

Cellular mechanisms involved in CO₂ and acid signaling in chemosensitive neurons

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Putnam, Robert W., Jessica A. Filosa, and Nicola A. Ritucci. Cellular mechanisms involved in CO₂ and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* 287: C1493–C1526, 2004; doi:10.1152/ajpcell.00282.2004.— An increase in CO₂/H⁺ is a major stimulus for increased ventilation and is sensed by specialized brain stem neurons called central chemosensitive neurons. These neurons appear to be spread among numerous brain stem regions, and neurons from different regions have different levels of chemosensitivity. Early studies implicated changes of pH as playing a role in chemosensitive signaling, most likely by inhibiting a K⁺ channel, depolarizing chemosensitive neurons, and thereby increasing their firing rate. Considerable progress has been made over the past decade in understanding the cellular mechanisms of chemosensitive signaling using reduced preparations. Recent evidence has pointed to an important role of changes of intracellular pH in the response of central chemosensitive neurons to increased CO₂/H⁺ levels. The signaling mechanisms for chemosensitivity may also involve changes of extracellular pH, intracellular Ca²⁺, gap junctions, oxidative stress, glial cells, bicarbonate, CO₂, and neurotransmitters. The normal target for these signals is generally believed to be a K⁺ channel, although it is likely that many K⁺ channels as well as Ca²⁺ channels are involved as targets of chemosensitive signals. The results of studies of cellular signaling in central chemosensitive neurons are compared with results in other CO₂- and/or H⁺-sensitive cells, including peripheral chemoreceptors (carotid body glomus cells), invertebrate central chemoreceptors, avian intrapulmonary chemoreceptors, acid-sensitive taste receptor cells on the tongue, and pain-sensitive nociceptors. A multiple factors model is proposed for central chemosensitive neurons in which multiple signals that affect multiple ion channel targets result in the final neuronal response to changes in CO₂/H⁺.

hypercapnia; brain stem; ventilation; peripheral chemoreceptor; glia; gap junction; glomus; channel; calcium; potassium; carbonic anhydrase; taste receptor; nociception

THE MAJOR STIMULUS for an increase in breathing is an elevation of CO₂/H⁺ (respiratory or hypercapnic acidosis, HA). For ventilation to be increased, this HA must be detected by specialized sensory elements. Cells that are sensitive to CO₂/H⁺, referred to as chemosensitive cells, have been identified both peripherally (glomus cells within the carotid body) (132, 271) and centrally (neurons localized within various regions of the brain stem) (68, 239, 241), although the central CO₂/H⁺-sensitive neurons appear to be quantitatively more significant for mediating increased ventilation in response to hypercapnia (241, 258). A major focus of research has been the characterization of cellular mechanisms involved in CO₂/H⁺ chemoreception. In this review, our goal is to highlight major issues and findings related to CO₂/H⁺ chemoreception and to give an overview of the current work on acid sensing, not to present an exhaustive review of all the work that has been done. We have summarized the considerable progress that has been made over the past decade in understanding the cellular signals and targets

involved in the response of central chemosensitive neurons to CO₂/H⁺. These neuronal responses are compared with those of nonneuronal cells that have been shown to be sensitive to either increased levels of CO₂/H⁺ or acid alone. Finally, a revised model of chemosensitive signaling in neurons is proposed, and future studies to expand our understanding of the cellular mechanisms involved in CO₂/H⁺ and acid signaling are suggested.

CO₂/H⁺-SENSITIVE CELLS

A variety of cells in the body are able to sense altered levels of CO₂ and/or H⁺. Some such cells are nonexcitable, like renal proximal tubule cells involved in blood acid-base regulation, which respond to HA with increased HCO₃⁻ reabsorption (412). Others include the glomus cells of the carotid body, which serve as the peripheral chemoreceptors for the control of ventilation (227), nociceptors responsible for pain sensation (388), and taste receptor cells that sense acid as a sour taste (339). These cells are easily identified in that they respond to an increase in CO₂ or H⁺ with an increase in the firing rate of their associated afferent nerves. The identification of central chemosensitive neurons is more problematic, and no neuron has unequivocally been identified as such. To be a central

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respiratory chemosensor, a neuron would: 1) have to respond to changes of CO_2/H^+ , 2) have axonal projections to a respiratory control center, and 3) ultimately lead to altered ventilation. Despite the lack of certain identification of any central chemosensitive neuron, a number of traits can be used to identify candidate neurons.

The first trait of a central chemosensitive neuron is that the firing rate of the neuron should be altered by changes in CO_2/H^+ (Fig. 1). Because of concerns that CO_2 can have a generally inhibitory effect on mammalian neurons (51), most of the studies on central chemosensitive neurons have focused on CO_2 -excited neurons. Nevertheless, a chemosensitive neuron could respond to increased CO_2/H^+ level with either increased or decreased firing rate, providing excitatory drive or removing inhibitory drive, respectively, either of which could lead to increased ventilation. In slices from the dorsal medulla, of the 50% of neurons that responded to increased CO_2/H^+ level, about half were stimulated and about half were inhibited by hypercapnia (159). Similar findings were made in explant cultures from the ventral medulla (389), brain slices from the medullary raphe (295), and raphe neurons in cell culture (383). In contrast, in the locus coeruleus (LC), a CO_2 -chemosensitive region of the pons, studies with brain slices revealed that >80% of the neurons responded to elevated CO_2/H^+ level, and all of these cells were stimulated (116, 262, 276). Thus a variety of brain stem regions contain CO_2/H^+ -sensitive neurons.

A second feature of central chemosensitive neurons is that they should be intrinsically responsive to changes of CO_2 and not simply respond to altered synaptic input from other neurons that are chemosensitive. This has most often been demonstrated by exposing neurons to altered levels of CO_2 in the presence of synaptic block media. With the use of such an approach, intrinsically CO_2 -sensitive neurons were demonstrated in the nucleus tractus solitarius (NTS) (83), the medullary raphe (295), the LC (262), the nucleus ambiguus (302), and the ventrolateral medulla (VLM) (389). Some caution in interpret-

ing these data needs to be exercised because it is often difficult to be sure that all possible neurotransmitter release is blocked. Furthermore, these studies do not rule out possible contributions from electrical synapses (gap junctions), which have been shown to exist among chemosensitive neurons (84, 159, 331, 333) (see *Gap junctions* in OTHER FACTORS IN CENTRAL CHEMOSENSITIVE SIGNALING). To determine that a neuron is truly intrinsically sensitive to CO_2/H^+ , it is necessary to show that it responds to an acid challenge in the absence of all possible input from both chemical and electrical synapses. Nevertheless, it is likely that many brain stem neurons that respond to a change of CO_2 are intrinsically chemosensitive.

A final feature of a putative central respiratory chemosensitive neuron is that it should reside in a region shown to alter ventilation when locally stimulated by acidification. Experimentally, local stimulation has been produced in at least two different ways: 1) focal injections of solutions containing acetazolamide, which produce a decremting sphere of acidification extending between 100 and 300–400 μm from the site of injection (67, 68, 245); and 2) focal microdialysis of a solution that is acidified by equilibrating with 25% CO_2 (208, 246, 247). Focal acetazolamide was injected into various brain stem regions in anesthetized animals, and the ventilatory response (as measured by an increase in phrenic nerve activity, which is a measure of the motor output to the diaphragm) was measured (240). Ventilation was increased by focal acetazolamide stimulation of the LC by 30% (compared with the ventilatory increase induced by the whole animal breathing 9% CO_2), of the NTS by 34%, of the VLM, including the retrotrapezoid nucleus (RTN), by 20%, and of the medullary raphe by 32%. Focal acidosis of the midline caudal raphe (23) and the pre-Bötzinger complex (332) also resulted in increased phrenic nerve discharge. These results indicate that no one area predominated, and it appears that several chemosensitive areas would need to be stimulated to get a full ventilatory response. Furthermore, the chemosensitivity of a given area appears to be state dependent. For instance, microdialysis of hypercapnic solution in the medullary raphe stimulated ventilation by 15% compared with the whole animal response, but only in unanesthetized animals and only when the animal was asleep (246). Regardless, there appears to be excellent agreement between regions in the brain stem shown to contribute to ventilatory control and regions that contain CO_2/H^+ -sensitive neurons. We thus refer to putative respiratory chemosensitive neurons in this review as neurons that fulfill all three criteria: 1) respond to alterations in CO_2/H^+ level with an increase in firing rate, 2) intrinsically chemosensitive, and 3) localized to a region known to contribute to ventilatory control.

It has been suggested that an additional criterion, the degree of chemosensitivity, need be considered (383, 384). In fact, the degree of chemosensitivity of neurons from various chemosensitive areas has not been compared under similar conditions by the same laboratory. Furthermore, there is no standard way in which to express the degree of chemosensitivity of a given neuron. In general, the firing rate response to an acid stimulus is indexed to the change of extracellular pH (pH_o), and not CO_2 level, to be able to compare the responses to different acid stimuli. In Table 1, we show a compilation of the degree of chemosensitivity for neurons from five different chemosensitive regions as estimated from appropriate references. We include three different estimates of chemosensitivity. If a

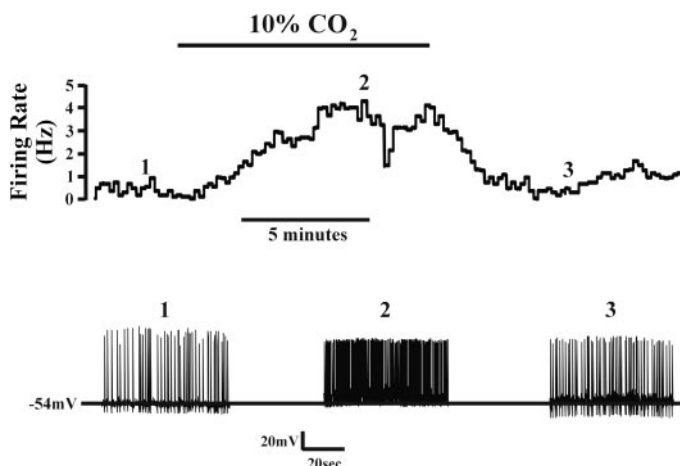


Fig. 1. Firing rate response to hypercapnia (10% CO_2) of a neuron from the retrotrapezoid nucleus, measured in a brain stem slice from a neonatal rat (postnatal day 11; P11) using a whole cell patch pipette at 35°C . The integrated firing rate (Hz, 10-s bins) is plotted at top. For the numbered regions (1, control, 5% CO_2 , pH_o 7.45; 2, hypercapnia, 10% CO_2 , pH_o 7.15; 3, recovery, 5% CO_2), a segment of the membrane potential trace is shown at faster speed (bottom), illustrating individual action potentials as vertical upward spikes.

Table 1. Chemosensitive responses to acidic stimuli of neurons from various brain stem regions of the rat

Brain Stem Region	FR (Initial)	%Increase in FR/0.1 unit pH _o	Slope, s ⁻¹ /ΔpH _o	Chemosensitivity Index	Acid Stimulus	Preparation	Temperature t, °C	Ref.
<i>Ventral medulla</i>								
Ventral medulla	4.0	25	9.9	156	IA	Slice (SB)	36	125
Ventral medulla	3.1	53	16.0	194	HA	Slice	36	120
	3.1	36	11.0	166	IA	Slice	36	120
Ventral medulla	3.7	42	15.5	184	HA	Slice	36	168
	3.7	23	8.6	146	IA	Slice	36	168
Ventral medulla	4.0	18	6.3	127	HA	Explant culture	37	250
	4.0	0	0.0	100	IA	Explant culture	37	250
Ventral medulla	1.0	27	6.1	167	HA	Organotypic culture	37	389
VLM	7.0	122	38.7	224	HA	Organotypic culture	32	394
<i>RVM/raphe</i>								
RVM	0.7	53	3.3	266	HA	Slice	RT	295
Medullary raphe	0.7	142	7.0	300	HA	Primary cell culture	RT	385
Medullary raphe	1.0	83	3.1	177	HA	Primary cell culture	RT	386
Medullary raphe	1.1	119	5.8	199	HA	Primary cell culture	RT	382
	1.2	39	4.8	170	IA	Primary cell culture	RT	382
Medullary raphe	1.1	94	6.8	270	HA	Primary cell culture	RT	383
<i>RTN</i>								
RTN	0.7	294	11.7	330	HA	Slice	37	307
<i>DM/NTS + DMN</i>								
DM	7.5	10	8.6	124	IA	Slice (SB)	36	125
NTS + DMN	0.8	47	3.3	150	HA	Slice	37	70
NTS + DMN	1.0	50	2.1	131	HA	Slice	37	238
<i>Locus coeruleus</i>								
LC	1.2	7	0.5	112	HA	Brain stem spinal cord	25	262
LC	1.8	16	2.3	121	HA	Slice	35	116
	1.3	10	1.5	117	IA	Slice	35	116
LC	1.7	26	4.2	135	HA	Slice	37	280
LC	0.7	18	1.3	134	HA	Slice	33	276

FR is the initial firing rate. %Increase in FR is a measure of chemosensitivity assessed as the percent increase in firing rate for every 0.1 unit increase in extracellular pH (pH_o), whereas slope represents the absolute increase (s⁻¹) in firing rate for a 0.1 unit increase in pH_o. CI indicates the chemosensitivity index as defined in Refs. 383 and 385. Acid stimulus was applied in conditions of either isocapnic acidosis (IA: constant CO₂, decreased HCO₃⁻) or hypercapnic acidosis (HA: elevated CO₂ constant HCO₃⁻). SB indicates a brain slice in synaptic block (low Ca²⁺, high Mg²⁺) medium; RT indicates room temperature. VLM, ventrolateral medulla; RVM, rostral ventral medulla; RTN, retrotrapezoid nucleus; DM, dorsal medulla; NTS, nucleus tractus solitarii; DMN, dorsal motor nucleus; LC, locus coeruleus.

neuron has a firing rate in control solution (FR_C) that increases in acidic solution (FR_A), then we calculate the chemosensitivity of this neuron as the percent increase in firing rate for a 0.1 pH unit acidification {using the equation [(FR_A - FR_C)/FR_C]/(ΔpH/0.1) × 100}. With this expression, a neuron that is not chemosensitive would have a 0% increase in firing rate. Alternatively, the absolute increase in firing rate per unit pH change could be calculated [using the equation (FR_A - FR_C)/ΔpH]. Finally, the chemosensitive index (CI) as described by Wang et al. (383) was calculated. The CI is based on the percentage of control firing rate to which firing rate increases in response to a 0.2 pH unit acid challenge. With this expression, a neuron that is not chemosensitive would have a value of 100% for CI. In general, the two methods based on percent increase in firing rate per pH change appear to be the most robust, being similar over a wide range of initial firing rates (Table 1). In contrast, the chemosensitivity determined from the slope of absolute firing rate vs. ΔpH are highly variable and very sensitive to differences in the initial firing rate (Table 1). Thus we propose that measures of the relative change in firing rate, rather than the absolute change, are better indicators of the relative chemosensitivity of neurons from different brain stem regions.

Despite the wide variation in responses, a few conclusions are evident. First, when directly compared using any of the measures of chemosensitivity (Table 1), the firing rate response of chemosensitive neurons to HA (increased CO₂, constant HCO₃⁻, and decreased pH_o) is greater than the response to isocapnic acidosis (IA; constant CO₂, decreased HCO₃⁻, and decreased pH_o) (116, 120, 168, 382). This response of chemosensitive neurons parallels the whole animal ventilatory response to these acid challenges (see HISTORY OF STUDIES OF CENTRAL CHEMORECEPTION). Second, there is reasonable agreement among different studies for the measured firing rate response in a given area. Third, there appear to be differences among the various areas in the degree of chemosensitivity of their neurons (Table 1). On average, neurons from the medullary raphe, and possibly the RTN, have the highest degree of chemosensitivity. The percent increase in firing rate per 0.1 pH unit is ~100 (i.e., it doubles), whereas the CI is ~240 (i.e., it increases 2.5-fold) for the raphe, and both are ~300 for the RTN, although the RTN values are based on only a few neurons (307). Neurons from the ventral medulla have a lower degree of chemosensitivity, with values of ~50 and 155, respectively. Neurons from the dorsal medulla, including the

NTS, have similar values (50 and 140). Finally, neurons from the LC have the lowest values, with the percent increase in firing rate per 0.1 pH unit averaging ~ 15 and the CI averaging ~ 125 .

These variations might give clues to the role played by neurons in these various regions. For instance, the LC is a region associated with pain sensation, attention, learning, and anxiety (9, 11, 154). The primary function of CO_2/H^+ -sensitive neurons within the LC may be to produce an aversive or anxiety response to elevated CO_2 levels (179, 326). In this regard, LC neuron response would be most significant at high levels of CO_2 , which is consistent with the relatively low level of chemosensitivity of neurons in this region (Table 1).

Finally, the chemosensitive response of the neurons from no one of these areas appears to be sufficient to explain the large ventilatory response to a 0.1 pH unit decrease, which is sometimes reported to be as high as an increase of 400–500% (114) (see HISTORY OF STUDIES OF CENTRAL CHEMORECEPTION). It is interesting that these high values for the ventilatory response to HA have been obtained for the most part in large animals, such as cats, dogs, goats, and humans (48, 114, 224, 253, 290, 322, 359, 373), whereas the cellular studies of chemosensitivity have been performed almost exclusively in rodents. A comparison of the ventilatory response of different animals to increased CO_2 levels reveals that the most commonly used animal model for the study of the cellular basis of chemosensitivity, the rat, appears to have a response that is two to three times lower than that of larger animals (192, 381) (Table 2). This means that most of the cellular studies performed to date have employed the least sensitive animal for studying chemosensitivity and suggests that studies of the cellular basis of chemosensitivity using neurons from more sensitive animals might be quite interesting. Nevertheless, these cellular findings are in general agreement with the focal stimulation studies discussed above and suggest that, assuming a linear relationship between chemosensitive neuron firing rate and increased ventilation, multiple central chemosensitive sites need to be stimulated to account for the full ventilatory response in the intact animal.

Table 2. Ventilatory responses to hypercapnia in different animals

Animal	%Increase in Ventilation/Torr CO_2	Baseline CO_2 , Torr	Source	Ref.
Rat	8.0	41	Arterial	192
	16.1	36	Arterial	381
Cat	17.9	30	End tidal	322
	45.3	35	Arterial	373
Dog	24.5	37	Arterial	224
Goat	32.3	38	Arterial	114
Human	27.3	36	End tidal	253
	23.0	42	End tidal	290
	26.4	39	Arterial	359
	27.3	38	End tidal	48

Ventilatory response data represent a relative measure of the increase in ventilation in response to hypercapnia, expressed as the percent increase in ventilation (ventilation at high CO_2 /ventilation at baseline CO_2) per Torr increase in CO_2 (either arterial or end tidal CO_2). Baseline CO_2 (in Torr, either arterial or end tidal) was measured when the animal was breathing room air.

