HIGHLIGHTED TOPIC | *Central CO_2 Chemoreception in Cardiorespiratory Control*

Glia modulation of the extracellular milieu as a factor in central CO_2 chemosensitivity and respiratory control

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Erlichman JS, Leiter JC. Glia modulation of the extracellular milieu as a factor in central CO_2 chemosensitivity and respiratory control. *J Appl Physiol* 108: 1803–1811, 2010. First published January 28, 2010; doi:10.1152/japplphysiol.01321.2009.—We discuss the influence of astrocytes on respiratory function, particularly central CO_2 chemosensitivity. Fluorocitrate (FC) poisons astrocytes, and studies in intact animals using FC provide strong evidence that disrupting astrocytic function can influence CO_2 chemosensitivity and ventilation. Gap junctions interconnect astrocytes and contribute to K^+ homeostasis in the extracellular fluid (ECF). Blocking gap junctions alters respiratory control, but proof that this is truly an astrocytic effect is lacking. Intracellular pH regulation of astrocytes has reciprocal effects on extracellular pH. Electrogenic sodium-bicarbonate transport (NBCe) is present in astrocytes. The activity of NBCe alkalinizes intracellular pH and acidifies extracellular pH when activated by depolarization (and a subset of astrocytes are depolarized by hypercapnia). Thus, to the extent that astrocytic intracellular pH regulation during hypercapnia lowers extracellular pH, astrocytes will amplify the hypercapnic stimulus and may influence central chemosensitivity. However, the data so far provide only inferential support for this hypothesis. A lactate shuttle from astrocytes to neurons seems to be active in the retrotrapezoid nucleus (RTN) and important in setting the chemosensory stimulus in the RTN (and possibly other chemosensory nuclei). Thus astrocytic processes, so vital in controlling the constituents of the ECF in the central nervous system, may profoundly influence central CO_2 chemosensitivity and respiratory control.

potassium homeostasis; acid-base regulation; lactate

GLIA HAVE BEEN VIEWED historically as passive bystanders contributing little to the dynamic landscape of neuronal function. More recently, astrocytic involvement in both the development and regulation of neuronal function has come to light. Glia are increasingly recognized as equal partners with neurons in brain function; "the long, dark period when the neuron concept dominated brain science" has come to an end (39). This view is, however, promulgated by researchers with vested interests in studies of glia—but skeptics abound (40), and with good reason. The majority of studies of glial processes have been done in reduced preparations (i.e., pure cultures of glia, coculture with neurons and astrocytes, brain slices, etc.), but it is rare to find studies of glia in intact animals either awake or asleep. Therefore, the idea that glia have the potential to influence neuronal function is widely available, but proof that the glia processes actually modify neuronal function and behavior in intact, unanesthetized animals is much harder to find.

Neuronal activity is modified by the intrinsic membrane characteristics of each cell and by synaptic inputs: this is the bedrock on which any analysis of the connection between neuronal activity and behavior is based. But the constituents of the extracellular fluid (ECF) also influence neuronal activity. Astrocytes play an essential role in creating the ECF bathing neurons, and in so doing, they have a surprisingly large role shaping respiratory behaviors that are more usually thought to be the sole domain of neurons. In addition to maintaining the ionic environment around each synapse, astrocytes remove neurotransmitters that diffuse out of the synapses, but they also release neurotransmitters and neuromodulators, so-called gliotransmitters, into the ECF. Glia also provide a variety of trophic factors essential for neuronal and synaptic development and remodeling. We will focus on astrocytic modification of the ionic content of the ECF; a review of neurotransmitter-related astrocytic processes is beyond the scope of this review. In the discussion that follows, we will describe the evidence supporting the importance of each glial process we have selected, discuss results relevant to respiration (where they exist), and also provide a skeptical analysis of the limitations of the evidence.

