude of the AHP was measured as the difference between the membrane potential prior to the spike and the membrane potential at a fixed time after the spike corresponding to the maximum AHP level when no baseline current injection was used.

In all cases, values expressed as \( x \pm y \) are means ± SD unless otherwise stated. Statistical significance was determined using a two-sample \( t \)-test assuming unequal variances.

Immunohistochemistry

After recording, photographs were taken of each neuron using a CCD camera to enable identification after immunostaining. Cells were fixed with 30% EtOH/1% acetic acid, followed by 60% EtOH/1% acetic acid, and then 95% EtOH/1% acetic acid for 30 min each. Coverslips were incubated for 48–72 h at 4°C with either a rabbit polyclonal antibody against serotonin (1:200;
Chemicon) or a mouse monoclonal antibody against tryptophan hydroxylase (TpOH; 1:2000; Sigma). The Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for processing. Neurons were visualized using the peroxidase method with diaminobenzidine as the chromogen. A total of 167 neurons were recorded and stained. Of these, 103 met the criteria for classification as stimulated, inhibited, or unresponsive to acidosis. Of the 103 classified neurons, 79 were recovered after immunohistochemistry (the remaining neurons underwent lysis or were dislodged from the coverslip during fixation and staining). The staining of neurons was evaluated by an individual who was blinded to the response of neurons to acidosis.

Immunohistochemistry for prepro-TRH (ppTRH) and substance P was performed on coverslips from which recordings had not been made. Coverslips were treated with colchicine (0.5–20 μM in culture medium) for 24 h before processing to increase cell body staining since substance P and ppTRH immunoreactivity was localized primarily in nerve processes in untreated cultures. The same protocol was used as above with a rabbit polyclonal antibody against ppTRH (1:1000; Accurate Chemical, Westbury, NY) or a rabbit polyclonal antibody against substance P (1:400; Chemicon, Temecula, CA).

As controls for each antibody, parallel coverslips were processed without addition of primary antibody. These controls uniformly resulted in no staining. In addition, on each coverslip stained with primary antibodies, staining was only seen in a minority of neurons, providing an internal control that these antibodies were specific for only a subset of neurons in culture. The specificity of the TpOH antibody was also confirmed by processing transverse slices from adult rat medulla, and the distribution of stained neuronal somata was consistent with the known location of serotonergic neurons (Steinbusch and Nieuwenhuys 1983). Photographs were taken with a Kodak DC290 camera, and printed with a Kodak 8670 thermal printer.

RESULTS

Acidosis-stimulated medullary raphe neurons were immunoreactive for serotonin

Immunoreactivity for serotonin, TpOH, ppTRH, and substance P was present in cultures prepared from the medullary raphe (Fig. 1), indicating that the major neurotransmitter phenotypes of raphe neurons were preserved under culture conditions. To determine whether some serotonergic neurons are stimulated by acidosis, the response of neurons to changes in CO₂ was determined using current-clamp recordings, and then cells were processed for immunohistochemistry using an antibody against serotonin. An example of an acidosis-stimulated neuron is shown in Fig. 2. Typical of acidosis-stimulated neurons (Wang et al. 1998), it had a larger multipolar soma. This neuron displayed a regular firing pattern (SD of rISI = 0.45; mean firing rate = 0.85 Hz), prominent postspike AHP, and a ramp interspike depolarization (Fig. 2A) characteristic of serotonergic neurons in vivo. Hypercapnic acidosis (i.e., simulated respiratory acidosis) over a physiologically relevant range resulted in an increase in firing rate, whereas alkalosis induced the opposite response (Fig. 2B). After recording, this neuron was found to be immunoreactive for serotonin (Fig. 2C). This was a consistent finding, with eight out of eight acidosis-stimulated neurons (mean CI = 265 ± 86%) also found to be immunoreactive for serotonin.

FIG. 1. Neurotransmitters found in neurons of the medullary raphe in vivo were present in culture. Neurons were microdissected from the rat rostral ventromedial medullary (VMM) and grown in primary cell culture. Coverslips were processed for immunohistochemistry using antibodies against serotonin (5-HT, A), tryptophan hydroxylase (TpOH, B), substance P (Sub P, C; colchicine 0.5 μM), and prepro-thyrotropin releasing hormone (ppTRH, D; colchicine 20 μM).
All acidosis-stimulated raphe neurons were immunoreactive for TpOH. It was next determined whether stimulation by acidosis was specific for serotonergic neurons in this culture preparation. For this set of experiments, an antibody against TpOH was used instead of an antibody against serotonin, because serotonin can be taken up by nonserotonergic neurons (Lebrand et al. 1996), which could potentially lead to false-positive results.