HISTORY OF STUDIES OF CENTRAL CHEMORECEPTION

A considerable amount of research over the past 100 years has focused on the signals that lead to increased ventilation in response to respiratory stimuli. The two most commonly studied respiratory stimuli are a decrease in O_2 (hypoxia) and an increase in CO_2 . Furthermore, when the level of CO_2 is elevated in the body, the levels of H^+ (i.e., pH) and HCO_3^- are also affected through the hydration reaction catalyzed by carbonic anhydrase (CA). Thus, in theory, the sensory components of ventilatory control could be responding to changes of O_2 , CO_2 , pH, or HCO_3^- levels, and all have been proposed to be involved in chemosensitive signaling. In the face of these numerous potential stimuli, a major effort in the field has been to find a unifying theory that would involve a single adequate stimulus to explain ventilatory control.

Winterstein (400) proposed that a change of pH is the adequate stimulus for the chemical control of breathing. His initial theory, called the reaction theory (at that time pH was referred to as reaction), was an attempt to unify the findings that both elevated CO_2 and decreased O_2 could stimulate ventilation. Winterstein, in the first version of his reaction theory, argued that it was decreased blood pH, due to either elevated CO_2 , producing H^+ through its hydration and dissociation reactions, or the accumulation of metabolic acids during hypoxia, that resulted in activation of the chemosensory mechanisms that controlled ventilation. Thus a single common signal was proposed to explain the ventilatory response to both stimuli. Interestingly, this model was proposed more than 15 years before the identification of the peripheral chemoreceptors and their importance in the ventilatory response to O_2 (153).

The reaction theory came under question over the years on the basis of a number of findings. When HCO_3^- is added to the blood, pH alkalinizes yet ventilation is increased (69). In contrast, addition of NH_4Cl causes blood acidification but reduction in ventilation, in seeming contrast to the reaction theory (188, 399). Winterstein was able to account for these findings based on the studies of Jacobs (166), who showed that at constant pH_o , starfish eggs acidified when exposed to HCO_3^- and alkalinized when exposed to NH_4Cl because of the membrane permeability of the uncharged partners CO_2 and NH_3 , respectively. Thus, in the second version of the reaction theory, Winterstein differentiated the effects of changes in blood pH (“hematogenic” effects) from “. . . a change of the center itself” (“centrogenic” effects) (399). It appears that by the “center itself,” Winterstein may have been thinking of the intracellular pH (pH_i) of chemosensitive neurons themselves (188), thus representing the first suggestion of pH_i as the adequate stimulus, although he may have been referring to the pH_o in the vicinity of the central chemosensitive neurons.

An alternative to Winterstein’s reaction theory was the multiple factors theory of Gray (133, 134). This theory focused on the factors that contribute to the eventual increase in ventilation; O_2 and especially CO_2 (both peripherally and centrally) are proposed to contribute to respiratory drive along with pH, i.e., multiple factors contribute to increased ventilation, not a single unifying adequate stimulus. On the basis of a mathematical model, Gray (133) described the partial effects of changes in O_2 , CO_2 , H^+ , and muscle reflexes on the overall ventilatory increase in response to stimuli like HA, metabolic acidosis, exercise, and high-altitude anoxia. Because we now

realize that control of ventilation does indeed involve input from central chemoreceptors, peripheral chemoreceptors, and reflex pathways, Gray's conception of a multiple factors theory can hardly be challenged, and as such, the theory does not directly address the site of chemosensitivity or the pathways of activation of these sites. However, Gray (133) implied multiplicity of chemosensitive stimuli when he stated that "... the present theory ... resolves the most persistent and controversial question in the field of respiration: Should H-ion or CO₂ be considered the true respiratory stimulus? From the standpoint of the multiple factors theory, this question should be framed as follows: To what extent does each of the two agents influence ventilation?" In addition to Gray's theory, two other lines of evidence supported the idea that CO₂ itself is a chemosensitive signal, independent of its effect on pH. The first was the finding that the effects of elevated CO₂ (HA) on ventilation often exceeded the effects of metabolic acidosis (31, 32). The other line of evidence was that elevated CO₂ increased ventilation by increasing both tidal volume and respiratory frequency, whereas metabolic acidosis resulted in increased ventilation by increasing tidal volume only (see DISCUSSION in Ref. 188).

It is clear that these early theories suffered from a lack of awareness of the complex relationships between pH in various compartments, including blood, cerebral spinal fluid (CSF), extracellular fluid pH in the brain, and intracellular fluid. For example, Gesell and Hertzman (128, 129) showed that injection of HCO₃⁻ into the blood system resulted in alkalinization of the blood but acidification of the CSF. Injection of NH₄Cl into the blood resulted in acidification of the blood but alkalinization of the CSF (399). It was proposed that a barrier appears to exist between the CSF and blood that allows passage of uncharged species predominantly and acts in an analogous fashion to the cell membrane, as described by Jacobs (166). Thus blood pH could be a poor reflection of CSF pH. Similarly, it was suggested that blood pH could also be a poor reflection of the pH_o in the brain (106). Furthermore, in many early studies (see, for example, Ref. 114), extracellular fluid pH (referred to in this review as pH_o but also referred to as pH_{cef} or pH_e) was estimated from the pH of the CSF during ventriculocisternal perfusion. However, this method of estimation was called into question because of the unknown effects of the volume and composition of endogenously produced CSF (28). Therefore, the relationship between ventilation and pH as determined in a number of early studies must be questioned because of uncertainty regarding the appropriateness of the estimated values of pH.

These concerns were eliminated by the introduction of reliable techniques to measure pH_o with the use of either pH-sensitive microelectrodes within the medulla oblongata (75) or macro pH-sensitive electrodes on the ventral surface of the medulla (5, 106, 322, 354). With the introduction of techniques to measure pH_o directly, studies were done to determine whether ventilation has a unique relationship to pH_o. In a review of central chemosensitivity and the reaction theory, Loeschcke (211) concluded that "... extracellular pH in the brain is the main chemical signal determining ventilation." However, to reach this conclusion, he had to postulate that the acid-sensitive mechanism is accessible to CO₂ but has limited accessibility to H⁺ from metabolic acids. More recently, numerous studies demonstrated a different ventilation-pH_o rela-

tionship for HA compared with metabolic acidosis (190, 322, 353). For instance, Eldridge et al. (106) showed that the frequency of phrenic nerve firing was two to four times higher at the same pH_o with HA than with metabolic acidosis. In other words, pH_o could not be uniquely associated with a given level of ventilation. Eldridge et al. (106) offered three possible alternative explanations for this disagreement with the reaction theory. The first is the limited access theory proposed by Loeschcke (211). The second is that CO₂ has an effect separate from the effect of H⁺ or that there are separate sites for sensing H⁺ and CO₂. This proposal is consistent with the multiple factors theory. The third proposal is that pH_i, and not pH_o, is the adequate stimulus for chemoreception, a suggestion also made by Shams (322). A variation of this latter concept was also proposed by Kiwull-Schöne and Kiwull (190) and Xu et al. (403), who suggested that it is the transmembrane pH gradient (the difference between pH_i and pH_o) that serves as the chemosensitive signal. Exposure of chemosensitive neurons to CO₂ or to metabolic acids was proposed to result in a larger fall of pH_o than of pH_i due to internal H⁺ buffering by proteins. This would result in a reduction of the transmembrane pH gradient and a stimulation of chemosensitive cells. This idea is supported by the findings of Nattie (242), who showed that exposure of the ventral medullary surface to diethyl pyrocarbonate, an agent that can reduce intracellular H⁺ buffering by binding to imidazole groups on histidines, eliminates the ventilatory response to elevated CO₂, although it is possible that diethyl pyrocarbonate is directly inhibiting a pH sensor that involves a histidine residue. The importance of changes in the pH gradient across the membrane is also proposed to explain the lack of a ventilatory stimulation during hypoxia-induced acidification (the hypoxia paradox, see Ref. 239), where the internally generated lactic acid is presumed to result in a greater fall of pH_i than of pH_o (403). Evaluation of the possibility that it is pH_i or the transmembrane pH gradient that is the adequate chemosensitive signal had to await reliable measurements of pH_i in chemosensitive neurons.

Many of these earlier studies suffer from various methodological concerns. As mentioned above, most of the earlier studies are hard to interpret because of doubts about the appropriate compartment in which to determine pH. Another issue is that many of these studies were done without accounting for contributions from peripheral chemoreceptors or other feedback mechanisms (106), which complicates the interpretation of the data regarding central chemoreception. Furthermore, under some of the experimental conditions used, it is unclear whether true "isocapnic" conditions were maintained (189). Finally, the vast majority of the earlier studies aimed at determining the adequate stimulus focused on pH_o measurements at the surface of the ventral medulla as the sole site of chemoreception, whereas the experimental treatments may well have affected other chemosensitive sites (such as increasing inspired CO₂ or intravenous infusion of acid as in Ref. 106). Thus the likely presence of multiple sites for chemosensitivity throughout the brain stem makes studies of the adequate stimulus far more difficult.

Despite these limitations, certain findings are so common that they are undoubtedly true and, as such, must be explained by any model of central chemosensitivity. One such observation is that acidosis induced by increased CO₂ levels appears to be a stronger stimulus to ventilation than is metabolic acidosis.

In other words, for a similar degree of extracellular acidification, it appears that the chemosensitive system is more sensitive to HA than to metabolic acidosis (see, for example, Ref. 106). Another common finding is that there is a very high degree of sensitivity of ventilation to changes of pH, at least in some animals (Table 2). The work of Fencl et al. (114) on goats is often cited as an example of the high degree of sensitivity of ventilation to changes of pH. In fact, these authors reported a five- to sixfold increase in ventilation for a 0.1 pH unit change of CSF pH. As mentioned above, the method used to calculate pH in their study has been questioned (28). Nevertheless, more recent work with cats, using reliable measures of pH_o , has still reported a considerable sensitivity with ventilation changing from twofold (5) to fourfold (322) for a 0.1 pH unit change of extracellular fluid pH. Finally, despite years of research, conflicting data seem to indicate that there may be no unifying theory for central chemosensitivity and that there may not be a single adequate stimulus.

REDUCED PREPARATIONS

Central chemosensitivity is clearly a complex process, involving numerous inputs and multiple possible signals. Thus, to study and define chemosensitive signaling at the cellular level, the use of reduced preparations is required. Several reduced preparations for the study of central chemosensitivity have been developed, including the isolated brain stem-spinal cord preparation, brain stem slices, and tissue culture.

Brain stem-spinal cord preparations. The entire brain stem, either with or without part of the spinal cord attached, can be removed and studied *in vitro*. Changes in "ventilatory output" in these preparations can be monitored by placing suction electrodes on the phrenic nerve root (with spinal cord attached) or on the hypoglossal rootlet (without spinal cord attached) (144), whereas chemosensitive neuron output can be measured with either extracellular or intracellular electrodes (92, 262). These preparations were originally taken from neonatal rats to minimize their size and therefore minimize problems with maintaining their oxygenation. They have been used to study a variety of questions related to central ventilatory control, including respiratory rhythm generation and control (347, 348), central chemoreception (144, 180, 262, 376), and the effects of anoxia on ventral medullary neurons (15, 386). An advantage of the brain stem-spinal cord preparation for studying central chemosensitivity is that it is possible to monitor a system output that is reflective of all the various central CO_2 chemosensitive areas without any peripheral inputs. The disadvantages of this preparation are that individual neurons, other than those near the surface, are relatively inaccessible to microelectrodes and that maintaining adequate oxygenation can be difficult, especially in the core of the preparation (for a discussion of this issue, see Ref. 87).

Two variations on the brain stem-spinal cord preparation have been described. In the first, the brain stem only was removed from an adult guinea pig (92, 234). In this preparation, ventilatory output was measured with suction electrodes on the hypoglossal roots (also reflective of respiration). Because this was a larger preparation, adequate oxygenation required both superfusion with artificial cerebral spinal fluid (aCSF) and perfusion by cannulation of the basilar artery. When this preparation was superfused alone (with aCSF equil-

ibrated with 95% O_2), tissue Po_2 dropped from 500 Torr at the surface to essentially 0 Torr at a depth of 400 μm (these brain stems were over 6 mm thick). However, when this preparation was perfused as well as superfused, surface Po_2 was 500 Torr but fell to a stable level of 200 Torr at a depth of 1 mm into the brain stem (92). This level of 200 Torr Po_2 was maintained throughout the remainder of the thickness of the brain stem. Although this finding indicates that in this preparation core hypoxia is not an issue, Dean et al. (87) recently pointed out that brain Po_2 *in vivo* is <40 Torr, and so the level of oxygenation in these brain stem preparations is considerably hyperoxic (at least at the surface in the superfused brain stem and throughout the entire perfused brain stem). The effect of this hyperoxia on the neuronal properties has yet to be determined.

The other preparation is the working heart-brain stem preparation (267). In this preparation, the animal is bisected below the diaphragm, decerebrated, and the dorsal surface of the medulla exposed and superfused. The descending aorta is cannulated, and the preparation is perfused. The heart continues to beat, receives venous return from the superior vena cava, and maintains a normal beat frequency. Respiratory activity is recorded by suction electrode from the phrenic nerve. This preparation gives a normal respiratory pattern, as evidenced by the cyclic bursting pattern in the phrenic nerve. In addition, intracellular recordings can be made from dorsal medullary respiratory neurons, and peripheral reflexes appear to be intact, because activation of peripheral chemoreceptors increases phrenic activity. This preparation has been used with both mice (267) and neonatal rats (101). Although use of this preparation requires considerable surgical skill and may be limited in studying the properties of individual neurons, it benefits by maintaining a nearly fully intact regulatory pathway (including central and peripheral inputs), allowing studies of respiratory control in the absence of anesthetics, and maintaining the preparation with adequate levels of oxygen to yield normal respiratory patterns and responses. This technique was exploited further recently by the ability to study ventral respiratory neurons with whole cell patch clamp (100) and the use of fluorescence recordings to study calcium transients in medullary respiratory neurons (38). This preparation appears to be a good preparation with which to study the mechanisms of central chemoreception (268).

The results of studies on central chemoreception using the brain stem-spinal cord preparation agree in general with results of earlier studies. For instance, Denavit-Saubié et al. (92) found different responses of the adult brain stem to hypercapnia in the superfusate vs. hypercapnia in the perfusate. In the former case, when only chemosensitive neurons in the surface layers were activated, there was an increase in the burst amplitude but a decrease in burst frequency in the output of the hypoglossal root. These findings agree with previous findings in intact animals exposed to hypercapnia on the medullary surface, showing superficial chemosensitive neurons in the medulla (211). When the superfusate was made acid by decreasing solution HCO_3^- content at constant Pco_2 (metabolic acidosis), burst frequency increased, indicating that these superficial chemosensitive neurons respond differently to acidosis induced by increased CO_2 level compared with metabolic acidosis. Denavit-Saubié et al. (92) and Morin-Surun et al. (234) further showed that when the perfusate was made hyper-

capnic, thus activating deeper chemosensitive neurons, there was an increase in burst frequency but a decrease in amplitude. The finding that deeper chemosensitive neurons give a different response to hypercapnia than superficial neurons suggests that there are differences in the response of chemosensitive neurons from different regions to the same stimulus.

Harada et al. (144) used a brain stem-spinal cord preparation from neonatal rats to study the effects of various acid challenges on overall ventilatory output (measured as integrated phrenic nerve activity). By comparing phrenic nerve output in brain stems exposed to solutions of varying pH at constant CO₂ (made by altering HCO₃⁻) with solutions of varying CO₂ at constant pH, these authors were able to differentiate the ventilatory effects of CO₂ from those of pH. They showed that pH and CO₂ had separate and additive effects on ventilatory output, suggesting that there are separate chemoreceptors for pH and CO₂, a conclusion that agrees with Eldridge et al. (106). This conclusion is further strengthened by the finding that decreased pH altered both phrenic burst magnitude and frequency, whereas increased CO₂ only increased phrenic burst magnitude. Also, the effects of altered CO₂ levels were transient, whereas those of altered pH were sustained. On the basis of these findings, Harada et al. (144) proposed that there are different sites of chemoreception for CO₂ and pH and that the response to altered CO₂ levels may be mediated by changes of pH_i as well as pH_o. Finally, their findings also suggested a small and transient effect due to altered HCO₃⁻.

Brain stem slices. Slice preparations have been used extensively to study cellular properties within the central nervous system (CNS). The brain stem can be sliced either transversely (82, 86, 295, 306) or horizontally (126, 168). Slices are often made as thin as possible (100–400 μm thick) to maintain adequate oxygenation and to easily visualize individual neurons (237, 306). A thicker brain stem slice preparation (600–700 μm thick) was used to maintain a spontaneous respiratory rhythm (328) that is synchronized between the pre-Bötzinger complex and the hypoglossal roots (286, 287). The advantage of such preparations is that individual neurons can often be easily seen for patching, there is reasonably good control of the microenvironment around the neurons, and neuronal association with glia are intact, as are most of the dendritic processes and synaptic connections.

Several disadvantages are also involved with the use of brain stem slices. During slicing there can be damage to neurons and glia, especially in the surface layers. This damage can arise directly from trauma induced by slicing, from ischemia induced during brain stem dissection and preparation for slicing (297), from the loss of endogenous antioxidants and subsequent oxidative damage (292, 293), and from acidification due to CO₂ accumulation (221). All of these insults appear to lead to a cytotoxic edema that often results in neuronal death within a few hours (221, 292, 297). A number of techniques have been developed in an attempt to minimize this damage. Richerson and Messer (297) prepared brain stem slices (100 μm) in a hyperosmotic solution to reduce slice swelling, with low Ca²⁺ plus kynurenic acid to prevent neuronal uptake of Ca²⁺ and Na⁺, which have been associated with neuronal injury. This technique resulted in reduced slice swelling and increased neuronal survival. Rice and colleagues (292, 293) showed that slices lose most of their endogenous ascorbate and glutathione during incubation after slicing and that this loss is associated

with slice edema. Slice edema and neuronal histology were markedly improved when the slices were made and incubated in aCSF to which 400 μM ascorbate had been added. Finally, MacGregor et al. (221) showed that the addition of the buffer *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) reduced slice (400 μm) edema, presumably due to reduced slice acidification. The use of a combination of these techniques will likely reduce slice damage during preparation.

The slicing procedure also undoubtedly results in some degree of dendritic pruning and the loss of synaptic input from neurons from more distant brain regions, eliminating either excitatory or inhibitory inputs. Recently, an additional concern was raised, i.e., the proper level of oxygenation to be used with slices (87). Originally, slices were bathed in solutions equilibrated with 95% O₂ to maintain oxygenation in the center of the slice. Because slicing procedures have improved and thinner slices are obtained, this oxygenation protocol has not been altered. Recent measurements of O₂ profiles within 300-μm brain stem slices superfused with aCSF equilibrated with 95% O₂ showed that the Po₂ at the surface of the slice was >400 Torr and at the center was nearly 300 Torr (237). These values are nearly an order of magnitude greater than Po₂ values seen in neural tissue *in vivo* (87). Given that oxidative stress has been shown to damage slices (292), an investigation of the appropriate level of O₂ with which to equilibrate the aCSF bathing slices is warranted (87).

Despite these disadvantages, brain stem slices have been used for nearly 30 years to study the cellular basis of central chemosensitivity (70, 82, 84, 116, 117, 121, 122, 125, 159, 168, 295, 304–306, 385), and many of the findings made using the brain slice preparation are reviewed below.