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THE GLIA-NEURONAL ENVIRONMENT

Glia have an intimate anatomic relationship with neurons, and many neurons are closely apposed to multiple astrocytic neighbors. Individual astrocytes are in turn coupled through gap junctions and form syncytia that surround multiple neurons. There are 10^8–10^9 gap junctions per cubic millimeter of cortical neuropil, and the distance between synapses and the nearest astroglial cell processes is often <2.5 μm (63). A single astrocyte can make contact with over 100,000 synapses and 300–600 neuronal dendrites (29), and hippocampal astrocytes are coupled through gap junctions to an average of 11 adjacent astrocytes (81). Due to the high density and large surface area of astrocytes, changes in the extracellular environment are sensed by astrocytes throughout most of the brain, and astrocytic transport processes, in turn, alter the ECF surrounding neurons. Astrocytes may, as a consequence, influence neuronal function over large volumes of the brain by controlling and manipulating the constituents of the ECF. The intimate association of synapses and astrocytes has led to the concept of the "tripartite synapse," in which glia are viewed as an integral element of the pre- and postsynaptic connections (3).

Gap junctions are ubiquitous between cells in the central nervous system. Astrocytes couple to astrocytes and to oligodendrocytes, and interneuronal gap junction coupling occurs frequently. Astrocytic coupling to neurons has also been described, but the evidence in favor of neuronal-astrocytic gap junctional coupling is weaker. Astrocytic to neuronal coupling has been described in the superior colliculi in rabbits on the basis of histological studies (65), in the locus ceruleus in rats based on electrophysiological and immunohistochemical data (2), and in the cerebellum of rats based on electrophysiological measurements (50). However, these findings have been disputed: light microscopy may not have sufficient resolution to identify the true cellular origin of gap junctional proteins, and electrophysiological measurements may be contaminated by local field potentials (45). Neuron-to-astrocyte gap junctions were not found (although interastrocytic, interneuronal, and astrocytic-to-oligodendrocytic gap junctions were found) using freeze-fracture electron microscopy and immunogold staining, a method with higher resolution than histology or immunohistochemistry performed with light microscopy (60). Thus the data supporting neuronal-glia interconnections by gap junctions in the neocortex and locus ceruleus have not been substantiated (45). Moreover, there are no data indicating that any of the respiratory effects of gap junctions discussed below depend specifically on neuronal-astrocytic gap junctions.

ASTROCYTIC HETEROGENEITY

Glia express a plethora of ion channels and membrane transporters that reflect both heterogeneity within this cell type, but also the similarity of astrocytes to neurons. There appear to be several subpopulations of astrocytes. One class of astrocytes (termed "passive") are coupled via gap junctions, display voltage-independent potassium leak currents, express glial fibrillary acid protein (GFAP) immunoreactivity, express glutamate transporters (but not ionotropic glutamate receptors), and express proteins associated with vesicular fusion and release of transmitters (71). The second class of astrocytes (termed "complex glia") are not coupled electrically, display time- and voltage-dependent Na$^+$ and K$^+$ currents, express functional α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, and express the protein S100β, and ~20% of these cells are also NG2-immunoreactive (77, 82). Passive glia are probably mature protoplasmic astrocytes, whereas complex glia may be a mixed population of immature astrocytes and NG2-immunopositive multipotent precursors (77, 82).

INHIBITION OF ASTROCYTIC FUNCTION IN INTACT ANIMALS

Fluorocitrate (FC) and fluoroacetate have been used in vitro and in vivo to study astrocytic function. FC administered in low concentrations within the central nervous system leads to reversible, selective dysfunction of astrocytic metabolism (53, 78), although FC is lethal to astrocytes after prolonged (hours) exposure. FC is selectively taken up into astroglia, inhibits aconitase, and halts the citric acid cycle in astrocytes, but leaves neuronal function unimpaired. During FC treatment, astrocytic mitochondria depolarize and ATP levels decrease, but neuronal ATP levels remain unchanged (74). To study the role of astrocytic modulation of central CO$_2$ chemosensitivity, we microperfused FC through push-pull cannulae placed in the retrotrapezoid nucleus (RTN) in anesthetized and awake animals (21, 32). Within minutes of application of FC, tissue pH decreased, and respiratory output increased significantly. Both ECF pH and respiratory activity recovered to control levels after cessation of FC administration. Neuroanatomic analysis of dead and dying cells in the region of FC exposure in the RTN revealed that astrocytes were selectively killed by this metabolic toxin. Largo et al. (41) also showed that astrocytes in the hippocampus depolarize in the presence of FC in a time-dependent manner, and this depolarization was associated with an acid shift in ECF pH and an increase in extracellular potassium. Therefore, the striking ventilatory stimulation after FC infusion into the RTN probably arose from stimulation of chemosensory neurons within the RTN from one of two mechanisms. First, the loss of metabolic integrity of the astrocytes was associated with a drop in ATP levels and partial collapse of the transmembrane potassium gradient. The associated increase in ECF potassium following FC treatment of the astrocytes is amplified by ongoing neuronal activity (and further release of K$^+$ into the ECF) and failed K$^+$ siphoning by the dysfunctional astrocytes. Elevated ECF K$^+$ partially depolarizes neurons and increases chemosensory neuronal activity for any level of hypercapnia. Second, the depolarization of astrocytic membrane potential in the RTN results in the movement of HCO$_3^-$ from the extracellular space into astrocytes through an electrogenic sodium-bicarbonate transporter (NBCe). This astrocytic depolarization-induced intracellular alkalosis leads to a reciprocal extracellular acidification (which we detected in the RTN at the site of microinjection). This acid shift increases the stimulus intensity for chemosensory cells and together with the increase in extracellular K$^+$, increases the neural activity of respiratory-related chemosensory cells in the brain stem and thereby increases the respiratory response to CO$_2$. Thus poisoning astrocytes revealed that disruption of the housekeeping functions of potassium homeostasis and pH regulation have quite profound effects on the autonomic regulation of ventilation.