After determining the effect of changes in CO₂ on firing rate of raphe neurons using current-clamp recordings, cells were processed for immunohistochemistry against TpOH. All acidosis-stimulated neurons tested (n = 22) were immunoreactive for TpOH (TpOH-IR). Examples are shown of two neurons that were stimulated by hypercapnic acidosis (Fig. 3, A and C). As an illustration of the methods used for quantification of chemosensitivity, the first neuron (Fig. 3A) had a mean firing rate at 5% PCO₂ of 0.41 Hz, and this increased to 1.45 Hz at 9% PCO₂, an increase to 354% of control. The extracellular pH changed an average of 0.19 pH units during the four transitions, and the CI for this neuron was 395%. Consistent with previous results, both neurons had a larger multipolar soma. Both of these neurons were strongly immunopositive for TpOH (Fig. 3, B and D).

Consistent with previous results (Wang et al. 1998), acidosis-stimulated neurons in this study had a high degree of chemosensitivity. On average, acidosis-stimulated neurons (n = 22 TpOH-IR neurons) increased their firing rate to 240 ± 93% of control in response to an increase in PCO₂ from 5 to 9% (P < 0.0001) and decreased their firing rate to 40 ± 38% of control in response to a decrease in PCO₂ from 5 to 3% (P < 0.0005). The mean firing rate for these neurons was 0.97 ± 0.55 Hz at 5% PCO₂, 2.01 ± 0.83 Hz at 9% PCO₂, and 0.51 ± 0.65 Hz at 3% PCO₂ (differences significant between 5 and 9%, P < 0.0001, and between 5 and 3%, P < 0.05). The mean CI was 327 ± 185% (P < 0.0001).

The majority of serotonergic medullary raphe neurons in this culture system were chemosensitive. From a total of 30 recordings from TpOH-IR medullary raphe neurons, 73% (22 of 30) met the criteria for stimulation by acidosis. Of the eight TpOH-IR neurons that did not meet the criteria for chemosensitivity, five had an increase in firing rate in response to acidosis that was either subthreshold or occurred less than four times. Two of these five TpOH-IR neurons had been in culture for 20 days, an age at which the full chemosensitive response may not have developed (Wang and Richerson 1999).

All acidosis-inhibited neurons were immunonegative for TpOH

Using the same approach, it was next determined whether acidosis-inhibited neurons were serotonergic. The effect of changes in CO₂ on the firing rate of two acidosis-inhibited neurons is shown (Fig. 4, A and C). The degree of chemosensitivity of acidosis-inhibited neurons was as large as that of acidosis-stimulated neurons but was opposite in sign. For those acidosis-inhibited neurons in which the mean baseline firing rate was maintained between 0.2 and 2 Hz (n = 12), an increase in PCO₂ from 5 to 9% resulted in a decrease in firing rate to 25 ± 23% of control (P < 0.0001). A decrease in PCO₂ from 5 to 3% resulted in an increase in firing rate to 170 ± 94% of control (P < 0.05). The mean firing rate for these neurons was 0.81 ± 0.45 Hz at 5% PCO₂, 0.26 ± 0.31 Hz at 9% PCO₂, and 1.34 ± 1.11 Hz at 3% PCO₂ (statistical significance between 5 and 9%, P < 0.005, and between 5 and 3%, NS).

Typical of acidosis-inhibited neurons (Wang et al. 1998), both neurons shown (Fig. 4) had a smaller fusiform soma. In
contrast to the results from acidosis-stimulated neurons, these neurons were not TpOH-IR (Fig. 4, B and D). This was also the case for all other acidosis-inhibited neurons tested (n = 16).

Normal baseline electrophysiological properties of serotonergic neurons were preserved in culture