Tissue and cell culture. Culture techniques have been employed in studies of central chemosensitivity as well. The use of cultured chemosensitive neurons offers some unique advantages, especially in the search for the adequate stimulus of central chemosensitivity. Among these advantages are the ease of performing electrophysiological measurements on these cells, the ready ability to control the external environment of these neurons, the elimination of excitatory and inhibitory synaptic input that can often complicate the interpretation of responses in more intact systems, and the ability of neurons to recover from slice damage (383).

Several laboratories have employed organotypic culturing of brain slices. This approach starts with a brain stem slice (225–500 μm). The slice can be minced into smaller pieces (250) or placed directly into culture in a chemically defined medium on permeable plastic or nylon grids (10, 27, 311, 393, 394) or on glass slides (389, 390). Slices are obtained from either neonatal rats [postnatal *days 0–6* (P0–P6)] (10, 27, 250, 393, 394) or rat fetuses (*embryonic day 16*) (389, 390). These various explant cultures are used in some cases after 8 days in culture (250) but mostly after 2–4 wk (10, 394). The explant slices usually flatten out, reducing their thickness to 50–200 μm (10), and have healthy-looking neurons that make synaptic contacts (27). When plated on glass slides, the slices exhibit outgrowths that are originally largely of glial origin but develop with time into a neural network (389, 390).

Primary cell culture has also been used to study central chemosensitive neurons. Cell cultures of CO₂-sensitive neurons have been derived from either fetal (119, 302, 303) or neonatal (343, 383) medullary brain stems. The technique

involves microdissection of the brain stem to include the areas of interest, followed by protease digestion and then plating of the resulting cells. These cells can be plated either on a monolayer of nonneuronal cells (119, 302, 303) or on laminin-coated coverslips in glia-conditioned medium (383). Neurons grow well under culture conditions and form either complex networks (119, 303) or individual cells (383). These cultured neurons are usually studied 2–5 wk after initial culturing (anywhere from 2 to 94 days), using standard electrophysiological and histological techniques. Rigatto et al. (302) observed that ~10–20% of the neurons cultured from either the nucleus ambiguus or the NTS exhibited regular spontaneous firing and responded to HA with an increase in firing rate (measured with whole cell patch techniques). The remaining neurons fired irregularly or were silent, and far fewer were stimulated by hypercapnic acidosis. Wang et al. (383) also found a proportion of their cultured medullary raphe neurons that exhibited regular spontaneous firing. Nearly 25% of the neurons studied were stimulated by HA (many of these exhibited regular firing patterns), ~25% were inhibited by HA, and the remaining 50% were unaffected. Wang et al. (383) further showed that the morphologies of HA-excited and HA-inhibited neurons were distinct. Thus it is clear that the use of cell culture yields neurons that exhibit a response to chemosensitive stimuli.

Cultured cell preparations can be problematic in terms of their representation of the *in vivo* preparation. For any given preparation, it must be shown that the prolonged period in culture during which neuronal growth occurs does not result in neurons with altered properties, such as the expression of ion channels or transporters that are not normally expressed *in vivo*. For example, the pH_i of hippocampal neurons cultured for 10–12 days was significantly lower than the pH_i of freshly dissociated neurons (285). If this is true in cultured chemosensitive neurons, it could be highly problematic, because these cells are likely sensitive to changes of pH_i (116). In addition, the lack of synaptic input itself suggests that cultured neurons may well respond differently to stimuli than they would in the context of the network *in vivo*. These cultured cells also have nearly unrestricted access to the bathing medium, a major advantage as stated above. However, in some cases, these neurons have been studied in solutions equilibrated with 95% O_2 (383), which should expose them to nearly 700 Torr of O_2 . Thus, to the extent that oxidative environments affect central chemosensitivity (238), such preparations should be highly affected. Interestingly, Rigatto et al. (303) studied their cultured neurons in growth medium that had a more normal P_{O_2} of ~60 Torr. In fact, their cultured neurons hyperpolarized and lost their spontaneous firing rate when exposed to medium equilibrated with 100% O_2 (640 Torr) (302, 303). The question of the appropriate level of O_2 to use with cultured neurons has been addressed, and it has been shown that cortical neurons grow better in culture medium equilibrated with 9% O_2 as opposed to 20–21% O_2 (40, 176). Therefore, the effect of the use of medium equilibrated with 95% O_2 to study cultured chemosensitive neurons needs to be examined. Finally, recent findings (see *Glial cells* in OTHER FACTORS IN CENTRAL CHEMOSENSITIVE SIGNALING) suggest that glial cells may well play a role in central chemosensitivity, and the removal of the association between glial cells and neurons in cell culture will undoubtedly

alter the response of cultured neurons compared with neurons *in vivo*.

Despite these concerns, cultured neurons often display many of the properties that they display in the brain slice (295, 383) and have been used to make important findings regarding the mechanisms of central chemosensitivity (298, 382).

SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS

There have been numerous proposed signals for central chemosensitivity, but the three most likely signals are changes in CO_2 , pH, and, in some cases, HCO_3^- (see HISTORY OF STUDIES OF CENTRAL CHEMORECEPTION). Many other agents have been suggested to play a role as well. We review the major findings for each of these possibilities.

Carbon dioxide. The hyperventilatory response to elevated CO_2 , especially when accompanied by extracellular acidosis (HA) can be substantial (see HISTORY OF STUDIES OF CENTRAL CHEMORECEPTION). In fact, the main argument for a role for molecular CO_2 as a signal in central chemosensitivity is the greater ventilatory response to HA compared with metabolic acidosis that is often observed (106, 144, 189, 322). In many of these studies, ventilation could not be plotted as a unique function of pH_o , and it was thus assumed that another factor, i.e., CO_2 , must be serving as an independent signal. The strong response to elevated CO_2 is also seen in reduced preparations (e.g., slice or cell culture), where putative central chemosensitive neurons respond to HA [e.g., slice (82, 116, 117); cell culture (250, 295, 303, 382, 383)]. In fact, a response to increased CO_2 is often taken as an indication that a neuron is chemosensitive, although this definition alone is undoubtedly too inclusive (for discussion, see CO_2/H^+ -SENSITIVE CELLS above and Refs. 294 and 384). There are two main difficulties in proposing that CO_2 per se is part of the cellular signaling pathway for central chemosensitivity. The first is that all of the earlier theories that proposed CO_2 to be a unique and independent signaling factor considered only the inadequacy of pH_o to explain the respiratory responses to various chemosensitive stimuli. However, no measurements of pH_i in central chemosensitive neurons existed even though cellular pH is markedly affected by CO_2 (279, 281). Furthermore, the effects of the various chemosensitive stimuli on pH_i , and thus the possibility that changes of pH_i serve as the adequate stimulus, were only occasionally considered (e.g., Ref. 106). The second major difficulty with CO_2 being a chemosensitive signal per se is that no appropriate cellular model has ever been proposed by which CO_2 could be a chemosensitive signal separate from its effects on pH_o and pH_i . However, we cannot rule out a CO_2 receptor, because one has been proposed for the response of renal proximal tubule cells to hypercapnia (412) and because receptors for other gases, such as nitric oxide (249), carbon monoxide (410), and oxygen (374), have been reported. Recently, increased molecular CO_2 , independent of its effects on pH, was proposed to activate L-type Ca^{2+} channels and raise intracellular Ca^{2+} in glomus cells (344–346) (see *Carotid body cells* in OTHER ACID-SENSITIVE CELLS). This effect involves elevated cAMP, and it has been suggested that molecular CO_2 could work through elevating intracellular HCO_3^- and activating soluble adenylylase, which has been shown to be HCO_3^- dependent (414) (Fig. 2). It is not known whether such a mechanism is at play in central chemosensitive neurons; how-

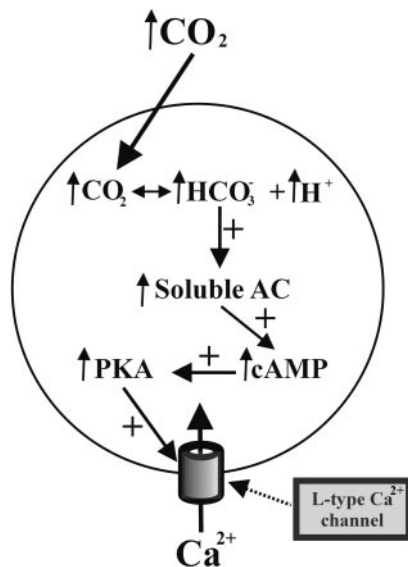


Fig. 2. Model of CO_2 activation of L-type Ca^{2+} channels in glomus cells from the carotid body. Hypercapnia elevates intracellular HCO_3^- level, which in turn activates soluble adenylate cyclase (AC). This in turn opens L-type Ca^{2+} channels by elevating cAMP and activating protein kinase A (PKA). This model is based on the findings of Summers et al. (346).

ever, in support of this possibility, it was recently shown in LC neurons that L-type Ca^{2+} channels are only activated when CO_2 is elevated, and not by decreased pH_i alone (117). Therefore, it will be of interest to determine whether molecular CO_2 can activate central chemosensitive neurons in a fashion that is independent of its effects on pH.

Bicarbonate. A potential role for HCO_3^- as a chemosensitive signal has not been extensively investigated. Studies of the role of HCO_3^- in chemosensitive signaling are complicated by the close interrelationship between HCO_3^- and pH. A small role for HCO_3^- in chemosensitive signaling was previously proposed (144). This suggestion was based on the observation that at constant CO_2 , elevating HCO_3^- in the superfusate occasionally resulted in a transient elevation of ventilation that was followed by depressed ventilation due to increased pH. However, these putative HCO_3^- effects are at most only transient and small in magnitude. Recently, a role for HCO_3^- in increasing the excitability of hippocampal neurons was described (42), so a role for HCO_3^- in chemosensitive signaling could reasonably be hypothesized.

Theoretically, there are several ways by which HCO_3^- could affect neuronal activity. The exchange of HCO_3^- and Cl^- across the blood-brain barrier is involved in the response of the brain extracellular space to respiratory acidosis in the intact organism (3, 4). At the cellular level, HCO_3^- -dependent transporters, including $\text{Cl}^-/\text{HCO}_3^-$ exchange, Na^+ -driven $\text{Cl}^-/\text{HCO}_3^-$ exchange, and Na^+ - HCO_3^- cotransport are involved in the regulation of pH_i in neurons and glial cells (90, 279, 304). It has been shown that HCO_3^- is needed for the recovery of synaptic transmission from anoxia and that this effect is independent of the effects of HCO_3^- on pH_i or pH_o (309). It was speculated that this effect could be mediated by the HCO_3^- dependence of the glutamate uptake transporter in astrocytes (35, 171), although it now appears that glutamate uptake is accompanied by movement of H^+ , not HCO_3^- (175, 411), or by

the ability of HCO_3^- to reduce free radical production by promoting the binding of iron to transferrin (177, 178, 288). This latter possibility is especially interesting, because increased production of free radicals was recently shown to selectively activate chemosensitive neurons within the NTS (238). As stated above, increased HCO_3^- can also activate soluble adenylate cyclase (414) and thereby increase cAMP and, ultimately, intracellular Ca^{2+} . Finally, the GABA_A channel has been shown to be permeable to HCO_3^- (172) with a selectivity for $\text{HCO}_3^-:\text{Cl}^-$ of 1:5 (173). Activation of the GABA_A channel can result in an efflux of HCO_3^- that leads to an intracellular acidification and an extracellular alkalinization (58, 172, 173). Thus changes in HCO_3^- in the extracellular solution may impact chemosensitive signaling by affecting neuronal pH_i , causing changes of pH_o , altering free radical production, changing cellular cAMP levels, and/or modifying the uptake of glutamate by astrocytes. A detailed examination of the role of changes of HCO_3^- in chemosensitive signaling would seem to be warranted.

Extracellular and intracellular pH. For nearly a century, H ions have been proposed to be the signal for chemosensitive cells in the control of ventilation (400). However, it has proved difficult to separate the effects of pH_o from pH_i . Much of the early work to differentiate these two possible signals employed comparisons of the effects of respiratory (hypercapnia and hypocapnia) vs. metabolic (weak acids and bases) acid-base changes on ventilation (e.g., Refs. 106, 190, 322, 353). Although these techniques often showed a difference between the ventilatory response to the stimuli, no information was available on the change of pH_i induced in putative chemosensitive neurons by respiratory vs. metabolic changes.

The study of signaling in chemosensitive cells has benefited greatly from the development of techniques to reliably study pH_i . Early techniques to measure pH_i in brain cells used the distribution of the weak acid dimethyl-2,4-oxazolinedione (312, 313) or nuclear magnetic resonance (26, 170, 378). Both of these techniques involved more global measurements of pH_i , and thus their relevance to changes of pH_i in chemosensitive neurons is problematic. However, on the basis of these findings, Lassen (199) made the first specific proposal that it is the change of pH_i , rather than the change of pH_o , that is the adequate stimulus for central chemosensitivity. Lassen's argument was based on the relative changes of pH_o , pH_i , and ventilation in response to hypercapnia (7% CO_2) and acetazolamide (an inhibitor of CA). It had been shown that both hypercapnia and acetazolamide result in a fall of brain pH_o of ~ 0.12 pH unit (26, 199). However, whereas hypercapnia (7% CO_2) resulted in a significant drop in brain pH_i of 0.06 pH unit (170), acetazolamide did not significantly affect brain pH_i when brain surface CO_2 was maintained constant (26, 378). In parallel with the changes of pH_i , but not the changes of pH_o , ventilation was markedly enhanced by hypercapnia but not by acetazolamide. These data strongly implicated changes of pH_i as the adequate stimulus for ventilation, but there is a concern that these measurements of brain pH_o and pH_i may not reflect the changes of these variables within chemosensitive neurons themselves. However, over the last decade, the emphasis of the studies of signaling pathways in central chemosensitivity has been on changes of pH_i .

The earliest measurements of pH_i in single respiratory-related neurons are those of Ballanyi et al. (14) and Cowan and

Martin (73). Ballanyi et al. (14) used double-barreled pH-sensitive microelectrodes (using a pH-sensitive resin for the pH electrode) to measure pH_i and membrane potential (V_m) in expiratory neurons of the ventral respiratory group. They found that when activity in the expiratory neurons was inhibited (presumably by input from the inspiratory neurons), the neurons rapidly (within 1–2 s) acidified by ~ 0.2 pH unit from resting pH_i levels of ~ 7.15 . This acidification was attributed to HCO_3^- efflux through $GABA_A$ channels, which are believed to be activated during inhibitory input. Interestingly, similar measurements in the axons of these neurons did not show alterations of pH_i with inhibition of neuronal output. These results suggest that pH_i changes in the axons (and presumably the dendrites) might well differ from those of the soma, and given the speed of the somatic acidification, $GABA_A$ channels must reside close to, if not on, the soma in these expiratory neurons. Furthermore, a few measurements from associated glial cells showed that these cells alkalize upon depolarization, and this alkalization was attributed to the voltage-sensitive $Na^+HCO_3^-$ cotransporter mediating HCO_3^- influx during glial depolarization (at resting V_m , this transporter is often outwardly directed, which would result in glial acidification). Cowan and Martin (73) also used double-barreled pH-sensitive microelectrodes (with a pH-sensitive resin) and measured V_m and pH_i simultaneously in individual neurons in slices of the dorsal respiratory group. Studying the effects of hypoxia on pH_i and V_m , these authors found that acidification of dorsal respiratory group neurons resulted in membrane depolarization, and they attributed this to effects of intracellular acidification on ion channels.

Trapp et al. (366) described a different method to simultaneously measure pH_i and V_m in dorsal vagal neurons. These neurons are from a region believed to contain chemosensitive neurons (159). To obtain simultaneous recordings of V_m and pH_i , the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was loaded into the neuronal cell body from a whole cell patch pipette. This study (366) showed that dye-loaded and patched neurons gave normal pH_i responses to exposure to NH_4Cl (33) or the base trimethylamine (TMA), indicating that neuronal pH_i was not "clamped" by buffer diffusing from the patch pipette. This technique was used to show that these neurons acidify in response to anoxia, alkalize in response to external buffer change from CO_2/HCO_3^- to HEPES, acidify in response to increased neuronal activity (366), and acidify in response to GABA and glycine, as a result of HCO_3^- efflux from a receptor-coupled channel (215). The response of these neurons to chemosensitive signals (e.g., HA) was not studied, but no marked change in firing rate was observed in response to anoxia-induced acidification or to alkalization induced by the buffer change (366). This could indicate that these cells were not chemosensitive or that the electrical response was washed out (88, 117, 295, 308) by the use of whole cell recordings.

Ritucci et al. (306) introduced a technique to measure pH_i in individual neurons in brain stem slices from putative chemosensitive regions using the pH-sensitive fluorescent dye BCECF. In this technique, cells were loaded with BCECF by incubating the slice in the membrane-permeable acetoxymethyl ester (AM) form of BCECF, BCECF-AM. This form of the dye readily enters the cell, where the AM groups are cleaved by internal esterases. The resulting BCECF is charged, and thus

relatively impermeable to the cell membrane, and its fluorescence is sensitive to pH_i . Slices loaded in such a fashion showed several large spheres of intense fluorescence and a diffuse background fluorescence, with the latter presumably due to dye loaded into neuronal processes, glial cells, and out-of-focus cells. The large spheres were shown to be dye-loaded neuronal cell bodies by immunocytochemical stains for neurons, by retrograde labeling of another fluorescent dye, and by direct electrical recordings. These cells were visualized and studied using fluorescence imaging microscopy.

By using this technique with brain stem slices from neonatal (P0–P12) rats, the response of neurons from two chemosensitive regions, the VLM and the NTS, were compared with the response of neurons from two nonchemosensitive regions, the inferior olive (IO) and the hypoglossal (305). Upon exposure to HA (10% CO_2 , 26 mM HCO_3^- , pH_o 7.15), neurons from the chemosensitive VLM and NTS regions acidified and remained acid during the entire exposure to HA, with pH_i returning to control values upon return to normocapnic solution (5% CO_2 , 26 mM HCO_3^- , pH_o 7.48). In contrast, neurons from the nonchemosensitive IO and hypoglossal regions showed pH_i recovery from the acidification induced by HA and an overshoot of pH_i , indicative of recovery during the acid exposure (281), upon return to normocapnic solution. Neurons from VLM and NTS were shown to have pH-regulating transporters by being exposed to isohydric hypercapnia (IH: 10% CO_2 , 52 mM HCO_3^- , pH_o 7.48). VLM and NTS neurons exhibited a brisk pH_i recovery from these solutions, and this recovery was unaffected by the HCO_3^- -dependent transport inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) but completely inhibited by the Na^+/H^+ exchange (NHE) inhibitor amiloride. These studies were extended to show that neurons from all four regions used NHE solely to recover from intracellular acidification (304).