A ROLE FOR ASTROCYTIC GAP JUNCTIONS IN RESPIRATORY CONTROL IN INTACT ANIMALS

Gap junctions provide a means of intercellular communication, and networks of interconnected passive astrocytes form a syncytia extending over a relatively large volume of brain tissue. The cell-cell channels formed by hexameric connexins are gated by the transjunctional voltage, pH, and various pharmacological agents. Reduced pH tends to inhibit conduction through gap junctions, and this might limit the contribution of gap junctions to respiratory functions such as central chemosensitivity. However, the pH-dependent inhibition of gap junctional conduction occurs at pH values below the physiological range (70), and significant changes in gap junctional resistance have not been observed in the physiological pH range (7.4) or the pH range associated with physiologically relevant hypercapnic stimuli (13, 33). Ionic and metabolic homeostasis is maintained throughout the astrocytic syncytium (e.g., spatial buffering of K$^+$, see below), and electrical coupling and intercellular signaling can, therefore, spread throughout the astrocytic network (e.g., Ca^{2+} waves can spread through astrocytes over surprisingly large distances). Gap junctional proteins have been identified in both neurons and glia in brain stem regions implicated in respiratory control in both neonatal and adult rodents (66). Connexin 43 (Cx43), Cx30, and Cx26 are the principal gap junctional proteins in astrocytes; Cx32 is expressed in oligodendrocytes; and Cx36 expressed in cortical (59) and brain stem neurons (66).

The exact role of gap junctions in respiratory rhythm generation has not been defined. In reduced preparations in which phrenic nerve activity has a decrementing pattern, which is typical of gasping, carbenoxolone, an inhibitor of gap junctions that is not specific to any particular connexin of class of intercellular gap junctions, decreases the frequency of respiratory activity and tends to decrease the amplitude of integrated inspiratory phrenic nerve activity (6, 17). In the isolated perfused rat brain stem preparation, carbenoxolone treatment increased the respiratory frequency in one study (67), but not in a virtually identical study in the same preparation (62). As discussed below, none of the transgenic animal models or human diseases involving connexin mutations has any identified abnormality of respiratory rhythm generation. It seems possible, therefore, that gap junctions are important in the neurogenesis of gasping or that their role in eupnea in more intact or more mature preparations is redundant and not apparent and is revealed only as the complexity of the preparation is reduced.

We microperfused carbenoxolone unilaterally into the RTN or nucleus tractus solitarius (NTS) to assess the role of gap junctions in central chemosensitivity in awake rats (31, 52). The RTN and NTS both contain chemosensory neurons and contribute to the ventilatory response to CO$_2$. In rats less than ~10 wk of age, carbenoxolone infused into either the RTN or the NTS decreased respiratory responses to CO$_2$ (indicating that the unblocked effect of gap junctions is to amplify the ventilatory response to CO$_2$). As the animals matured, these responses waned in both areas, and carbenoxolone microperfusion in the RTN actually stimulated ventilation after 12 wk of age. Thus the role of gap junctions in the ventilatory response to CO$_2$ evolved from an excitatory to an inhibitory function over the course of development. These studies implicate gap