To determine how closely raphe neurons in primary cell culture resemble their counterparts in situ, which is important for interpretation of the results from this in vitro approach, the baseline electrophysiological properties of cultured raphe neurons were characterized. Most serotonergic neurons in culture had a highly regular firing pattern (Fig. 2A). When the regularity of firing was quantified, the SD of the rISI of neurons that were TpOH-IR was significantly lower than that of neurons that were not immunoreactive for TpOH (0.51 ± 0.23 vs. 0.94 ± 0.56; P < 0.001; Fig. 5). In addition, there was no difference in the SD of the rISI of TpOH-IR neurons that were stimulated by acidosis and TpOH-IR neurons that did not meet the criteria for an acidosis-stimulated neuron. The present results also confirmed the previous finding (Wang et al. 1998) that the SD of the rISI of acidosis-stimulated neurons was significantly lower than that of acidosis-inhibited neurons (0.50 ± 0.19 vs. 1.22 ± 0.60; P < 0.001; there was no overlap between the current data set and the previously published data set). The SD of the rISI for the group of neurons that were not immunoreactive for TpOH and also not responsive to acidosis (TpOH-/NR) was intermediate (0.77 ± 0.48) and significantly different from the group of neurons that were inhibited by acidosis (1.22 ± 0.60; P < 0.05) and from the group of neurons that were TpOH-IR (0.51 ± 0.23; P < 0.05). The TpOH-/NR neurons with the lowest SD of the rISI also had a higher firing rate than TpOH-IR neurons or acidosis-inhibited neurons, which also distinguished them from these other two groups (Fig. 5). The highly regular firing pattern of serotonergic neurons measured on a short time scale was not always associated with a constant firing rate over a longer time. For example, in many neurons the firing rate at 5% PCO2 varied over the duration of recording.

Serotonergic neurons in culture had wide action potentials that were followed by a prominent AHP (Fig. 6A). The mean action potential width of serotonergic neurons was 4.55 ± 0.5 ms (n = 9 TpOH-IR neurons). The AHP was broad and rounded and rose smoothly into a ramp depolarization that led to the next spike (Fig. 2A). The reversal potential of the AHP (Penington and Kelly 1993) was measured in serotonergic neurons using recordings in current-clamp mode. The resting membrane potential was biased to different levels with constant baseline current injection, and action poten-
tials were induced with a brief depolarizing current pulse (Fig. 6B). The AHP reversed and became an afterdepolarizing potential at resting membrane potentials more negative than $-272.8 \pm 5.3$ mV ($n = 10$ TpOH-IR neurons), 25.2 mV more positive than the calculated Nernst potential for K$^+$ of $-98.0$ mV.

Serotonergic neurons in cell culture displayed spike frequency adaptation. When TpOH-IR neurons were depolarized from resting potential, they responded with a rapid increase in firing rate that then decreased over time (Fig. 7; $n = 10$ TpOH-IR neurons). Following the depolarizing pulse, there was a prolonged AHP that was dependent on the magnitude of depolarization (Fig. 7; $n = 10$ TpOH-IR neurons).

Serotonergic raphe neurons in cell culture had prominent A-current. In current clamp when TpOH-IR raphe neurons were given a hyperpolarizing prepulse followed by a depolarizing pulse, there was a delay before the first action potential that was dependent on the duration and magnitude of the hyperpolarizing prepulse (Fig. 8A; $n = 2$ TpOH-IR neurons, and 10 neurons that were not processed for immunohistochemistry). In voltage clamp, there was a transient outward current induced on depolarization that was dependent on the level of preceding hyperpolarization (Fig. 8B; $n = 14$ of 14 TpOH-IR neurons).

The mean amplitude of the transient component of outward current was $1,040 \pm 1,016$ pA (range 85–3,740 pA; $n = 14$ TpOH-IR neurons). Bath application of 4-aminopyridine (4-AP; 3 mM) blocked the transient outward current by $89 \pm 9\%$ ($n = 3$ TpOH-IR neurons) and also decreased the effect of hyperpolarization on the delay until the first action potential ($n = 2$ TpOH-IR and 4 neurons that were not processed for immunohistochemistry).

**DISCUSSION**

It is demonstrated here that all acidosis-stimulated neurons within the VMM are serotonergic and that the majority of serotonergic medullary raphe neurons are stimulated by acidosis. These results directly link chemosensitivity to a specific neuronal phenotype within a nucleus proposed to contain central respiratory chemoreceptors. This is the first time that the neurotransmitter content of a putative central chemoreceptor neuron has been directly determined. Defining serotonin as a downstream mediator of chemoreceptor neurons may help to elucidate the cellular mechanisms by which an increase in blood CO$_2$ initiates a response to restore pH homeostasis.
Preservation of intrinsic properties of serotonergic neurons in primary cell culture

Development of a culture system of serotonergic neurons offers many experimental advantages. However, to generalize results obtained using this approach, it is critical that the neurons studied maintain normal properties. Many neurons in culture “largely resemble their counterparts in situ” (Banker and Goslin 1991), including expression of the correct neurotransmitters and ion channels. This was also true for the serotonergic raphe neurons studied here since they express chemosensitivity (Richerson 1995; Veasey et al. 1995), neurochemical content, and baseline electrophysiology characteristic of serotonergic neurons in vivo (Mason 1997).