The question arose as to how all four regions contained a pH recovery transporter capable of responding to cellular acidification, but this recovery was not evident in neurons from the chemosensitive VLM and NTS regions during HA. This question was answered by Ritucci et al. (304), who showed that the NHE from VLM and NTS neurons was far more sensitive to inhibition by a fall in pH_o than was the NHE in neurons from the nonchemosensitive IO and hypoglossal regions. NHE from IO and hypoglossal neurons were completely inhibited at pH_o of 6.7–6.8, whereas NHE from VLM and NTS neurons were completely inhibited by pH_o of only 7.0–7.1. These findings agree with the results of studies on the effect of anoxia on pH_i in neurons from the same regions (54). In neurons from nonchemosensitive regions (IO and hypoglossal), the fall of pH_i induced by anoxia was greater in the presence of amiloride, suggesting that NHE activity blunted the anoxia-induced acidification. In contrast, amiloride did not affect anoxia-induced acidification in neurons from chemosensitive medullary regions (VLM and NTS), indicating that NHE was inhibited during anoxia (presumably by decreased pH_o) in these neurons. Together, these studies suggest that a maintained fall of pH_i is an important part of the chemosensitive signaling pathway but that a fall of pH_o also appears to be important in the maintenance of a decreased pH_i .

Similar findings of a maintained fall of pH_i in response to HA have been made in cultured medullary raphe neurons (36), in RTN neurons from brain stem slices (255), and in neurons

from organotypic medullary cultures (393). The latter study involved the use of a number of acid challenges to compare their effects on pH_i (measured in BCECF-loaded neurons) and their effects on firing rate (measured separately). A procedure that acidified both pH_o and pH_i (HA) resulted in an increase in firing rate. Replacing CO_2/HCO_3^- buffer with HEPES, which involved no change of pH_o and a slow decrease of pH_i , also resulted in an increase in firing rate. Exposure to NH_4Cl , which leads to increased pH_i (at constant pH_o), resulted in decreased firing rate, whereas removal of NH_4Cl , which leads to decreased pH_i (at constant pH_o), resulted in increased firing rate (393). These findings implicated changes of pH_i in chemosensitive signaling. This conclusion was supported by Wang et al. (382), who varied external CO_2 , pH_o , and HCO_3^- independently and measured the resultant increase in firing rate in cultured raphe neurons (they did not measure pH_i). Exposure to HA (9% CO_2 , 26 mM HCO_3^- , pH_o 7.17), IH (9% CO_2 , 40 mM HCO_3^- , pH_o 7.4), IA (5% CO_2 , 15 mM HCO_3^- , pH_o 7.16), and acidified HEPES-buffered medium (pH_o 7.2) all induced an increase in firing rate, indicating that a change of CO_2 , pH_o , or HCO_3^- is not required for increased firing rate and suggesting that a change of pH_i may be the primary stimulus for chemosensitivity (382).

Filosa et al. (116) developed a new technique for simultaneously measuring V_m and pH_i in CO_2 -sensitive neurons from LC. This technique involved the use of perforated patch pipettes to measure V_m . The membrane-permeable form of BCECF (BCECF-AM) was placed in the patch pipette, and loading occurred through the perforated patch. The AM groups were cleaved by intracellular esterases, thus generating the pH-sensitive and membrane-impermeable form of the dye, BCECF. In this way, with the use of fluorescence imaging microscopy, the firing rate and pH_i changes induced by various solutions could be measured simultaneously. The protocols were similar to those used by Wang et al. (382). Exposure of LC neurons to HA, IH, IA, and acidified HEPES solutions all resulted in an increase in firing rate. A plot of the magnitude of the measured change of pH_i vs. the magnitude of the increased firing rate showed a good correlation (Fig. 3A). The change in firing rate was poorly correlated with the change of CO_2 , the change of HCO_3^- , or the pH_i - pH_o gradient (Fig. 3B). Furthermore, the rate of change of pH_i was more closely correlated with the rate of change of firing rate than was the rate of change of pH_o (116). This was especially clear when comparing neuronal response to HA exposure vs. IA exposure. HA resulted in a fast change of pH_o , pH_i , and firing rate, whereas IA resulted in a fast change of pH_o but a slow change of pH_i and firing rate. These findings strongly implicate changes of pH_i as the primary stimulus for chemosensitivity but do not rule out a role for changes of pH_o .

The picture that emerges from all of these studies is that a maintained fall of pH_i may be the adequate stimulus to chemosensitive neurons. Although there is excellent evidence that a fall of pH_i is a major part of the stimulation pathway, there are several problems. The first problem is that a maintained fall of pH_i in response to HA (i.e., no apparent pH_i recovery from acidification) is not a unique property of chemosensitive neurons. In both the NTS (305) and the medullary raphe (36), virtually all neurons showed maintained acidification in response to HA, but only 20–40% of the neurons respond to HA with an increased firing rate (82, 383). Nottingham et al. (255)

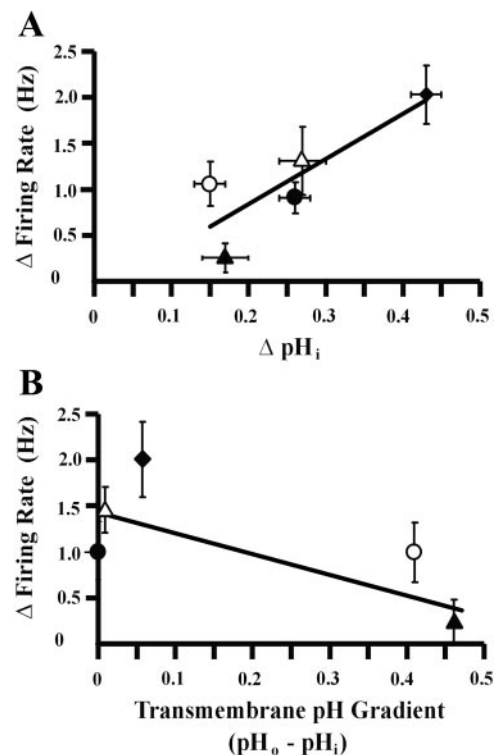


Fig. 3. A: relationship between integrated firing rate (Hz, 10-s bins) and the magnitude of the change of intracellular pH (ΔpH_i) in locus coeruleus (LC) neurons in response to a variety of acid challenges. Firing rate and pH_i were measured simultaneously in individual LC neurons in a brain stem slice from neonatal rats by using perforated patch pipettes loaded with the membrane-permeable form of the pH-sensitive dye BCECF-AM (116). There is a significant correlation between the degree of intracellular acidification and the increase in the spontaneous firing rate: firing rate = $4.90(pH_i) - 0.14$, $R^2 = 0.71$, $P < 0.05$. B: relationship between integrated firing rate and the transmembrane pH gradient ($pH_o - pH_i$, where pH_o is extracellular pH) under the same conditions as in A. The relationship between firing rate and the transmembrane pH gradient is not significant: firing rate = $-2.10(pH_i) + 1.37$, $R^2 = 0.33$, $P > 0.05$. The various acid challenges were as follows (all at 35°C): isohydric hypercapnia (\circ : 15% CO_2 , 77 mM HCO_3^- , pH_o 7.45), weak acid propionate (\blacktriangle : 50 mM propionate, 5% CO_2 , 26 mM HCO_3^- , pH_o 7.45), hypercapnic acidosis (\triangle : 15% CO_2 , 26 mM HCO_3^- , pH_o 6.8), isocapnic acidosis (\bullet : 5% CO_2 , 7 mM HCO_3^- , pH_o 6.8), and acidified HEPES-buffered solution (\blacklozenge : nominal absence of CO_2/HCO_3^- , pH_o 6.8). Data are represented as means \pm SE of between 5 and 24 values. [Adapted from Fig. 9 of Filosa et al. (116) with permission from The Physiological Society.]

further demonstrated that neurons from the nonchemosensitive hypoglossal region exhibited pH_i recovery in response to HA when taken from young rats (<P11), in agreement with the findings of Ritucci et al. (305), but neurons from the same region lacked apparent pH_i recovery from HA-induced acidification when taken from older animals (>P15). This indicates that there is a developmental shift in the pH_i response to hypercapnia-induced acidification. Although it is clear that all chemosensitive neurons studied display a maintained acidification in response to HA, this response is not unique to chemosensitive neurons. Thus, as previously discussed (36, 255), altered pH_i regulation alone is not sufficient to make a neuron chemosensitive, and a neuron that shows maintained acidification in response to HA cannot be assumed to be chemosensitive.

A second problem with change of pH_i as the adequate stimulus is that the firing rate of LC neurons does not appear to

always be a fixed function of pH_i . Although the changes of pH_i and of firing rate are well correlated upon exposure to acid stimuli, they are often poorly correlated upon return to normal solutions (116). For instance, in response to removal of an acidified HEPES solution, LC neuron firing rate returns to normal quickly, whereas the neuron remains acid for a prolonged period of time. In contrast, upon removal of IH, pH_i returns to normal and even exhibits an alkaline overshoot, but firing rate remains elevated, only slowly returning toward initial values (116). Furthermore, upon exposure to IH, LC neurons become acid but then exhibit pH_i recovery. However, IH induces an increase in firing rate with no evidence for an adaptation of the firing rate associated with pH_i recovery. Finally, exposure to propionate induces acidification of LC neurons comparable to that seen with IH exposure (116), but there is no increase in firing rate (Fig. 1A). In fact, the firing rate increases slightly upon removal of propionate when pH_i becomes alkaline. These findings clearly suggest that although pH_i is an important chemosensitive signal, it is not the sole determinant of the neuronal firing rate.

This conclusion is strengthened by the phenomenon termed the “hypoxia paradox” (239). Hypoxia-induced intracellular acidification of medullary neurons does not stimulate ventilation in the absence of peripheral chemoreceptors, even though ventilation will still increase in response to a change of pH_i induced by hypercapnia during the hypoxia (189, 401). As discussed above, this observation was in part the reason why the transmembrane pH gradient was proposed to be the adequate signal for chemoreception (see HISTORIES OF STUDIES OF CENTRAL CHEMORECEPTION, but also see Fig. 3B). This paradox has not been fully studied at the cellular level, but VLM and NTS neurons, when exposed to anoxia, acidify to the same extent as when exposed to hypercapnia (10% CO_2), and this acidification is maintained (54). Although we do not have comparable firing rate responses, VLM neurons have been shown to hyperpolarize in response to anoxia (15), and brain stem neurons from neonates (as used in the pH_i studies, e.g., Refs. 54, 305) show only a modest depolarization in response to hypoxia (141). A possible explanation for this paradox is that during anoxia/hypoxia, reduced ATP levels result in opening of the ATP-sensitive K^+ (K_{ATP}) channel, which suppresses neuronal firing rate (73, 142, 193). This issue needs to be

studied in greater detail, but, again, it suggests that intracellular acidification alone is not the only factor determining neuronal firing rate.

Another issue with the assumption that changes of pH_i are the adequate stimulus for chemosensitivity is that all previous measurements of neuronal pH_i are from the cell body. It is possible that the key processes that make a cell chemosensitive reside in the dendrites, and not the soma (e.g., see Ref. 12). It has been shown in neurons that the changes of pH_i in the dendrites to a given stimulus are larger and faster than the changes of pH_i in the soma (398). It will be important in the future to measure the changes of pH_i in the dendrites of chemosensitive neurons in response to acidic stimuli. Finally, a critical experiment to fully test the role of changes of pH_i as the adequate chemosensitive stimulus has never been done. If a change of pH_i alone is the adequate chemosensitive signal, then changes of CO_2 or changes of pH_o , without a change of pH_i , should not result in increased firing rate in chemosensitive neurons. An increase of CO_2 is always associated with a fall of pH_i in chemosensitive neurons, and the pH_i in these neurons is frequently highly correlated with pH_o (36, 304) so that a fall of pH_o will usually also result in a fall of pH_i .

Two approaches could be used to clamp intracellular pH in the face of CO_2 and/or pH_o changes. The first is to use the null point technique that has been used in the past to determine steady-state values of pH_i (37, 105, 349). With this technique, a cell is exposed to mixed solutions of weak acids and bases to create a condition in which the effects of the two on pH_i are equal and opposite, resulting in no change of pH_i . Because CO_2 in solution is a weak acid (carbonic acid), a suitable weak base would need to be added to the solution. The feasibility of such an approach is shown in Fig. 4. An LC neuron was exposed to HA (15% CO_2 , pH_o 6.9) and pH_i showed a maintained acidification, as expected. When the HA exposure was repeated with a solution that also contained 20 mM of the weak base TMA, there was at most a slight change of pH_i , even though external CO_2 was increased and pH_o was decreased. Although this approach achieved a degree of pH_i clamping, the weak base TMA resulted in a dramatic neuronal depolarization (probably due to TMA entry through K^+ channels) that precluded assessing the effects of the treatment on firing rate. For this approach to ultimately work, a weak base will need to be

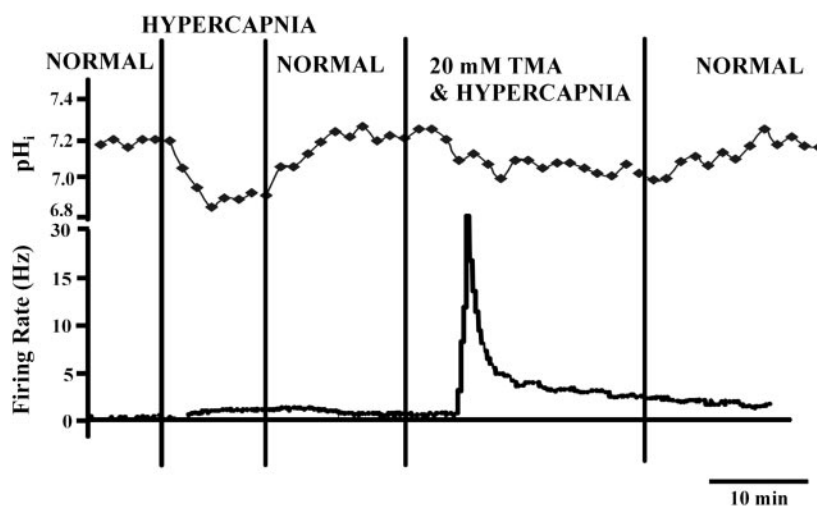


Fig. 4. Simultaneous recording of pH_i and integrated firing rate (Hz, 10-s bins) in a LC neuron in a brain stem slice from a neonatal rat. Hypercapnic acidosis resulted in intracellular acidification and a reversible increase in firing rate. Simultaneous exposure to hypercapnic acidosis and the weak base trimethylamine (TMA; 20 mM) resulted in essentially no change of pH_i (pH_i clamp) in the face of decreased pH_o and hypercapnia. However, the TMA itself resulted in a large depolarization and marked increase in firing rate.

found that can clamp pH_i and yet have minimal direct effects on V_m .

A second approach to pH_i clamping involves the use of whole cell pipettes. With whole cell recordings, there is direct diffusional exchange between the pipette solution and the cytoplasm. Normal changes of pH_i in response to HA have previously been observed using whole cell recordings (215, 280, 308). However, it is possible that, by filling the whole cell pipette with a concentrated solution of buffer, a substantial pH_i clamping could be achieved (at least in the neuronal soma). An alternative approach to pH_i clamping using whole cell (or perforated patch) pipettes was described by Grinstein et al. (138). With this technique, the whole cell pipette (and thus the cell) is loaded with a high concentration of a weak acid or base (e.g., NH_3/NH_4^+) (138) that is only permeable to the membrane in the uncharged form. By setting the concentration of the same compound outside, any desired pH_i can be achieved and clamped. The eventual successful use of such a technique to clamp pH_i while varying CO_2 and pH_o offers great promise in finally determining whether pH_i is the single adequate stimulus for chemosensitive signaling.

OTHER FACTORS IN CENTRAL CHEMOSENSITIVE SIGNALING

A number of other factors besides changes of pH have been implicated as being involved in chemosensitive signaling. Several of these are briefly discussed below.

Calcium. A number of studies have indicated that Ca^{2+} may play a role in chemosensitive signaling. An increase in extracellular Ca^{2+} has been shown to decrease the integrated respiratory output in the brain stem-spinal cord preparation and also to reduce the response to hypercapnia (197). However, it is not clear whether this effect is on central chemosensors or on the rhythm generator. In rat organotypic medullary cultures, Ca^{2+} channel blockers (verapamil, flunarizine, and Cd^{2+}) blocked the generation of the respiratory rhythm and decreased the responsiveness to hypercapnia (10). Using the Ca^{2+} -sensitive fluorescent dye fura-2 {1-[2-(5-carboxyoxazol-2-yl)-6-amino benzofuran-5-oxy]-2-(2'-amino-5-methylphenoxy)ethane- N,N,N',N' -tetraacetic acid}, these authors also showed that the generation of respiratory rhythms was associated with intracellular Ca^{2+} oscillations. They concluded that Ca^{2+} oscillations resulted from both Ca^{2+} influx through surface Ca^{2+} channels and second messenger-induced release from internal stores, and that these Ca^{2+} oscillations were required for the generation of the rhythmic discharge in these neurons.

A few studies have indicated that Ca^{2+} may play a role in the response of chemosensitive neurons themselves to hypercapnia. Although Kawai et al. (180) showed that Cd^{2+} (200 μM) did not block HA-induced depolarization of chemosensitive ventral medullary neurons, Wellner-Kienitz et al. (390) found that Cd^{2+} (50 μM), but not Ni^{2+} , did block the increased firing rate response to HA in medullary neurons in organotypic culture. It is unclear why these studies had different results with Cd^{2+} , but it could reflect differences in the neurons, possibly due to culturing. In the latter study, the effects of Cd^{2+} were attributed to blockage of Ca^{2+} entry through L-type Ca^{2+} channels, which thereby inhibits activation of Ca^{2+} -activated K^+ (K_{Ca}) channels. It was further proposed that HA blocked either L-type Ca^{2+} channels or K_{Ca} channels directly.

In LC neurons, voltage-activated Ca^{2+} channels have been described, and these channels are believed to play a role in the activation of the K_{Ca} channels in these neurons, as well (260, 397). In fact, Ca^{2+} entry and activation of K_{Ca} channels have been proposed to be a negative feedback mechanism that controls excitability in LC neurons (2).

The potential role of Ca^{2+} channels in chemosensitive signaling in LC neurons was recently addressed (117). In these neurons, blockage of Na^+ spikes with TTX revealed the presence of small, broad spikes that are believed to be caused by opening of dendritic Ca^{2+} channels (8, 116, 117, 395, 397). Filosa and Putnam (117) showed that these TTX-insensitive spikes could be blocked by Co^{2+} (2 mM) or nifedipine (50 μM) and that their frequency was increased by HA or IH, but not by exposure to propionate. It was concluded that acidic stimuli that involved increased CO_2 levels, but not other acidic stimuli, could activate L-type Ca^{2+} channels. This conclusion appears to be at odds with the usual finding that a decrease in either pH_i or pH_o results in Ca^{2+} channel inhibition (321, 363). Activation of L-type Ca^{2+} channels by acidic stimuli in LC neurons could be mediated by membrane depolarization or, possibly, by activating second messengers such as protein kinases, as shown for hypoxia-induced activation of Ca^{2+} channels in chemosensitive glomus cells (344–346) (see *Carotid body cells* in OTHER ACID-SENSITIVE CELLS). The finding that HA and IH, but not propionate, activated Ca^{2+} channels (117), which paralleled the effects of these stimuli on firing rate (Fig. 3A) (116), suggested that activation of Ca^{2+} channels might be involved in chemosensitive signaling. This possibility was tested directly. Filosa and Putnam (117) showed that nifedipine (50 μM) inhibited HA-induced increased firing rate by 40% and completely blocked IH-induced firing rate. These findings strongly suggest that activation of Ca^{2+} channels and/or the accumulation of intracellular Ca^{2+} play a role in chemosensitive signaling. A role for intracellular Ca^{2+} is suggested by the observation that reduction of the Ca^{2+} chelator EGTA from 10 mM to 0.4 mM in the pipette filling solution of whole cell pipettes reduced the washout (88, 295, 308) of the electrical response to hypercapnia in chemosensitive neurons (117), although no study has ever shown an increase of Ca^{2+} in chemosensitive neurons in response to hypercapnia.