junctions in respiratory control, but they are limited in several respects. First, carbenoxolone has nonspecific effects unrelated to gap junctions. Second, the inhibition of gap junctions was not restricted to a particular cell type; we cannot distinguish the effects of carbenoxolone on gap junctional coupling between neurons or glia in this study. There are extensive in vitro data demonstrating electrical and transcellular coupling (e.g., movement of ions, cyclic nucleotides, etc.) among astrocytes in the brain, which suggests that the ventilatory effects are mediated by glia. But of course, neurons in the brain stem may also be coupled by gap junctions (33). Third, what particular function of astrocytes or neurons or pericytes or endothelial cells—all of which express gap junctions—may be altered by blocking gap junctions to produce these ventilatory changes also remains to be explored. It is worth noting in this context that carbenoxolone does not seem to alter the intrinsic chemosensitivity of CO$_2$-sensitive neurons in the NTS (10), although gap junctions may enhance the overall chemosensitivity of the locus ceruleus (30). The interested reader may find further reading on this topic in the following reviews (12, 68).

Gene deletion might be a powerful tool for probing the functional role of astrocytes in vivo, and a Cx43 knockout mouse has been made. Unfortunately, deletion of Cx43 is lethal shortly after birth. As is often the case, the cause of death is not related to astrocytic function (the reason this knockout might interest us), but related to defective cardiac development. A conditional knockout (cKO) of *Cx43* in mice has been created. The *Cx43*cKO mice are viable and show no apparent neuroanatomic abnormalities in the brain (72). They demonstrate several physiological abnormalities of brain function, including accelerated hippocampal spreading depression (72) and impaired Ca^{2+} wave propagation in neocortex (28); increased exploratory behavior; altered motor/locomotor capacity; and changes in brain acetylcholine level (72). In addition, *Cx43cKO* mice in combination with a null mutation of *Cx30*, another astrocytic connexin, have impaired spatial K$^+$ buffering and a reduced threshold for the generation of epileptiform events in the hippocampus in situ (76). Gap junctions clearly are important in neuronal function; however, none of the knockout animals has any apparent respiratory phenotype (although this has not been explored in detail so far as we know). A similar problem exists in humans: mutations of Cx26 cause sensorineural hearing loss (a clear neuronal effect of gap junctions), but so far as we know, none of the patients with Cx26 mutations has abnormal breathing (38). Similarly, x-linked Charcot-Marie-Tooth Disease, which is associated with central and peripheral myelination dysfunction and sensory and motor neuron loss in the periphery, results from mutations of Cx32, yet there are no described abnormalities of central respiratory function (5). Use of genetically modified animals may provide more specific tools to explore the impact of gap junctions in the astrocyte syncytium on respiratory function in the future, but to date, the knockout animals created have not been sufficiently specific to astrocytes and gap junctions or have not demonstrated a respiratory phenotype.

EXTRACELLULAR POTASSIUM HOMEOSTASIS: SPATIAL BUFFERING AND K$^+$ SIPHONING

A role for glia in potassium buffering in the brain ECF was first proposed by Orkand over 40 years ago (49) but has only

recently been studied at the cellular level. The extracellular K^+ concentration in the ECF of the brain is maintained close to 3 mM and is largely unaffected by fluctuations in serum K^+ levels (37). Neuronal stimulation can, however, increase extracellular K^+ levels three- to fourfold, and during pathological conditions, the extracellular K^+ can increase to 50–80 mM (69). The transmembrane K^+ concentration difference has a significant impact on a variety of neuronal functions, including membrane potential, activation/inactivation kinetics of ion channels, neurotransmitter release, and electrogenic transport mechanisms.