FIG. 5. Serotonergic neurons in culture displayed a regular firing pattern. Acidosis-stimulated neurons tended to have a combination of a low SD of the relative interspike interval (rISI) and low firing rate compared with neurons that were inhibited by acidosis. This property was shared by TpOH-IR neurons that did not meet the criteria for an acidosis-stimulated neuron. Neurons that did not respond to acidosis and were not TpOH-IR had a SD of the rISI that was intermediate to TpOH-IR and acidosis-inhibited neurons. A regular firing pattern is characteristic of serotonergic neurons in vivo (Mason 1997).

FIG. 6. Action potentials of serotonergic neurons in culture were similar to those of serotoninergic neurons in vivo and in slices. A: TpOH-IR neurons had wide action potentials (4.55 ± 0.5 ms; n = 9). Shown is the mean (—) and mean – SD (•••) of action potentials from 9 TpOH-IR neurons. B: the afterhyperpolarization (AHP) reversed and became an afterdepolarizing potential at a voltage 25.2 mV more positive than the calculated Nernst potential for K⁺. A short depolarizing pulse (●) was superimposed on tonic current injection used to control the baseline membrane potential. The AHP reversed and became an after-depolarizing potential at −72.8 ± 5.3 mV (n = 10 TpOH-IR neurons).

FIG. 7. Serotonergic medullary neurons in culture had spike frequency adaptation and a slow AHP after a depolarizing pulse. A: in this neuron, current injection led to an increase in firing rate that was maximum at the onset of the pulse with a gradual decrease over time. After the end of the pulse, there was a prolonged AHP. B: the initial and steady-state firing rates were dependent on the amount of current injection (mean ± SE for 5 neurons). C: the level of AHP was dependent on the amount of current injection (mean ± SE for 6 neurons).

FIG. 8. Serotonergic medullary neurons in culture had spike frequency adaptation and a slow AHP after a depolarizing pulse. A: in this neuron, current injection led to an increase in firing rate that was maximum at the onset of the pulse with a gradual decrease over time. After the end of the pulse, there was a prolonged AHP. B: the initial and steady-state firing rates were dependent on the amount of current injection (mean ± SE for 5 neurons). C: the level of AHP was dependent on the amount of current injection (mean ± SE for 6 neurons).
FIG. 8. Serotonergic raphe neurons in culture had A-current. A: in current clamp, raphe neurons had delayed activation on depolarization. This TpOH-IR neuron was hyperpolarized with −10 pA of current to prevent baseline firing. Then a hyperpolarizing current pulse of −300 pA was given for a variable length of time (0 ms to 34 ms; 2-ms steps), followed by a depolarizing pulse of 100 pA. For each trace, a single action potential was generated with the earliest action potential corresponding to the trace without a prepulse. Similar results were seen in 11 other raphe neurons. B: in voltage clamp, depolarization produced a rapid, transient outward current whose size was dependent on the magnitude of a hyperpolarizing prepulse. The hyperpolarizing voltage pulses in this example ranged from −47 to −78 mV in steps of 2 mV, and the depolarizing pulse was to +10 mV. Similar results were seen in 14 TpOH-IR neurons.

neurons in culture was similar to that previously determined for acidosis-stimulated neurons in culture (Wang et al. 1998) and for serotonergic neurons in vivo (Mason 1997). Using a similar approach, a discriminant function is able to distinguish serotonergic neurons from nonserotonergic neurons in the raphe in vivo (Mason 1997). In culture there was slightly more overlap between serotonergic and nonserotonergic neurons, which might be explained by the inclusion of neurons from parts of the VMM outside of the raphe proper that were not included in the in vivo study.

It has previously been shown that the baseline membrane potential of acidosis-stimulated neurons in culture was the same as that in brain slices (Richerson 1995; Wang et al. 1998). The effect of CO₂ on membrane potential was also the same using either of these two preparations, with an increase in firing rate being associated with an increase in the slope of the interspike ramp depolarization without an effect on the mean resting potential. Here it was found that serotonergic neurons in culture maintained other electrophysiological properties characteristic of serotonergic raphe neurons in situ, including A-current (Aghajanian 1985; Segal 1985), wide action potentials, spike frequency adaptation, a long AHP after a depolarizing pulse (Bayliss et al. 1997a), and a postspike AHP with a reversal potential 25 mV more positive than would be predicted by dependence solely on potassium current (Penington and Kelly 1993).