In summary, there is accumulating evidence for Ca^{2+} as a chemosensitive signal. It is unclear whether this is true for chemosensitive neurons from all regions or not. Furthermore, it is not clear whether it is the activation of the Ca^{2+} channel per se or the accumulation of intracellular Ca^{2+} , or both, that is crucial for signaling. It is anticipated that direct measurements of the changes of intracellular Ca^{2+} , most likely employing fluorescence imaging techniques, in chemosensitive neurons in response to acidic stimuli will be forthcoming, and such data will be important for the understanding of the role of Ca^{2+} in chemosensitive signaling. Another important unanswered question is the mechanism by which acidic stimuli activate Ca^{2+} channels in central chemosensitive neurons. Thus there is a clear need for more detailed studies of the role of Ca^{2+} in central chemosensitive signaling.

Gap junctions. Neurons from the solitary complex (NTS and dorsal motor nucleus of the vagus) (84, 159), the LC (6, 13, 62, 63, 161, 261, 369), and the pre-Bötzinger complex (289, 332), putative chemosensitive regions, have been shown to be coupled by gap junctions. For instance, in the solitary complex,

only ~12% of the neurons were shown to be coupled by gap junctions by both dye coupling and electrophysiological measurements, but 86% of these coupled neurons increased their firing rate in response to hypercapnia (84, 159). This finding indicates that, at least in the solitary complex, the chemosensitive neurons are preferentially coupled. Using immunohistochemical techniques, Solomon et al. (333) demonstrated the presence of connexins, the proteins that form gap junctions, in chemosensitive neurons. Specifically, both connexin32 (Cx32) and Cx26 were found in neurons from the ventrolateral medulla, the dorsal medulla (including the NTS and dorsal motor nucleus of the vagus), the medullary raphe, and the dorsal pons (including the LC) (333). More recently (329), Cx36 was also found in both the soma and dendrites of neurons from all of these chemosensitive regions. Thus the proteins that form gap junctions have been demonstrated in many of the same chemosensitive areas where functional coupling is found. Furthermore, Dean et al. (85) found that coupling was maintained in the face of very high levels of hypercapnia (to at least 35% CO₂) and intracellular acidification (down to pH_i 6.7) in solitary complex and LC neurons. Cell-cell coupling also appears to play a role in synchronizing the rhythmic output of LC neurons (8, 63). Finally, inhibition of gap junctions with carbenoxolone has been shown to alter respiratory rhythm (34, 330), suggesting a role for gap junctions in the generation of the respiratory rhythm.

The role of gap junctions in respiratory control was recently reviewed (81, 331), and despite considerable study over the last several years, many important questions remain. It is not clear whether neurons from all chemosensitive regions exhibit cell-cell coupling and whether it is neuron-neuron coupling or neuron-glia coupling that is most important. The role of cell-cell coupling is also not clear. It could be that coupling serves to amplify the response to a chemosensitive signaling, possibly through metabolic coupling, or it could reduce the response to a signal by reducing the input resistance of a given chemosensitive neuron. In the latter case, a more widespread or stronger stimulus would be required to get a chemosensitive response. Finally, the developmental pattern of gap junction expression is not clear. It is generally believed that gap junctions are involved in early neurodevelopment and decrease with age (137), but connexins have been found (137, 333) and electrotonic coupling has been reported (84) in both neonatal and adult rats. However, it is not clear that functional gap junctions are fully active at all developmental stages. The answers to questions such as these should add greatly to our understanding of the role of cell-cell coupling in central chemosensitivity.

Carbonic anhydrase. Because chemoreception involves a response to CO₂ that is probably mediated in part by changes of pH, a role for CA in central chemoreception would be expected. CA has been demonstrated in neurons as well as in glia and capillary endothelial cells, using histochemical techniques (299, 300, 382). It is present in neurons from chemosensitive regions, including a small area of positively stained neurons in the ventrolateral medulla (299), the solitary complex (250, 264), and the medullary raphe (382). However, a neuronal presence of CA is not unique to chemosensitive neurons, because CA-positive neurons have also been demonstrated in the IO (299, 382), the hypoglossal region, the hippocampus, and the cerebellum (382), all nonchemosensitive

regions. Thus the presence of CA in a neuron does not indicate that the neuron is chemosensitive.

Neurons have been shown to contain both CA II and CA IV (382), and with the use of CA II-deficient mice, neurons have been shown to contain a membrane-bound form of CA as well as the cytosolic form of CA II (301, 364). The presence of membrane-bound CA suggests that this isoform may play a role in the regulation of pH_o, which could play a role in central chemosensitivity.

It would be expected that inhibition of CA might slow the rate of response to elevated CO₂ but not alter the eventual magnitude of the change, because CA only catalyzes the hydration of CO₂ and does not alter its equilibrium. Indeed, the ventilatory response to hypercapnia is slowed in the presence of the CA inhibitor acetazolamide (67, 360). However, in other studies respiratory drive was either decreased (7) or increased (67, 68, 359, 402) by CA inhibitors. The interpretation of these studies is complicated by the fact that acetazolamide injection can result in acidification, presumably due to decreased buffering of metabolic H⁺ by CO₂/HCO₃⁻ buffering (320). In fact, local injections of acetazolamide into various brain stem regions to produce focal acidosis have been used as a probe for chemosensitive areas (67, 68, 239, 241).

An interesting indication of a role for CA in central chemosensitivity came from the work of Dean and Reddy (88). They found that the electrical response of solitary complex neurons to hypercapnia was washed out when whole cell recording pipettes were used. To test whether the cellular component that was washed out was CA, they added CA II to the whole cell pipette. Under these conditions, the electrical response to hypercapnia was not washed out. These findings suggest that soluble CA plays a role in central chemosensitivity.

The most direct tests of a role for CA in central chemosensitivity were recently performed in medullary slices (248) and cultured raphe neurons (382). In the case of the slices, the increased bursting frequency (measured as a respiratory-related output in hypoglossal roots) in response to CO₂ was not altered by exposure of the slice to 1 mM acetazolamide (248). Similarly, the electrical response of cultured raphe neurons to hypercapnic acidosis was not affected by 0.1 mM acetazolamide (382). These data suggest that CA plays no role in central chemoreception.

On the basis of previous studies, a role for CA in central chemoreception appears unlikely. However, a detailed examination of the role of CA is probably warranted. The use of knockout animals would seem to be an especially attractive possibility for such studies.

Oxidative stress. It has been known for many years that hyperoxia can lead to increased ventilation (19, 74, 127, 231), although this observation has received little attention. Hyperoxia is often used to suppress the peripheral chemoreceptors (24, 77, 91, 230), although this suppression may only be partial and is unlikely to be the only effect of hyperoxia on ventilation (52, 277, 310). The possibility that hyperoxia can stimulate central chemoreceptors has received little attention. The observation that hyperoxia suppresses peripheral chemoreceptors and yet may increase ventilation suggests that it can activate central chemoreceptors and/or the rhythm-generating center itself. This hypothesis was recently directly demonstrated for chemosensitive neurons from the solitary complex (238). A subset (~40%) of solitary complex neurons was shown to

increase their firing rate in response to hyperbaric oxygen (HBO₂; up to 2,500 Torr). The interesting finding was that 90% of these HBO₂-sensitive neurons were also sensitive to hypercapnia; i.e., the oxygen-sensitive neurons were also the CO₂-sensitive, or chemosensitive, neurons (238). Furthermore, these neurons were stimulated by chemical oxidants, and their activation by HBO₂ could be blocked by an antioxidant, suggesting that solitary complex neurons are activated by the production of oxygen free radicals. Finally, the response of these neurons to hypercapnia was unaffected by antioxidants, indicating that the pathway of activation of solitary complex neurons by hyperoxia differs from the pathway of activation by hypercapnia. These new findings need to be repeated with chemosensitive neurons from other regions of the brain stem to determine to what extent activation of chemosensitive neurons by oxidative stress is a generalized phenomenon. However, these findings point to a need to reevaluate studies examining changes in ventilation in animals exposed to hyperoxia, with the intent to eliminate peripheral chemosensitive input, because central chemoreceptors may well have been directly stimulated by hyperoxia in these studies. Finally, the relevance of hyperoxic hyperventilation is unclear. Increased ventilation in the face of hyperoxia should increase oxygen delivery to the tissues and is therefore of questionable physiological significance. As suggested by Mulkey et al. (238), hyperoxic hyperventilation may not be adaptive but, instead, may represent an early expression of oxygen-induced damage to the CNS.

Glial cells. Glial cells, especially astrocytes, have been shown to play an active role in regulating the extracellular environment and in functioning to modulate neuronal excitability (146), and they would therefore be expected to be involved in chemosensitive signaling. This could occur in several ways. Glial cells are believed to contribute to the regulation of the extracellular pH of the brain (57, 90), and because extracellular pH is likely to be part of the chemosensitive signal (see *Extracellular and intracellular pH* in SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS), glial cells should be able to modulate the neuronal response to chemosensitive stimuli. Furthermore, glial cells can regulate the concentration of extracellular glutamate with a transporter that cotransports glutamate with a proton (175, 411), thereby modulating neuronal function (148). This mechanism would not only regulate extracellular glutamate but would also contribute to the regulation of extracellular pH due to the associated movements of H⁺. Another way in which glial cells can modulate neuronal function is by metabolic coupling, largely through the exchange of lactate (89). Finally, glial cells can inhibit neuronal excitability by the release of ATP (251), which is of particular interest because purinoceptors have been implicated in chemosensitive signaling (336, 356, 357). Thus there are numerous ways in which glial cells can play a role in chemosensitive signaling.

Despite these possibilities, the role of glia in chemosensitivity has received little attention. Glial cells are known to contain high levels of CA (299) and thus might play a role in the response to hypercapnia. Putative chemosensitive neurons in the ventral medulla of rats have been suggested to be located in association with a glial layer (257). Furthermore, the brain response to acute hypercapnia has been suggested to be dependent on the regulation of p*H*_o by glial cells (177), with neuronal p*H*_i being regulated only after p*H*_o is regulated by glial cells.

These findings are in agreement with the observed lack of p*H*_i recovery in medullary neurons when p*H*_o is reduced and with the presence of active neuronal p*H*_i regulation when p*H*_o is maintained constant (305). However, the best evidence for a role of glial cells in chemosensitivity is the finding of altered ventilation *in vivo* when medullary glial cells are impaired by fluorocitrate (110, 156). When fluorocitrate was injected into the RTN, ventilation was increased (110), as was the ventilatory sensitivity to CO₂ (156).

Very few studies have examined the effects of CO₂/H⁺ on glial cells. Neocortical and spinal glial cells were shown to depolarize in response to hypercapnia (202). The responses of medullary glia were more complicated. In the ventral medulla, >40% of the glial cells depolarized in response to acid stimulation, whereas one-third of dorsal glial cells hyperpolarized in response to acid stimulation (123). The acid-induced depolarization of the ventral glia could be prevented by the use of medium containing low Ca²⁺ and high Mg²⁺ levels, suggesting that the depolarization is caused by synaptic activity, possibly mediated by acetylcholine (123). Finally, medullary glial cells were shown to alkalinize upon depolarization, presumably because of depolarization-induced activation of electrogenic Na⁺-HCO₃⁻ cotransport (14), which was recently demonstrated in medullary glia (203). It thus appears likely that glial cells play a role in central chemosensitive signaling.

Synaptic activity/neuropeptides. Chemosensitive neurons from several regions, including the NTS (82), the raphe (295), and the LC (180), are intrinsically chemosensitive, responding to hypercapnia in the absence of chemical synaptic transmission. In addition, cultured chemosensitive neurons, which do not make synaptic contacts, also exhibit intrinsic chemosensitivity (119, 303, 382, 383). Although this would seem to argue against a role for chemical synaptic transmission in chemoreception, it is likely that synaptic transmission modulates the response of all chemosensitive neurons. Theoretically, certain neurotransmitter receptors can alter p*H*_i and p*H*_o, and their binding can be highly sensitive to pH, thus providing a basis for altered synaptic transmission in response to elevated CO₂/H⁺.

Early studies suggested that cholinergic synapses are involved in central chemoreception (239). Acetylcholine superfused across the surface of the VLM increased ventilation (96). This elevated ventilation was blocked by atropine, as was the slope of the ventilation-inspired CO₂ curve, suggesting that muscarinic receptors (subtypes M₁ and M₃ have been implicated) (50, 223) are involved in the central CO₂ response (95). A role for acetylcholine in central chemosensitivity, but not respiratory rhythm generation, was also shown with the isolated brain stem-spinal cord preparation (233) and with VLM neurons in brain stem slices (124). Finally, cholinergic systems are believed to play a role in changes of the control of ventilation during sleep vs. wakefulness (20), and a decrease in muscarinic receptors in the arcuate nucleus has been found in infants that have died from sudden infant death syndrome (186). These data argue that cholinergic synaptic activity is involved in central chemoreception, at least in the VLM.

A number of amino acid neurotransmitters have effects on ventilatory control (181). The excitatory neurotransmitter glutamate stimulates ventilation (207, 244) and glutamate antagonists decrease ventilation (244) when injected into the rostral VLM. Similar findings were made with glutamate injection

into the raphe (22). Furthermore, some CO_2/H^+ -sensitive neurons from the VLM contain glutamate (174). A potential role for glutamate in chemosensitivity is also suggested by the high sensitivity of *N*-methyl-D-aspartate (NMDA) receptors for changes of pH_o , making these receptors potential targets for chemosensitive signals. NMDA receptors are cation channels with permeability to both Na^+ and Ca^{2+} , and they have a molecular architecture that is similar to that of K^+ channels (55, 265). Relatedly, Low et al. (213) recently hypothesized that the basis for pH sensitivity of NMDA receptors is similar to that of K^+ channels.

NMDA receptors are inhibited by extracellular acidification with an IC_{50} value of 7.3 for cerebellar neurons (367, 368). Thus, at physiological values of pH_o , these NMDA receptors are partially inhibited by protons, suggesting that a small change in interstitial pH could alter their function (213). This pH sensitivity of NMDA receptors has been suggested to have a neuroprotective effect (65, 362). An increase in synaptic activity, as seen during seizures or ischemia, can decrease pH_o by 0.2–1.0 pH unit (324), which could limit the degree of neurotoxicity by inhibiting NMDA receptors. A similar process might function in chemosensitive neurons, where the degree of NMDA receptor expression could modulate the overall response of the neuron to a given acidic stimulus by altering the degree of membrane excitability. The pH sensitivity of NMDA receptors and its role in chemoreception is an area that deserves further study.

The neurotransmitters glycine and GABA can acidify rat ventral medullary neurons by activating HCO_3^- efflux (see *Bicarbonate* in SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS), and intracellular acidification can activate these neurons (215). Microdialysis of the GABA_A receptor agonist muscimol in the rostral VLM or the raphe significantly reduces the ventilatory response to hypercapnia (78, 229). In addition, in the posterior hypothalamus, GABAergic inhibition, but not glutamate activation, appears to be involved in modulating the respiratory response to hypercapnia (158, 269). Finally, GABA receptors are pH sensitive, with their response to GABA binding being either decreased (194, 387) or increased (194), depending on the receptor subunit composition. These findings suggest a role for these neurotransmitters in modulating chemosensitive signaling, but their precise role is currently unknown.

Monoamines have also been implicated in central chemoreception. Noradrenergic agents applied to the brain stem-spinal cord preparation with the pons transected exhibit an increase in respiratory frequency (169), and systemic acidification activated the catechol signal in the rostral VLM (291), suggesting that adrenergic VLM neurons may play a role in acid sensing. Furthermore, catecholamines can block Ca^{2+} action potentials in chemosensitive LC neurons by inducing hyperpolarization, and these effects appear to be mediated by α_1 -adrenoceptors (395, 396). Finally, in the medullary raphe, all acid-stimulated neurons are serotonergic, and most, but not all, serotonergic neurons are stimulated by acid (386).

Neuroactive peptides have also been shown to be involved in respiratory control. Local injections of substance P into the pre-Bötzinger complex resulted in increased respiratory frequency (136). When substance P was complexed with the cytotoxic agent saporin and bilaterally injected into the pre-Bötzinger region, there was near complete ablation of neurons expressing the neurokinin-1 receptor. Rats so treated exhibited

severe ataxia (irregular breathing with apneic periods) and altered respiratory control (135). These data suggest that a normal breathing pattern depends on substance P, or at least on neurons expressing the neurokinin-1 receptor.

The most suggestive evidence for a role of neuromodulators in central chemoreception is for a role of ATP and purinoceptors in the response of VLM neurons to hypercapnia (335, 336, 355). Bilateral injections of the P2 purinoceptor antagonist suramin into the VLM markedly reduced the phrenic nerve activity in response to increased inspired CO_2 (356). Because injection of ATP excited medullary respiratory neurons and this effect could be blocked by a P2X receptor antagonist, it has been proposed that ATP is involved in central chemoreception, with its effect most likely mediated by binding to P2X purinoceptors (357). The possible involvement of ATP in central chemoreception is also suggested by the identification of P2X receptors in chemosensitive petrosal neurons, afferent terminals of the carotid body (278), and respiratory neurons of the VLM, including the pre-Bötzinger complex (336, 355). Furthermore, ATP binding to P2X receptors is enhanced by acidification (185), as would occur during exposure to hypercapnia. A role for P2X receptors in central chemosensitivity would be strongly indicated by showing that hypercapnia induces increased extracellular ATP in chemosensitive regions *in vivo*.

Although there is considerable evidence that a variety of neurotransmitters can alter central chemoreception (49), the precise role of neurotransmitters in the cellular response to hypercapnia from different brain stem regions is lacking.

POSSIBLE TARGETS IN CENTRAL CHEMOSENSITIVE SIGNALING

The original model for central chemosensitivity proposed that hypercapnia-induced increased neuronal excitability is mediated by inhibition of a K^+ channel. pH-sensitive K^+ channels have been a particular focus of studies of chemosensitive signaling because there is considerable evidence for changes of pH acting as a primary signal in the chemotransduction pathway (see *Extracellular and intracellular pH* in SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS). There are at least two ways by which these channels can be pH sensitive. A change of pH, either intracellular or extracellular, could directly affect the channel by altering the charge on titratable groups, altering the channel conformation. Alternatively, a change of pH could alter some modulatory agent that affects channel activity (315). Regardless of the mechanistic basis, a number of different K^+ channels have been shown to be sensitive to changes of either pH_i or pH_o . These include members of the inwardly rectifying K^+ channel family (K_{ir}) (97, 318, 352, 371, 404, 405, 408), calcium-activated K^+ channels (K_{Ca}) (216, 317, 390), the voltage-sensitive K^+ channel family (K_v) (21), and TWIK-related K^+ channels (16, 99, 232). The pH sensitivity of these channels in neurons is of particular interest in terms of chemoreception, because modulation of any of these channels could alter neuronal excitability and therefore firing rate.

K_{ir} channels. K_{ir} channels stabilize the resting V_m and help to determine the interspike slope (ramp depolarization before an action potential), thus playing a major role in defining cell excitability in cardiac and neuronal cells (252). These channels

are made from subunits with two transmembrane segments and are unique in that they show inward or anomalous rectification (252, 315). K_{ir} channels are modulated by a number of cytosolic and membrane factors such as G proteins (79), second messengers, phosphatidylinositol 4,5-bisphosphate (PIP_2), ATP (162), V_m (167), and changes in $pH_{i/o}$, acting either directly (283, 405, 408, 413) or indirectly (276). The pH-sensitive K_{ir} channels include those from the K_{ir1} , K_{ir2} , K_{ir4} , and K_{ir5} families (Table 3). It is important to emphasize that most studies that have demonstrated pH sensitivity of K_{ir} channels have been done with channels expressed in *Xenopus* oocytes. Thus there is a need for more information on the electrophysiological properties of the native channels to verify whether properties of the expressed channels correlate with those found in their normal environment.

The site and mechanism of pH modulation of K_{ir} channels appears to differ among the different types of K_{ir} channel subfamilies, suggesting different sites of H^+ action or different pH sensors. $K_{ir1.1}$ channels are inhibited by intracellular acidification with a pK between 6.5 and 7.2 (Table 3) (60, 228, 315, 404). This inhibition is believed to involve a lysine on the NH_2 terminus near the first membrane-spanning domain (113). In $K_{ir1.1}$ channels, intracellular pH-inhibition appears to be mediated through suppression of the channel's open probability (P_o), with no change in the single-channel conductance (60, 113, 228, 370).

$K_{ir2.3}$ channels are inhibited by either pH_i or pH_o , with a pK of 6.8 and 6.8–7.2 (Table 3), respectively (72, 282, 283, 413). This implies that there are at least two pH-sensitive domains in $K_{ir2.3}$ channels. An extracellular pH-sensitive domain has

been demonstrated in these channels (72) that involves a histidine group on the extracellular loop after the first membrane-spanning domain. In addition, an intracellular pH-sensitive domain has been shown that involves a motif on the NH_2 terminus and is centered on a threonine (283, 413). The presence of two distinct pH sensors in $K_{ir2.3}$ channels is further supported by the fact that extracellular acidification reduces $K_{ir2.3}$ channel activity by reducing single-channel conductance (72), whereas a fall of pH_i reduces both the single-channel conductance and the P_o (413). Interestingly, the intracellular pH sensor site was also found to be the site for PKC phosphorylation, suggesting that PKC might play a role in shifting the pK for pH sensitivity in $K_{ir2.3}$ channels (413).

$K_{ir4.1}$ and $K_{ir4.2}$ channels are inhibited by intracellular acidification with a pK of ~6 (273, 404, 405, 408) and 7 (273) (Table 3), respectively. The pH sensitivity of $K_{ir4.1}$ appears to involve both a lysine in the NH_2 terminus and a glutamic acid residue near the second membrane-spanning domain (404, 405), whereas the greater pH sensitivity of $K_{ir4.2}$ involves an additional pH sensor in the COOH terminus (404). The major effect of pH_i on K_{ir4} channels is a reduction of P_o (405, 407), but in some cases a small increase in single-channel conduction with intracellular acidification is seen (407).

$K_{ir5.1}$ is somewhat different. It does not form homomeric channels, but it does have a pH-sensitive motif similar to that found in $K_{ir2.3}$ (404, 408), suggesting that $K_{ir5.1}$ is also pH sensitive. Although $K_{ir5.1}$ does not form a channel itself, it can readily form a heteromeric channel with $K_{ir4.1}$ (273, 352, 404, 408). Interestingly, the heteromeric $K_{ir4.1}$ - $K_{ir5.1}$ channel is

Table 3. Properties of various pH-sensitive ion channels

pH-Sensitive Channel	pH-Induced Response	~pK	Conductance, pS	Ref.
<i>Inwardly rectifying K^+ channels</i>				
$K_{ir1.1}$	Inhibited by ↓ pH_i	6.5–7.2	31–39	60, 228, 315, 370, 405
$K_{ir2.1}$	Inhibited by ↓ pH_i	5.0	23	72, 282, 283
$K_{ir2.3}$	Inhibited by ↓ pH_i	6.8	13–17	282, 283, 413
	Inhibited by ↓ pH_o	6.8–7.2		72, 413
$K_{ir4.1}$	Inhibited by ↓ pH_i	6.0–6.1	22–23	273, 315, 404, 405, 407, 408
$K_{ir4.2}$	Inhibited by ↓ pH_i	6.7–7.1	25	273
$K_{ir4.1-5.1}$ (hybrid)	Inhibited by ↓ pH_i	6.8–7.5	50–59	76, 273, 352, 404, 408
$K_{ir4.2-5.1}$ (hybrid)	Inhibited by ↓ pH_i	7.6	54	273
<i>Ca^{2+}-activated K^+ channels</i>				
K_{Ca}	Inhibited by ↓ pH_i	6.4–6.7	184–200	61, 196, 210
<i>Voltage-activated K^+ channels</i>				
$K_v1.2$	Inhibited by ↓ pH_o	4.9		160, 338
$K_v1.4$	Inhibited by ↓ pH_i	7.5		263
	Inhibited by ↓ pH_o	6.3		160
$K_v1.5$	Inhibited by ↓ pH_o	6.2–7.2		338, 365
$K_v2.1$	Inhibited by ↓ pH_o	<6.2		365
<i>TWIK-related K^+ channels</i>				
TASK-1	Inhibited by ↓ pH_o	7.3–7.4	40	99, 204, 236
TASK-2	Inhibited by ↓ pH_o	8.2–8.3	14	236, 254
TASK-3	Inhibited by ↓ pH_o	6.0–6.7	27–100	184, 284
TREK-1	Activated by ↓ pH_i	6.3		222
<i>Ca^{2+} channels</i>				
HVA (L-type)	Inhibited by ↓ pH_o	6.5		321
HVA (N-type)	Inhibited by ↓ pH_o	7.3		187
LVA	Inhibited by ↓ pH_o	6.9		321

HVA, high voltage activated; LVA, low voltage activated.

sensitive to pH changes in the physiological range, with a pK of 6.8–7.5 (76, 273, 352, 404, 408) (Table 3).

All of the above-described effects of pH on K_{ir} channels are believed to be due to direct effects of protons on the channel. An alternative basis for the pH-sensitive response of K_{ir} channels has been proposed. In LC neurons, the effect of intracellular acidification was proposed to be mediated through changes of the charged state of endogenous polyamines found in these neurons (276, 315). Polyamines are known to block outward K_{ir} currents in a voltage-dependent manner (315). During intracellular acidosis, polyamines become protonated, which increases their voltage-dependent blockade of K_{ir} channels (276).

K_{ir} channels have been associated with the chemosensitive response of certain neurons, such as the LC (276) and VLM (319). LC neurons exposed to 50–300 μ M $BaCl_2$ (K_{ir} channel inhibitor) showed a decrease in outward currents. VLM neurons similarly exhibited a reduced activity in response to hypercapnia upon exposure to $BaCl_2$ and to more specific inhibitors of K_{ir1} and $K_{ir4.1}$ (319). Although these data suggest that K_{ir1} and/or $K_{ir4.1}$ are involved in central chemosensitivity, the apparent pK values for these channels appear to be too low for them to play a role in chemosensitivity (Table 3). Two possibilities exist. The pK values may be shifted in the alkaline direction under the influence of intracellular modulators, such as PKC (413) or PIP_2 (408). The other possibility is that the major channel in chemosensitive responsiveness is a heteromeric $K_{ir4.1}$ - $K_{ir5.1}$, which has a pK in the physiological range (273, 352, 404, 408) (Table 3). This possibility is strengthened by the fact that $K_{ir4.1}$ is mainly expressed in the brain stem, including the LC (39). In *Xenopus* oocytes expressing $K_{ir4.1}$ or coexpressing $K_{ir4.1}$ with $K_{ir5.1}$, exposure to 5, 10, and 15% CO_2 (constant HCO_3^-) produced a concentration-dependent inhibition of whole cell K^+ currents (404). These effects were mediated by changes of pH_i . However, although heteromeric 4.1–5.1 channels are especially sensitive to inhibition by pH_i (Table 3) or hypercapnia (76) compared with other K_{ir} channels, including homomeric 4.1 channels (pK 6.03), and thus are especially attractive as a target of chemosensitive signaling, there is still no direct evidence for functional heteromeric channels in CNS neurons. Because a number of pH-sensitive K_{ir} channels are expressed in the brain stem (39), and because many of the nuclei in this region are chemosensitive, future electrophysiological studies on the role of pH sensitivity of native K_{ir} channels will help us to understand the role of these channels as targets of chemosensitive signals. In addition, studies on the distribution of the various K_{ir} isoforms and on the role of these channels in the CO_2 response of brain stem neurons are needed.

K_{Ca} channels. K_{Ca} channels play a fundamental role in excitable cells, contributing to both the repolarization and the afterhyperpolarization (AHP) of the action potential. The induction of an action potential results in a rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which activates K_{Ca} channels. These channels are made from subunits containing six transmembrane segments (BK channels have a 7th membrane-spanning domain), and they contribute to a biphasic AHP. The initial faster phase of the AHP is due to the activation of large BK channels (which are gated by both Ca^{2+} and V_m), whereas the slower phase is due to the activation of SK channels (which are gated solely by Ca^{2+}) (29). Because K_{Ca} channels form

part of the action potential repolarization and AHP, pH effects on these channels are important, since they undoubtedly modulate cell excitability (64, 65).

Intracellular acidification inhibits both the fast and slow phase of the AHP, suggesting that low pH_i inhibits both BK and SK channels (64). The effect of pH_i on BK channels has been well studied. Intracellular acidification from pH 7 to pH 6 decreases BK channel activity (200) with a pK (Table 3) of 6.4–6.7 (196, 210). Decreased pH_i inhibits BK channels by competing with Ca^{2+} for binding to the channel (61, 71, 196, 200), thereby decreasing P_o (41, 200, 201, 210). In addition, decreased pH_i lowers single-channel conductance of BK channels from cortical neurons (201) but not from glial cells (41). Furthermore, intracellular H^+ may also bind to a modulatory site on BK channels that alters channel affinity for Ca^{2+} (200). In contrast, virtually nothing is known about the pH sensitivity of SK channels, although it is likely that decreased pH_i also inhibits SK channels, because intracellular acidification reduces the slow phase of the AHP in hippocampal neurons (64). Given the likely role of SK channels in determining neuronal firing rate (270), studies on the effect of pH on SK channels would be most interesting.

Inhibition of K_{Ca} channels by intracellular acidification is probably involved in chemosensitive signaling. In peripheral chemoreceptors (type I cells of the carotid body), Peers and Green (272) showed that decreased pH_i selectively inhibits K_{Ca} channels. K_{Ca} channels are also likely to be involved in central chemosensitive responses. BK channels are expressed in dorsal vagal motor neurons (270), and subunit 3 of SK channels (SK3) is expressed in neurons from numerous chemosensitive regions, including the NTS, dorsal vagal motor nucleus, LC, raphe (341), rostral VLM, and pre-Bötzing complex (30). A direct role for K_{Ca} channels in central chemosensitivity was demonstrated in cultured neurons of the fetal rat medulla (390). Hypercapnia resulted in an increase in the interspike slope and an increase in firing rate in rat medullary cultured neurons. High CO_2/H^+ also reduced the amplitude of the AHP and prolonged the repolarization phase of the action potential waveform. Application of the K_{Ca} channel inhibitors tetraethylammonium (TEA) or Cd^{2+} resulted in the loss of the changes in the action potential waveform induced by HA (390). These findings suggest that K_{Ca} channels are inhibited during HA. Although this study suggests a contribution of K_{Ca} channels to central chemosensitivity, the results are weakened by two factors. First, they were obtained with organotypic cultured neurons, which may lack or have altered chemosensitive pathways. Second, TEA is not a specific inhibitor of the K_{Ca} channel. It would be of interest to repeat these studies on chemosensitive neurons under more nearly physiological conditions, using more specific blockers of K_{Ca} channels. In summary, it appears likely that K_{Ca} channels are involved in central chemosensitive signaling, but it will be of interest to determine what role is played by BK vs. SK channels and to determine how sensitive these channels are to decreased pH_i in vivo.

K_V channels. Another candidate target for acidification in chemosensitive neurons is the voltage-gated K^+ channel family (K_V). K_V channels, made from subunits containing six transmembrane segments, are regulated by membrane voltage changes. There are numerous subtypes of K_V channels, including K_V1 through K_V9 , but the two main functional types of K_V

channels in neurons are the fast-inactivating A-type K^+ currents and the delayed-rectifying K^+ currents (K_{DR}) (225). These K_V channels contribute to the repolarization phase of the action potential and thus help determine the action potential waveform and firing rate of neurons (225). The molecular subunits that make up A-type and K_{DR} channels in chemosensitive neurons are currently unknown. However, many of the K_V channel subunits, including 1.2, 1.4, 1.5, and 2.1, have been shown to be inhibited by acidification (66, 160, 209, 263, 338, 365).

Inhibition of the K_V channel subunits is mediated by a decrease in pH_o (although see Refs. 21 and 263, which describe a pH_i effect), but the sensitivity to changes of pH varies widely among subunits (Table 3). $K_{V1.2}$ and $K_{V2.1}$ are relatively insensitive to inhibition by decreased pH_o , with a pK of ~ 5 (160, 338) and <6.2 (365), respectively. In contrast, $K_{V1.4}$ and $K_{V1.5}$ are more highly sensitive to inhibition by decreased pH_o , with a pK of 6.3 (160) and 6.2–7.2 (338, 365), respectively. In most cases, the inhibition of K_V channels by decreased pH_o is mediated by enhanced channel inactivation. Interestingly, $K_{V1.4}$ appears to be very sensitive to inhibition by decreased pH_i , with a pK of 7.5 (263). A major problem with all of these studies is that they were done on K_V channels expressed in either *Xenopus laevis* oocytes or HEK-293 cells. In coronary and pulmonary vascular cells, Berger et al. (21) showed K_V channel inhibition by intracellular acidification. One interesting observation from this study was the effects of decreased pH_i on K_V channels differed in vascular smooth muscle cells from different regions, suggesting that additional intracellular modulatory factors play a critical role in defining the overall response of K_V channels from a given cell to a stimulus such as acidification. It is clear that studies of the types and pH sensitivity of K_V channels in neurons from chemosensitive regions are needed.

The role of K_V channels in central chemoreception has not been adequately addressed. That these channels show pH-sensitivity to both changes in pH_i and pH_o makes them possible targets under conditions of hypercapnia. Also, the K_V channel inhibitors TEA and 4-aminopyridine have been shown to reduce the effect of hypercapnia on chemosensitive neurons from the LC (117) and the NTS (83), suggesting the involvement of K_V channels in chemoreception. Furthermore, the β -subunit of K_V channels is highly expressed at all developmental stages in brain stem neurons (98). Finally, a recent mathematical model of chemosensitivity in the snail (see *Central chemosensitive neurons from the snail* in OTHER ACID-SENSITIVE CELLS) suggests that both A-type and K_{DR} channels are involved in the response to CO_2/H^+ of chemosensitive snail neurons (56). Together, these findings indicate the likelihood that K_V channels are involved in signaling in central chemosensitive neurons and suggest that detailed studies of the role of these channels in chemosensitive neurons will be fruitful.

TWIK-related K^+ channels. In recent years a new gene family of K^+ channels has been identified. These channels have four transmembrane segments and are referred to as “leak” or background channels (99, 118, 157, 205, 206, 222, 232, 327). These channels tune the action potential duration and modulate the responsiveness to synaptic inputs (266). Several members of this gene family are sensitive to changes of pH, including the TWIK-related K^+ channel (TREK-1) (232)

and the TWIK-related acid-sensitive K^+ channels (TASK-1, TASK-2, and TASK-3) (16, 236, 254).

TASK-1 and TASK-3 channels share many properties, including being expressed in the brain (especially TASK-3), are unaffected by changes of intracellular pH, and are inhibited by decreased pH_o (Table 3). A major difference between these channels is their sensitivity to inhibition by decreased pH_o . TASK-1 channels are especially sensitive to pH_o with a pK of 7.3–7.4 (99, 204), whereas TASK-3 channels are considerably less sensitive with a pK of 6.0–6.7 (183, 184, 284). TREK-1 channels are also highly expressed in the brain, but unlike TASK channels, TREK channels are modulated by changes of pH_i and are activated by neuronal acidification (157, 222, 232). The pK for activation by pH_i is ~ 6.3 (222), but it was recently shown that at the low values of PO_2 normally seen in the brain, acidification has no effect on TREK channels (232). Because intracellular alkalization still inhibits TREK channels in low O_2 , it has been proposed that these channels are involved in the response of neurons to respiratory and metabolic alkalosis (232). TREK channels thus are unlikely to be involved in the increased firing rate induced by HA in central chemosensitive neurons.

TASK channels have been proposed to be targets of chemosensitive signaling in brain stem respiratory neurons as well as in peripheral chemoreceptor cells (16, 47, 140, 145). In situ hybridization studies have revealed high levels of expression of TASK channels throughout the CNS, including respiratory and nonrespiratory areas in the brain stem and spinal cord (16). In the LC, both TASK-1 and, to a lesser extent, TASK-3 channels were found to be expressed (351). In addition, TASK-1 channels have been characterized in peripheral chemoreceptors such as carotid body type I cells and neuroepithelial bodies from the lung (47, 145). Because TASK channels are believed to contribute to resting V_m , inhibition of these channels by extracellular acidification, under conditions of HA, would shift the resting V_m closer to threshold without changing the action potential waveform per se. In this way, HA could increase firing rate in chemosensitive neurons expressing TASK channels. The facts that TASK channels are expressed in chemoreceptor cells, including the LC, and that these channels (especially TASK-1) can be inhibited by even a small extracellular acidification suggest that TASK channels could be involved in chemosensitive signaling.

Ca^{2+} channels. Because the response of many chemosensitive neurons to stimuli such as hypoxia and hypercapnia results in increased levels of Ca^{2+} , it has been hypothesized that Ca^{2+} channels might be modulated during an acid challenge as well (1, 116, 117). There are two types of high-voltage-activated (HVA) Ca^{2+} channel families, Ca_{v1} (L-type channels) and Ca_{v2} (P/Q-type, N-type, and R-type channels), and one low-voltage-activated (LVA) Ca^{2+} channel family, Ca_{v3} (T-type channels) (53). To date, only voltage-gated L-type Ca^{2+} channels have been implicated in chemoreception.