The characteristics of raphe neurons studied here are not unique to serotonergic neurons. For example, a highly regular firing pattern is present in other monoaminergic neurons. A-current and spike frequency adaptation are also a common property of CNS neurons. However, cultured serotonergic raphe neurons possessed all of these properties in combination, and thus expressed the same properties as serotonergic neurons in situ. There are clearly some differences between serotonergic neurons in culture and those in vivo, such as their two-dimensional anatomy in a monolayer. The absence of projections to the raphe from other nuclei would also presumably have effects. For example, there would be a lack of noradrenergic input, which normally induces increased firing of raphe neurons (Vandermaelen and Aghajanian 1983), which might explain why acidosis-stimulated neurons in culture have a lower incidence of spontaneous firing than raphe neurons in brain slices (Wang et al. 1998). However, the intrinsic properties of these neurons were preserved, and those were the properties that were of interest here.

Significance of chemosensitivity in medullary raphe neurons

Within the region studied, stimulation by acidosis was a property that was unique to serotonergic neurons. Furthermore serotonergic raphe neurons were relatively homogeneous in their combination of basic electrophysiological properties, response to acidosis, and morphology (Wang et al. 1998). This specificity and homogeneity suggests that chemosensitivity is a phenotypic specialization of serotonergic neurons that serves a physiological role rather than simply being a nonspecific response.

Based on the criteria used here, 27% of serotonergic neurons were not stimulated by acidosis. These criteria were designed to reduce false positives and to remain consistent with previously published work (Richerson 1995; Wang and Richerson 1999, 2000; Wang et al. 1998). In the current study, it was more important to minimize false positives at the expense of generating false negatives. However, some of the “unresponsive” serotonergic neurons actually did have a response to acidosis that was small or inconsistent, and some may have been too young to have developed a robust response (Wang and Richerson 1999). Thus the percentage of mature serotonergic neurons that have some degree of chemosensitivity is actually greater than 73%. Since such a large percentage of serotonergic neurons are stimulated by acidosis and many medullary raphe neurons project to nonrespiratory brain stem and spinal cord nuclei, these chemosensitive serotonergic raphe neurons are likely to contribute to nonrespiratory effects of hypercapnia.

The subset of raphe neurons that are inhibited by acidosis have not yet been studied in the same detail as those that are stimulated. Their response is as large as that of acidosis-stimulated neurons but opposite in direction, suggesting that they may also be chemoreceptors. It is unclear whether they represent a homogeneous population of cells. However, the observed differences in SD of the rISI between the different subsets of VMM neurons indicate that acidosis-inhibited neu-
rons actually are a phenotypic subtype of neurons that are distinct from other neurons from this region. If they provide a tonic inhibitory influence on respiratory output that is reduced as CO₂ rises (Richerson 1995), their role would be analogous to inhibitory chemoreceptors present in the alligator (Powell et al. 1988) and in birds (Hempleman and Burger 1985).

**Role of serotonin in the CNS response to changes in CO₂**

The major feedback mechanism for long-term regulation of ventilation relies on sensation of blood CO₂ levels, mediated primarily by central chemoreceptors within the brain stem (Cunningham et al. 1986; Mitchell et al. 1963; Schlaefke 1981). Although there are now many candidate regions for the central chemoreceptors (see INTRODUCTION), it remains unclear whether all of these regions play a role in controlling blood CO₂ (Nattie 1999).

Neurons of the medullary raphe have many properties that would be expected for chemoreceptors. For example, the raphe nuclei lie in a region of high blood flow, and raphe neurons have an anatomical relationship with blood vessels that would enable them to rapidly sense changes in blood CO₂ (Felten and Crutcher 1979; Scheibel et al. 1975). Serotonergic raphe neurons have widespread projections throughout the medulla and spinal cord, with dense innervation of respiratory and autonomic nuclei (Holton et al. 1987; Jacobs and Azmitia 1992; Smith et al. 1989; Steinbusch 1981). Serotonin and the co-localized TRH and substance P (Dean et al. 1993; Iverfeldt et al. 1989) each stimulate ventilation in vivo (Holm et al. 1986, 1987; Horita et al. 1976; Kraemer et al. 1977; Lalley 1986; Millhorn et al. 1980; Mueller et al. 1984; Murakoshi et al. 1985; Schaefer et al. 1989). Substance P also induces an increase in frequency of rhythmic activity generated by the preBötzinger Complex in vitro (Gray et al. 1999), and TRH induces bursting pacemaker activity within the respiratory division of the nucleus tractus solitarius (Dekin et al. 1985). Each of these neurotransmitters also depolarize respiratory motor neurons (Bayliss et al. 1992, 1997c; Ptak et al. 2000; Talley et al. 2000). Thus the substrate exists for raphe neurons to stimulate respiratory output through a variety of mechanisms, and at many sites within the respiratory network, when they are stimulated by respiratory acidosis in vivo.