Ca^{2+} channels are modulated by a number of different factors (1, 53). Although Ca^{2+} channels are believed to be modulated by changes of pH, the pH sensitivity of these channels appears to differ in nonchemosensitive vs. chemosensitive cells. In general, Ca^{2+} channels in nonchemosensitive cells are inhibited by acidification (Table 3). For example, both HVA and LVA Ca^{2+} channels are inhibited by intracellular acidification in CA1 hippocampal neurons (361), whereas they

are inhibited by extracellular acidification in rat thalamic relay neurons (321). Intracellular acidification also inhibits Ca^{2+} channels in catfish horizontal cells (350), mammalian vascular smooth muscle cells (191), chick sensory neurons (187), and porcine tracheal smooth muscle cells (406). In contrast, HA appears to activate Ca^{2+} channels in chemosensitive cells such as carotid body cells (314, 316, 344–346) (see *Carotid body cells* in OTHER ACID-SENSITIVE CELLS), central chemosensitive neurons in the snail (108) (see *Central chemosensitive neurons from the snail* in OTHER ACID-SENSITIVE CELLS), and LC neurons from neonatal rats (117) (see *Calcium* in OTHER FACTORS IN CENTRAL CHEMOSENSITIVE SIGNALING). These findings suggest that acidic stimuli can activate Ca^{2+} channels.

This apparent paradox might be explained if the activation of Ca^{2+} channels in chemosensitive neurons is governed by a pathway not directly related to changes of pH but, rather, by other secondary messengers. Such a pathway has been proposed for carotid body cells (Fig. 2). Activation of Ca^{2+} channels in these cells by HA results from the activation of soluble adenylylase by elevated intracellular HCO_3^- and does not reflect a direct activation of Ca^{2+} channels by acidification (see *Carotid body cells* in OTHER ACID-SENSITIVE CELLS). It will be of interest to see whether the activation of Ca^{2+} channels in chemosensitive snail neurons and LC neurons by hypercapnia is similarly mediated by soluble adenylylase. Regardless, these data suggest that although Ca^{2+} channels are typically inhibited by acidification, modulatory pathways that are enhanced by acidic stimuli may overcome the pH-induced inhibition of these channels and activate them instead.

A REVISED MODEL FOR CENTRAL CHEMORECEPTION

On the basis of our review of studies of chemosensitive signaling and targets, we propose a revised model for central chemosensitive signaling. This model is essentially a cellular version of the multiple factors theory of Gray (133). We propose that there is not a single adequate stimulus for central chemosensitive neurons but that, rather, there are multiple stimuli and targets. Thus our model might also be referred to as the multiple signals/multiple targets theory. Based on our findings discussed above, such a model for chemosensitive signaling in LC neurons is shown in Fig. 5. In response to HA, multiple signals are generated, including a fall of pH_i , a fall of pH_o (116), and an increase in CO_2 . These signals, in turn, appear to activate multiple targets, including at least two possible K^+ channels as well as L-type Ca^{2+} channels (117). We propose that it is the sum of these effects that leads to the final observed increase in neuronal firing rate. Thus the activation of LC neurons by multiple pathways appears to be additive.

There are some features of this model that should be addressed. First, increasing the magnitude of one pathway of stimulation might be sufficient to overcome inhibition of another pathway. For instance, during IH, if the hypercapnic stimulus is increased to high levels of CO_2 (>20% CO_2), then firing rate may still be observed with IH in the presence of nifedipine. Although such an experiment has not been performed, it could further strengthen the model prediction that the effects of the various stimulation pathways are additive. Second, the specific signals or targets may differ in neurons

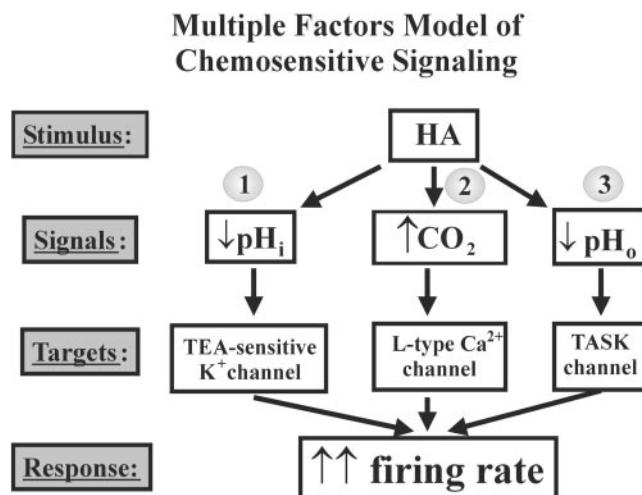


Fig. 5. Multiple factors model of chemosensitive signaling based on findings in LC neurons. In the multiple factors model, an acid stimulus such as hypercapnic acidosis (HA), results in multiple signals, including decreased pH_i , decreased pH_o , and increased CO_2 level. These signals are proposed to affect multiple ion channel targets, including various K^+ channels [TASK and tetraethylammonium (TEA)-sensitive K^+ channels] as well as Ca^{2+} channels. It is the overall effect of these multiple signals affecting multiple channels that determines the magnitude of the final response of the neuron, i.e., increased firing rate.

from different chemosensitive regions. For example, if neurons from another chemosensitive region lack certain pH-sensitive ion channels (e.g., TASK channels) or certain soluble activators of Ca^{2+} channels, those neuron would have to rely on other parts of the multifactor model for activation. In this regard, motor neurons and LC neurons show higher expression of pH-sensitive TASK-1 channels compared with non-motor neurons from other chemosensitive areas (351). Third, other pathways not observed in LC neurons may play a major role in the chemosensitive response of neurons from other regions. For example, a unique acid-sensitive cation channel has been proposed to be involved in chemosensitive signaling in raphe neurons (298, 358), and this channel would then have to be included in a model of chemosensitivity in raphe neurons. Finally, such a multifactor model would be ideal for mathematical modeling. A mathematical model of mammalian central chemoreception has not been produced, but a model is being developed for chemosensitive neurons from the snail (56). This model suggests the involvement of three different K^+ channels in the central chemosensory response of snail neurons (see *Central chemosensitive neurons from the snail* in OTHER ACID-SENSITIVE NEURONS).

The only signal that could serve as an adequate stimulus is the change of pH_i . Our multiple factors model would be greatly strengthened by showing that chemosensitive signals can result in increased chemoreceptor firing without a change of pH_i as long as other signals are sufficiently strong. Experimental approaches to achieving this objective were discussed above (see *Extracellular and intracellular pH* in POTENTIAL SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS). If such experiments ultimately show that chemoreceptors can be stimulated without a decrease of pH_i , this would argue strongly that changes of pH_i by themselves are neither necessary nor sufficient to result in a chemosensitive response.

Finally, the high degree of sensitivity of chemosensitive neurons to even small changes of pH is difficult to explain at the cellular level (see Table 1). It was previously proposed that the steep pH sensitivity of ventilatory control could arise at the cellular level from the sum of a number of conductance changes, each of which has a modest pH dependence (294), i.e., that there are multiple targets of chemosensitive signaling. Thus the multiple factors model of chemosensitivity could potentially also help explain the high degree of pH sensitivity of respiratory control.

OTHER ACID-SENSITIVE CELLS

There are many other types of CO_2/H^+ -sensitive cells besides central chemosensitive neurons. These can be subdivided into two categories: 1) other types of CO_2 -sensitive cells involved in respiratory control and 2) acid-sensitive cells that are unrelated to respiratory control. We briefly review studies of acid signaling in three types of cells from the first category: glomus cells from the carotid body (that serve as peripheral chemosensors), central chemosensitive neurons from a nonvertebrate model system, and intrapulmonary chemoreceptors from birds. We also review studies of acid signaling in two types of cells from the second category: acid-sensing taste receptor cells on the tongue and acid-sensitive sensory elements responsible for pain sensation (nociceptors).

Carotid body cells. There are several recent reviews of various aspects of the function of, and signaling in, carotid body cells (147, 163, 212, 241). The carotid body, the primary site of peripheral chemoreception, has two types of cells: catecholamine-containing type I cells (glomus cells) and type II cells (sustentacular cells), which are like glial cells. Numerous studies have shown that it is the type I glomus cells that are sensitive to both hypercapnia and hypoxia (115). Because these cells are easily identified and isolated, studies of the cellular mechanisms of CO_2/H^+ chemoreception in glomus cells preceded similar studies in central chemoreceptive neurons. We briefly outline the progress that has been made in studying cellular chemoreceptive signaling in glomus cells. It is noteworthy how similar the findings in glomus cells are to those reviewed above in central chemoreceptors (see SIGNALS IN CENTRAL CHEMOSENSITIVE NEURONS and OTHER FACTORS IN CENTRAL CHEMOSENSITIVE SIGNALING).

Because carotid bodies respond to both hypercapnia and hypoxia, changes of pH have been suggested to be important for chemosensitive signaling (377). Hanson et al. (143) presented strong evidence that it is the change of pH_i and not pH_o that is important for glomus cell response to hypercapnia. They showed that hypercapnia-induced increased firing of the carotid sinus nerve (CSN), the nerve that carries the carotid output to the CNS, is slowed when a membrane-permeant inhibitor of CA is used but not when a membrane-impermeant form is used. The regulation of pH_i and its response to hypercapnia in glomus cells were studied in detail by Buckler et al. (45, 46). Unlike central chemoreceptive neurons (304, 305), glomus cells were shown to contain numerous pH-regulating transporters, including NHE, Na^+ -driven $\text{Cl}^-/\text{HCO}_3^-$ exchange (NDCBE), and Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange (AE) (45, 46), as well as HCO_3^- channels (337). Despite this large complement of pH-regulating transporters, the pH_i response to chemosensitive stimuli in glomus cells was

similar to that of central chemosensitive neurons (116, 305). Thus HA resulted in a sustained fall of pH_i with no evident pH recovery, resulting in a strong relationship between pH_i and pH_o ; IH resulted in a rapid initial fall of glomus cell pH_i followed by pH recovery; and IA resulted in a sustained fall of pH_i that was slower than seen with HA (45, 46). Furthermore, as with central chemosensitive neurons (116), these changes of glomus cell pH_i correlated well with changes in CSN firing rate induced by HA, IH, and IA (45, 46, 198). These findings, coupled with the observations that CA is only localized to the glomus cells (256) and that inhibition of CA reduced the response of glomus cells to hypercapnia (164), strongly argue that decreased pH_i is an important cellular signal for the response of glomus cells to hypercapnia. This strong case for a role of pH_i as a signal and the likely similarity between the signaling mechanisms in peripheral and central chemoreceptors were pointed out by Nattie (243). Interestingly, inhibition of CA did not inhibit the response of glomus cells to hypoxia, suggesting that changes of pH_i are not directly involved in the glomus cell response to hypoxia (163).

$[\text{Ca}^{2+}]_i$ has also been implicated in chemosensitive signaling in glomus cells. Buckler and Vaughan-Jones (43, 44) showed that $[\text{Ca}^{2+}]_i$ changes in a fashion similar to the changes of pH_i . Thus, in response to HA, there is a maintained increase of $[\text{Ca}^{2+}]_i$, whereas in response to IH, there is a transient $[\text{Ca}^{2+}]_i$ increase. With IA, there is a slower but maintained increase of $[\text{Ca}^{2+}]_i$. These findings led Buckler and Vaughan-Jones (43, 44) to propose a model for chemosensitive signaling in glomus cells. In this model, an acid stimulus is proposed to cause a decrease in pH_i , which inhibits a K^+ channel, resulting in glomus cell depolarization. This depolarization is proposed to activate voltage-gated L-type Ca^{2+} channels and lead to elevated $[\text{Ca}^{2+}]_i$. The elevated $[\text{Ca}^{2+}]_i$ then results in increased neurosecretion from glomus cells and increased firing rate of CSN. Thus, in this model, the changes of pH_i and $[\text{Ca}^{2+}]_i$ occur in series.

The K^+ channel target of the decreased pH_i in this model was initially suggested to be the K_{Ca} channel, which was shown to be inhibited by a fall of pH_i (272). However, K_{Ca} channels were only inhibited by ~20–30% with a substantial fall of pH_i (272). Furthermore, an inhibitor of K_{Ca} channels does not alter the response of glomus cells to hypercapnia (259). This suggests that K_{Ca} channels are not an important target of chemosensitive signaling in glomus cells. Recent studies have demonstrated the presence of TASK-1 channels in glomus cells that are inhibited by a fall of pH_o (47), leading to the suggestion that changes in “. . . both internal and external pH will play a role in mediating the response to acid stimuli.”

The model also predicts that hypercapnia will ultimately increase L-type Ca^{2+} channel activity. However, Peers and Green (272) showed that these channels were not activated by intracellular acidification, suggesting that, in contrast to the prediction of the model (43, 44), acidification does not appear to result in sufficient membrane depolarization to activate these channels. Nevertheless, Ca^{2+} channel blockers completely eliminated the carotid body response to hypercapnia (and hypoxia) (314), showing the importance of Ca^{2+} channel activation in glomus cells. Note this differs from the response seen in central neurons, where Ca^{2+} channel blockers only partially inhibit the response of LC neurons to hypercapnia (117).

The pathway by which L-type Ca^{2+} channels are activated in glomus cells was recently studied (345, 346). Hypoxia was shown to activate a number of intracellular signaling pathways in glomus cells, including stimulation of phospholipase C and increased cAMP production (345), prompting the suggestion that hypoxia activates L-type Ca^{2+} channels through a PKC-dependent mechanism. Interestingly, hypercapnia was shown to activate L-type Ca^{2+} channels, but intra- or extracellular acidification inhibited these channels (346). Furthermore, hypercapnia, but not acidification, elevated cAMP in glomus cells. Summers et al. (346) proposed that it is molecular CO_2 that activates L-type Ca^{2+} channels in glomus cells, through a cAMP and protein kinase A (PKA) pathway (see Fig. 2). The pathway by which molecular CO_2 could increase cAMP is unknown, but it was suggested that it might be through intracellular HCO_3^- activation of soluble adenylylase (414). Regardless, these findings would support a multiple factors theory for chemosensitive signaling in glomus cells as well, with hypercapnia signaling cell responses through changes of both pH (pH_o and pH_i) and CO_2 .

If L-type Ca^{2+} channels are activated by hypoxia through a PKC-dependent mechanism, but by hypercapnia through a PKA-dependent mechanism, it might be expected that the two stimuli are additive. Indeed, both Ca^{2+} currents (346) and $[\text{Ca}^{2+}]_i$ (80) were increased significantly more by the combination of hypoxia and hypercapnia than by either one alone, further suggesting that each stimulus works through a different signaling pathway.

In addition to various K^+ and L-type Ca^{2+} channels, Cl^- channels have been implicated in chemosensitive signaling in glomus cells. With the use of the Cl^- channel and/or $\text{Cl}^-/\text{HCO}_3^-$ exchange inhibitors anthracene-9-carboxylic acid or DIDS, the initial fast, transient responses of CSN to hypercapnia were eliminated, suggesting a role for the channel or exchanger in the chemosensitive response (165). More direct evidence comes from patch-clamp studies of glomus cells, in which an acid-activated Cl^- current that is inwardly rectified was demonstrated (274). Extracellular acidosis (pH dropped from 7.8 to 7.0) increased the channel current by 35%, and this activation was proposed to contribute to intracellular acidification and depolarization, most likely by promoting HCO_3^- efflux through the channel (274). The role of Cl^- channels in chemoreception in glomus cells warrants further study.

Three other aspects of chemoreception in glomus cells are reminiscent of findings in central chemosensitive neurons. First, glomus cells are highly sensitive to hypercapnic stimuli, increasing CSN firing rates from 70–360% for a 0.1 pH unit decrease of pH_o (compare with values in Table 1) (198, 259, 314). Second, glomus cells are extensively gap junction coupled, not just with each other but with type II cells and with the CSN as well (111). Furthermore, hypercapnia results in uncoupling, which is speculated to increase the neurotransmitter release from glomus cells (112). This latter point is at odds with findings in some central chemosensitive neurons that maintain coupling even at fairly high levels (~30%) of CO_2 (85). Regardless, there appears to be a role for gap junction coupling in chemosensitive signaling in both central and peripheral chemosensitive cells. Third, there is evidence in glomus cells that adenosine and, in particular, P2X purinoceptors play a role in chemosensitive signaling (195, 278). Adenosine has been shown to enhance the response to hypercapnia of

glomus cells from neonatal rats, in part by increasing intracellular cAMP (195). P2X purinoceptors have been shown to be expressed in CSN nerve terminals that are apposed to glomus cells, and it is believed that they are involved in the chemosensitive response of the carotid body (278). Thus it appears that adenosine and P2X purinoceptors play a role in both peripheral and central chemoreception, although the precise nature of that role is currently unclear.

Finally, though a mathematical model of carotid body response has been generated (372), it treats the cellular transduction mechanisms of chemoreception largely as a black box, focusing instead on a quantitative descriptor of the overall output of the carotid body. Just as with central chemosensitive neurons, it would seem that a mathematical model of the cellular mechanisms involved in chemosensitivity in glomus cells would be timely.

In summary, it appears that there are many similarities between the cellular signaling mechanisms involved in central chemosensitive neurons and glomus cells, although differences certainly exist. It also seems that the multiple factors theory is as appropriate for peripheral chemoreception as it appears to be for central chemoreception.

Central chemosensitive neurons from the snail. The best studied nonmammalian central chemosensitive neurons are those from the terrestrial pulmonate snails, *Helix pomatia* and *Helix aspersa*. Chemoreception in the snail was compared with chemoreception in air-breathing vertebrates by Erlichman and Leiter (108). A limited number of intrinsically CO_2 -stimulated neurons were identified in the subesophageal ganglia of these snails. These neurons were similarly chemosensitive as mammalian neurons, increasing their firing rate 43% for a 0.1 unit decrease in pH_o (compare to values in Table 1) (109). Stimulation of these neurons led to increased open area and frequency of the pneumostome, which allows increased ventilation of the mantle (109). In experiments in which NH_4Cl prepulses were used to alter pH_i at constant pH_o , pneumostomal opening followed pH_i , being greater at lower pH_i and smaller at higher pH_i (107). These findings suggest that, as in mammalian chemosensitive neurons, changes of pH_i are important for chemosensitive signaling in snail neurons as well. The pH_i responses to various chemosensitive stimuli were studied extensively in snail chemosensitive neurons (109, 131) and compared with the findings in mammalian neurons (304, 305). The snail responses were found to be very similar, with HA resulting in a maintained acidification in snail chemosensitive neurons, whereas IH resulted in acidification followed by pH_i recovery (109, 131). As with mammalian chemosensitive neurons, snail neurons exhibited pH_i recovery when pH_o was held constant, and this pH recovery was largely mediated by NHE, although some neurons also seem to contain NDCBE (275). Interestingly, Ca^{2+} channel blockers, like nifedipine, seem to reduce pH_i recovery from acidification in snail neurons, suggesting a possible role for Ca^{2+} in chemosensitivity in snail neurons (275). As with mammalian neurons, snail neurons from nonchemosensitive regions exhibited pH_i recovery in response to HA (109, 131). Snail chemosensitive neurons could also be stimulated by changing pH_o (from 7.8 to 7.3), although the change in pH_i under these conditions is not known (94). Finally, as with mammalian neurons, modification of histidine groups blocked the chemosensitive response of snail neurons, suggesting the importance of the state of protonation

of histidine groups to chemosensitivity (214). These data suggest that both pH_i and pH_o may serve as chemosensitive signals in snail neurons as they are proposed to do in mammalian chemosensitive neurons.

A major difference between the response of snail and mammalian chemosensitive neurons to acid stimuli is that the action potential in snails is a Ca^{2+} spike, whereas it is a Na^+ spike in mammalian neurons (108, 109). However, this difference may not be so major, because it was recently shown that acidic stimuli activate Ca^{2+} channels in mammalian chemosensitive cells (117, 346). Thus Ca^{2+} channels appear to be activated in both snail and mammalian chemosensitive neurons by acidic stimuli.

Multiple targets for these signals have been proposed in chemosensitive snail neurons. With the use of voltage-clamp techniques, extracellular acidification has been shown to block currents attributed to an A-type K^+ channel and the K_{DR} channel (93, 94). Recently, a mathematical model of chemosensitive signaling in snail neurons was described (56). This model involved three different outward K^+ channels (A-type, K_{DR} , and K_{Ca}) and two inward currents (a Na^+ current and a Ca^{2+} current). The model predicted that alterations of all three K^+ channels by acidic stimuli would contribute to the chemosensitive response of these neurons. Thus it seems that the cellular mechanisms of chemosensitive signaling in snail neurons are substantially similar to those in mammalian neurons and that a multiple factors theory may be just as appropriate for snail neurons as it is for mammalian chemosensitive neurons.

Intrapulmonary chemoreceptors. Intrapulmonary chemoreceptors (IPC) are found in the lungs of birds and reptiles and are believed to give feedback control to ventilation and to match ventilation with metabolism (323). Airway chemotransducers in mammals, called neuroepithelial bodies, which are believed to be analogous to IPC, were recently reviewed by Kemp et al. (182). The best studied of the IPC are those from the avian lung. Avian IPC are vagal afferents that innervate the lung and are CO_2 sensitive (151, 152). In fact, IPC are highly CO_2 sensitive, increasing their firing rate $\sim 160\%$ for a 0.1 pH unit increase in plasma pH (compare with values in Table 1) (18). The actual cellular component that is CO_2 sensitive is unknown, and there is evidence that the afferent fibers innervate a separate chemosensor (102, 103). Regardless, IPC responses to CO_2 are of interest because they are backwards compared with the responses of many other chemoreceptors, although not all (298); hypercapnia decreases IPC firing rate and hypocapnia increases it (18). IPC responses to CO_2 are also marked by partial spike frequency adaptation with prolonged exposure (102) and near complete inhibition of the CO_2 response by CA inhibitors (152).

Given the unusual responses of IPC to CO_2 , the cellular signaling pathways that mediate these responses should be quite interesting. However, studies of these mechanisms have been hindered by the inability to unequivocally identify the specific cells that are responding to CO_2 . Nevertheless, progress has been made in understanding the cellular signaling pathways. Because the CO_2 response of IPC is inhibited by a membrane-permeable inhibitor of CA (acetazolamide) but not by a membrane-impermeable one (benzolamide), it was suggested that it is pH_i , and not molecular CO_2 , that is the proximal cellular chemosensitive signal (152). This conclusion is supported by the reduced CO_2 sensitivity of IPC in birds

exposed to chronic hypercapnia (18). The acclimatization to chronic elevated CO_2 levels resulted in reduced ventilation, an increased IPC discharge rate for a given CO_2 level, and a metabolic compensation that resulted in HCO_3^- retention and blood alkalization. Thus, at a given CO_2 level, IPC are assumed to have an alkaline pH_i and, therefore, an increased firing rate (18). The importance of pH_i for chemosensitive signaling in IPC is also shown by the response of IPC to inhibitors of pH regulating transporters. The $\text{Cl}^-/\text{HCO}_3^-$ exchange inhibitor DIDS, which would be expected to alkalize IPC, increased the discharge rate of IPC at a given CO_2 level (323), whereas the Na^+/H^+ exchange inhibitor dimethyl amiloride, which would be expected to acidify IPC, decreased the discharge rate of IPC at a given CO_2 level (149). These findings have been combined into a model of signaling in IPC that emphasizes the relationship between pH_i and IPC firing rate, where pH_i is modeled as a kinetic balance among the hydration/dehydration of CO_2 (mediated by CA), acid extrusion by pH-regulating membrane transporters, and intracellular buffering (150). This model was able to successfully replicate the actual IPC discharge rate seen in response to various challenges.

The cellular targets of chemosensitive signaling in IPC are unknown, but it is presumed that these targets are also unusual. Very little is known about the ion channels in IPC, but to explain the backwards relationship between pH_i and IPC firing rate, it has been hypothesized that IPC contain K^+ channels that are opened by decreased pH_i and/or Ca^{2+} or Na^+ channels that are inhibited by decreased pH_i (149, 150). The development of an experimental cellular model of IPC would be most helpful in further characterizing the signaling in these unusual CO_2/H^+ -responsive cells and would allow direct measurements of pH_i and the channels involved in chemosensitive signaling.

IPC do not appear to be present in mammals, but mammalian airway epithelia do contain neuroepithelial bodies (NEB). NEB are most prevalent in neonates and are believed to serve both a neuroendocrine function, promoting lung development, and a chemosensory function (375). NEB are highly sensitive to hypoxia and are therefore oxygen sensors (182). The CO_2 sensitivity of NEB has not been determined but would be an interesting future study.

Acid-sensitive taste receptor cells. Sour taste is one of the four main taste modalities, and it arises from acidic stimuli (recently reviewed in Refs. 325, 339). Taste arises from taste receptor cells (TRC), which are elongated neuroepithelial cells that join together into multicellular aggregates called taste buds (325, 339). TRCs are polarized, with their apical membranes forming microvilli that face the oral cavity, and are exposed directly to substances taken orally. TRCs are joined by a tight junction that has selective permeability, and on their basolateral side, they make contact with an axon from a taste nerve. In the case of the anterior two-thirds of the tongue, TRCs connect to a branch of cranial nerve VII, the chorda tympani (CT) (325). Thus, somewhat analogously to carotid body cells, TRCs respond to the primary stimulus and generate action potentials in a secondary neuron, whose activity can be recorded.

Because sour TRCs respond to acid, the same question has arisen in studies of the cellular responses to taste as with central chemoreception: Is it pH_o or pH_i that is being sensed?

There is a poor correlation between the perception of a sour taste (or CT response) and the pH of the solution being tested (17, 217). This suggests that pH_i is the critical signal for sour taste. With the advent of fluorescence techniques to measure pH_i in TRCs in isolated taste bud fragments using BCECF (217, 220), this hypothesis was tested directly. The pH_i of TRCs was found to track pH_o to a high degree. When pH_o was acidified, pH_i acidified without pH recovery (220). Furthermore, exposure to HA solutions led to a maintained acidification of TRCs, but exposure to IH solutions resulted in a transient acidification with rapid pH_i recovery (220). These findings are very reminiscent of those made in central chemosensitive neurons (e.g., Ref. 305). The IH findings may be of significance to taste sensation in that they suggest that HCO_3^- , secreted by the salivary gland, may lead to normalization of TRC pH_i and suppression of sour taste.

A role for changes of pH_i in sour taste transduction was supported by the correlation between the response of pH_i in TRCs and the increased activity in the CT (217, 340). Using a variety of stimuli (weak acids and strong acids), Lyall et al. (217) showed that the CT response correlated with the change of pH_i . They showed that the time course of the pH_i change was similar to the time course of the response of CT, further supporting a role of pH_i in sour taste transduction. In fact, Lyall et al. (217) concluded that changes of pH_i are the proximate stimulus for sour taste transduction.

The evidence for a major role of pH_i in sour taste is very strong. There is, however, considerable evidence that this response can be modulated. The effects of weak acids on TRC pH_i are not voltage sensitive, nor are they affected by the Na^+ channel and NHE inhibitor amiloride, suggesting that weak acids affect pH_i by direct influx of the uncharged protonated form of the weak acid (340). However, the weak acid CO_2 affects pH_i in a voltage-sensitive fashion (217). This has led to the suggestion that TRCs contain a HCO_3^- conductance that alters their pH_i response to CO_2 (217). Finally, the strong mineral acids (like HCl) also acidify TRCs, but the pathway of H^+ entry is unknown (217). H^+ entry can be increased by elevated cAMP in TRCs (218). Normally, TRCs respond to HCl only when pH_o is below 4, but it is proposed that cAMP opens an apical entry pathway (possibly an H^+ channel) and thereby increases the effect of HCl on pH_i . This effect is further strengthened by the ability of cAMP to inhibit a pH recovery mechanism, probably isoform 3 of NHE, i.e., NHE3 (218). Thus, upon exposure to HCl, if cAMP is elevated in TRCs, then they will acidify more and be less likely to undergo pH_i recovery, both of which contribute to an increased CT activity in response to the acid stimulus.

TRC responses to acid stimuli can also be modulated in the other direction. Elevating intracellular Ca^{2+} in TRCs by exposing them to the calcium ionophore ionomycin results in stimulated pH_i recovery in response to an acid stimulus (218). The pH-regulating transporter that is stimulated by Ca^{2+} may be NHE1 (218). Associated with this pH recovery is an adaptation of the increased CT activity (218). Thus the stimulation of pH_i recovery by increased $[Ca^{2+}]_i$ is directly linked to an adaptation of the sour taste sensation, and this may be a major modulatory pathway for sour taste (218). It will be of interest to see whether cAMP and elevated $[Ca^{2+}]_i$ likewise modulate the responses of central chemosensitive neurons.

The marked correlation between the effects of cAMP and intracellular Ca^{2+} in modulating pH_i in TRCs and in modulating CT activity represents additional strong evidence that a change of pH_i is the proximate signal for sour taste in TRCs. However, just as with central chemosensitive neurons, no pH_i clamp experiment has been performed to determine whether elevated $[Ca^{2+}]_i$, acidic pH_o , or other stimulus, in the absence of a change of pH_i , can increase CT activity or whether a change of pH_i is a truly necessary and adequate stimulus.

Finally, salt taste is another strong taste modality. The sensation of salt involves Na^+ entry into TRCs across an apical epithelial Na^+ channel (ENaC) (219, 325, 339). Because this channel, under certain conditions, can allow H^+ permeation, the suggestion was made that there may be similarities between the cellular mechanisms of Na^+ and acid taste sensation (130). There is ample evidence, however, that the pathways for acid and salt sensing are distinct pathways. CT responses to NaCl are voltage sensitive and can be inhibited by the amiloride analog benzamil (which is highly specific for ENaC), whereas, as stated above, the CT response to acid is voltage insensitive and unaffected by amiloride and benzamil (340). It has recently been shown that, although distinct, the responses to salt and acid do interact. In the presence of an acid stimulus, the decreased pH_i inhibits ENaC and reduces the response to NaCl (219). Thus TRC acidification appears to be a negative modulator of salt sensation. This is likely to be just one example of the ways in which the responses to the various taste stimuli interact.

In summary, studies of the acid signaling mechanism in TRCs show many similarities to other CO_2/H^+ -sensitive cells, including a major role for changes of pH_i , a lack of pH_i recovery during the acidic stimulus, a role for changes of $[Ca^{2+}]_i$, and a dependence on ion channels as targets of the pH changes. Of all acid-sensitive cells, the case for changes of pH_i as the proximate stimulus is probably the strongest for TRCs, because even very large changes of pH_o do not stimulate acid taste and modulation of pH_i changes both upward and downward result in comparable changes in CT activity. It remains to be determined exactly what ion channels are affected by changes of pH, but as with central chemosensitive neurons, there are many candidate channels and it is likely that more than one will ultimately be shown to be involved in the taste response to acids.

Pain sensation by nociceptors. An extracellular acidosis is associated with a number of pathological conditions, including ischemia, inflammation, and malignant tumors (379, 380, 388, 409). These conditions are also associated with the sensation of pain from peripheral tissues. On the basis of these findings, combined with the observation that injection of acid into peripheral sites can cause the sensation of pain (e.g., Ref. 155, 388), it has been proposed that extracellular acidification is a primary signal for pain sensation in peripheral nociceptors.

The effects of acidification in mediating pain are believed to involve ion channels, and the role of ion channels in nociception was recently reviewed (104, 226). The acid-sensitive ion channel (ASIC) has been implicated in this nociceptive response. ASIC channels, which belong to the amiloride-sensitive Na^+ channel/degenerin family, are transiently activated by rapid extracellular acidosis, are permeable to Na^+ , Ca^{2+} , and K^+ , and are located in sensory neurons (for reviews of ASIC, see Refs. 25, 379, 391). It has been proposed that these

channels mediate the pain sensation associated with extracellular acidification (379, 380, 409). The details of this mechanism are not fully understood but, because of an interest in the ability to modulate pain perception, are currently being intensively studied. The perception of pain can be suppressed by the Ca^{2+} channel blocker diltiazem (388) and, paradoxically, by hypercapnia (139). However, these effects are more centrally mediated, involving suppression of neuronal activity in a fashion similar to local anesthetics or by the release of central opioids, respectively. Pain perception is increased by increased levels of cAMP, but it is not clear that this involves ASIC at all (155).

In addition to their role in nociception, ASIC have been found to be widely distributed throughout the brain (379, 380, 392), although their role in brain function is not fully known. Synaptic activity results in altered pH_o (59), and as pointed out by Waldmann et al. (380), “. . . pH fluctuations are not just a by-product of neuronal activity, but rather are a way of communicating within the central nervous system.” It has thus been proposed that in the brain, ASIC channels serve a neuromodulatory role (380). This proposal is strengthened by the observation that ASIC are localized to areas of the brain with a high density of synapses (e.g., amygdala, cerebral cortex, olfactory bulb, and nucleus accumbens) (392). In fact, ASIC are suggested to play a role in neuromodulation associated with fear conditioning (392). Thus it appears that acid-sensing neurons play a role in both nociception and central neuromodulation.

SUMMARY AND CONCLUSION

Considerable progress has been made, especially over the past decade, in understanding the cellular basis for central chemosensitivity. What has become clear is that changes of pH_i play a major role in neuronal responses to increased CO_2/H^+ levels, not just in central chemosensitive neurons but in many acid-sensitive cells. It is also clear that changes of pH_o and often $[\text{Ca}^{2+}]_i$, as well as other possible agents, are likely involved in cellular signaling in acid-sensitive cells. In addition, there appear to be not a single chemosensitive ion channel but, rather, numerous ion channels (including K^+ , Ca^{2+} , Na^+ , and perhaps Cl^- and HCO_3^- channels) seem to be involved in the chemosensitive response to acidic stimuli. These findings led us to propose a new multiple factors theory of central chemosensitive signaling, stressing that the neuronal response to CO_2/H^+ involves multiple cellular signals and that multiple ion channels are the targets of these various signals.

While much has been accomplished, there is much to be done, including many new avenues of research in central chemosensitivity. A major test of the multiple factors theory will be the determination of central chemosensitive neuron responses to acidic stimuli under intracellular pH clamp conditions. If a change of pH_i is not an adequate and necessary signal for the response of chemosensitive neurons, then, in response to acidic stimuli, the combination of decreased pH_o and increased $[\text{Ca}^{2+}]_i$, in the absence of a change of pH_i , should be sufficient to elicit an increased firing rate.

Apart from this critical test, many issues remain. Given that neurons from many different brain stem regions have been identified as putative central chemoreceptors, a comparison of the cellular responses to acidic stimuli in neurons from these different regions is likely to give insight into the role that the

neurons from each area play in respiratory control. Furthermore, the development of chemosensitivity has been studied in neurons from only two areas, the LC (342) and the medullary raphe (385), and a different pattern was seen in the two areas. Stunden et al. (342) found that the firing rate response to hypercapnia of LC neurons was the same in slices from rats ages P1–P16, whereas Wang and Richerson (385) found an increase in both the number of chemosensitive neurons and the response of individual neurons to hypercapnia in cultured raphe neurons between 3 and 24 days in culture. It is expected that these developmental studies will be extended to neurons from other chemosensitive regions such that the cellular development of chemosensitivity can be compared with the organismic development of the chemosensitive response of ventilation (342).

Much of what we know about chemosensitive signaling was done using reduced preparations and often using tissue from neonates. It remains to be shown that findings based on studies using the developing CNS are equally applicable to the functioning of the adult CNS.

Recent studies have also suggested new aspects of central chemosensitive neuron signaling not previously suspected. For instance, there seems to be a role for gap junction coupling in the response of central chemosensitive neurons to hypercapnia, but the nature of this role and its cellular basis is unknown. Furthermore, recent evidence suggests that glial cells may play a role in central chemosensitivity, but this area also remains largely unstudied. Several studies have implicated a role for Ca^{2+} in central chemosensitivity, but unlike in peripheral chemoreceptors, its role is largely unexplored. Finally, the modulatory role of synaptic transmission on central chemosensitive neurons is not well understood. This issue raises the broader issue of the modulation of central chemosensitivity in general, and very little is known regarding the mechanisms of modulation. This is of particular importance given the likelihood that alteration of central chemosensitivity may play a role in a number of pathological conditions, including congenital central hypoventilation syndrome (334), sudden infant death syndrome (296), and sleep apnea (235). All of these aspects of chemosensitive signaling are likely to receive more concentrated study in the future.

A key feature of central chemosensitivity that has been slowly accepted over the last decade is that there are numerous regions that contain putative chemosensitive neurons. It will be of interest to compare and contrast the cellular mechanisms of chemosensitivity among these areas to see whether they all share the same basic mechanism or whether marked differences exist among the regions, which might then give us some insight into the role each region plays in central chemoreception. This effort will be greatly aided by the creation of a mathematical model to predict chemosensitive behavior for a given acidic stimulus. Such a model would be based on the various chemosensitive signaling pathways and the channel targets in a given cell. If these targets and channels differ among neurons from different chemosensitive regions, the model should be able to predict differences in the responses to acidic stimuli among the neurons of these different regions. A successful model would also need to incorporate the nature of the interactions among the various signals and targets, i.e., whether they are merely additive in their effects or whether they are synergistic, giving us further insight into how the

signals and targets interact. Such an analysis might also help to explain another puzzling aspect of central chemosensitivity: the exquisite sensitivity of ventilation to even small changes of pH. This is hard to explain at the cellular level, but as previously proposed (294), the large overall response may represent the summation of many small changes. Thus a highly CO₂/H⁺-sensitive neuron could arise from the combination of smaller cellular responses to changes of CO₂, pH_i, and pH_o. Alternatively, the high degree of sensitivity of ventilation to small pH changes may result from the integration and amplification of inputs from numerous chemosensitive regions, each of which has a smaller response to the pH change.

Finally, it probably should come as no surprise that the response of central chemosensitive neurons to CO₂/H⁺ seems to involve multiple signaling pathways. The most common stimulus for respiration is HA (a respiratory acidosis), which involves increased CO₂ at constant HCO₃⁻. Such a stimulus thus involves increased CO₂, decreased pH_o, and decreased pH_i. Thus multiple signals are essentially unavoidable under normal physiological conditions. Furthermore, given the close relationship between pH_o and pH_i, it would be virtually impossible to observe a decreased pH_o without a decreased pH_i in vivo, rendering somewhat moot the debate as to whether a change in pH_o or pH_i is the adequate stimulus. Nevertheless, from a cellular signaling perspective, the concept that the response to CO₂/H⁺ in chemosensitive neurons arises from multiple factors is profoundly different from the assumption that the response is dependent on a single adequate stimulus.

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