A disturbance in pH/CO₂ normally induces a variety of nonrespiratory effects, such as changes in cardiac output, vasomotor tone, cerebral blood flow, and level of arousal (Berry and Gleeson 1997; Daly 1986; Madden 1993; Millhorn and Eldridge 1986; O’Regan and Majcherzcyk 1982). These changes are important for a homeostatic response that must accomplish more than just an increase in ventilation. For example, arousal is a critical element in the response to hypercapnia (Berry and Gleeson 1997). The mechanisms of the nonrespiratory effects of hypercapnia have not been defined, but the current data suggest that raphe neurons contribute to them. The raphe and serotonin have been associated with a variety of brain functions other than respiration, including cardiovascular control, cerebrovascular control, and sleep (Jacobs and Azmitia 1992; Jacobs and Fornal 1991; McCall and Harris 1987; McCall et al. 1987; Mravvitch and Sercombe 1996). The widespread projections of serotonergic neurons suggest that they not only induce an increase in ventilation but also alter autonomic output and affect other brain functions in response to an increase in CO₂. Thus these neurons may contribute to a global homeostatic response to perturbations of blood CO₂ levels aimed at restoring acid/base balance.

The results presented here do not address the related question of how chemosensitive neurons in other brain stem regions contribute to the response to hypercapnia. For example, the locus coeruleus contains chemosensitive neurons (Pineda and Aghajanian 1997) and has been linked to respiratory and autonomic control as well as modulation of forebrain function (Steriade et al. 1993). The relative contribution of each of the brain stem regions containing chemosensitive neurons to the overall response of the whole animal to CO₂ has not been determined. It is possible that all these regions are simply redundant in their roles in responding to hypercapnia. Alternatively, each type of chemosensitive neuron may play a distinct role, based on differences in specific targets, downstream effects, the magnitude of the stimulus needed to initiate a response, and/or the magnitude of the response to small changes in CO₂. In some neurons, the response to changes in CO₂ may play a relatively minor role compared to their other primary functions, whereas in others sensation of CO₂ may be their primary function. In addition, the importance of each type of neuron in the response to changes in CO₂ may vary depending on the state of arousal (Nattie 1999).

**Effect of sleep on raphe neurons and respiration**

There is an intriguing and undefined relationship among sleep, raphe neurons, and respiration. During sleep, there is a decrease in sensitivity of ventilation to increased CO₂, changes in respiratory pattern, and a decrease in upper airway tone (Douglas 2000; Kubin et al. 1996; Orem and Kubin 2000; Phillipson and Bowes 1986). These changes in breathing at the systems level during sleep parallel a decrease in firing rate of raphe neurons (Jacobs and Fornal 1991). This correlation suggests that the decrease in serotonergic and peptidergic input to respiratory neurons during sleep might lead to a decrease in upper airway muscle tone, changes in rhythmogenesis, and blunting of chemoreception.

Some cases of sudden infant death syndrome (SIDS) may be due to a delay in maturation of the normal response to a rise in CO₂ during sleep (Hunt 1989; Richerson 1997). In infants who died of SIDS, a decrease in muscarinic receptor binding has been reported in the human arcuate nucleus (Kinney et al. 1995), which is homologous to the medullary raphe of the cat and rat (Filiano et al. 1990). A decrease in LSD binding has also been found within serotonergic nuclei located throughout the ventral medulla of SIDS victims, suggesting that an abnormality of the medullary raphe/serotonergic system is involved in the pathogenesis of SIDS (Panigrahy et al. 2000). In rats, the percentage of raphe neurons that are chemosensitive and the magnitude of their response to hypercapnia increases during the first 3 wk after birth (Wang and Richerson 1999). Taken together, these findings suggest that a normal delay in development of chemosensitivity of serotonergic neurons, perhaps coupled with other insults to these neurons, may contribute to the pathophysiology of SIDS.
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ACIDOSIS-STIMULATED NEURONS ARE SEROTONERGIC