Potassium clearance from the extracellular space occurs by two mechanisms: K^+ uptake and spatial buffering (69). During K^+ uptake, extracellular potassium ions are either transported or passively diffuse across the cell membrane through potassium channels. To prevent sustained cellular depolarization, K^+ influx is often accompanied by either the inward movement of anions (e.g., Cl^-) or outward movement of a counter cation (e.g., Na^+). Spatial buffering of potassium, as originally proposed (49), had two defining principles. First, astrocytes had to form a functional syncytium in which K^+ accumulated from a point source could move down its concentration gradient through the astrocytic syncytium so that astrocytes could function as a K^+ sink. This transcellular movement of K^+ is probably the domain of "passive" astrocytes. Second, the astrocytic membrane had to be highly and selectively permeable to K^+ to allow the ion to enter the intracellular compartment from the extracellular point source and exit from the astrocytic syncytium at some distant site. The process of potassium redistribution depends on inward rectifying K^+ channels (Kir, see below) and gap junctions (35, 58). The Kir channels show pH-dependent transport kinetics (i.e., pH decreases conductance through these mechanisms), which is paradoxical given that the potassium siphoning mechanism, in which these channels are thought to play a role, is likely to be inhibited when it is most needed, for example during ischemia (81).

Inward rectifying channels have a high open probability at resting membrane potential, and their conductance increases as the extracellular K^+ level increases (58). Kir is expressed widely throughout the brain in astrocytes, oligodendrocytes, Bergmann glia, and Mueller cells. Neusch et al. (47) studied respiratory activity in neonatal mice [postnatal *day 6* (P6) through P11] lacking Kir4.1. Older animals could not be used in these studies because Kir 4.1 $-/-$ mice die of progressive motor impairment within the first 2 wk of life. In the brain stem, Kir 4.1 is expressed in astrocytes surrounding capillaries and neurons in the ventral respiratory group. Kir 4.1 in wild-type neonatal animals was initially expressed in astrocytic cell bodies but migrated to astrocytic processes in older animals as the protein was redistributed during development. The membrane potential was relatively depolarized in astrocytes in the Kir4.1 $-/-$ mice compared with wild-type controls (-47 mV vs. -71 mV). In addition, K^+ uptake from the extracellular space was nearly abolished in the knockout mice even when the V_m was clamped to normal membrane potentials. Thus Kir4.1 plays an important role in K^+ efflux and uptake and in setting the resting membrane potential in astrocytes. Interestingly, the fictive, rhythmic respiratory activity arising from the ventral respiratory group in brain slices was not different between knockout and wild-type mice. However, the rhythm in the brain stem slice is probably gasping—a rhythm that is actually initiated and sustained by elevated ECF K^+ levels. This study does not, therefore, provide an adequate test of the effect of loss of Kir on normal eupneic respiratory activity or the network that generates eupnea. Nonetheless, these findings suggest that astrocytes and Kir4.1 are involved in K^+ homeostasis. It seems likely that potassium homeostasis is important in a variety of central respiratory-related functions, but the evidence to support this contention in intact animals is lacking.

ASTROCYTIC MODIFICATION OF ECF PH

The intracellular pH of vertebrate glial cells ranges from 6.9 to 7.6 when measured in CO_2/HCO_3^- buffer at an extracellular pH of 7.2–7.5. Astrocytes in respiratory-related sites maintain an intracellular pH $\sim$ 0.1–0.2 pH units more acidic than extracellular pH, and the intracellular pH is significantly more alkaline than would be expected from the passive distribution of H^+ ions across the cell membrane (54). It has been recognized for nearly 100 years that changes in pH alter ventilatory output (79). Although the cellular compartment (intracellular or extracellular) and the respiratory target(s) of changes in pH are still being studied (and different ionic and receptor targets probably exist in the multiple sites of central CO_2 chemosensitivity), pH/CO_2 is the principal determinant of breathing on a moment to moment basis, especially in anesthetized preparations, whether particular CO_2 chemosensors respond to intracellular or extracellular pH (16, 56). Consequently, studying astrocytic pH-regulatory mechanisms at sites involved in respiratory control in the brain stem may provide insight into cellular transduction mechanisms underlying the chemosensory response to CO_2.

There are two pH-regulatory processes of particular interest: electrogenic sodium-bicarbonate transport (NBCe; SLC4A4) and sodium-proton exchange (NHE). The reversal potential of NBCe in vertebrate astrocytes ranges from -86 mV to -63 mV (7, 48) but is often near the resting membrane potential (V_m) of glial cells (approximately -70 mV). Although mRNA for the NBCe has been isolated in neurons (64), only glia are thought to express functional NBCe activity (44). The stoichiometry of NBCe is likely 1:2 in astrocytes, and NBC transport is, therefore, electrogenic (15). As a consequence, the direction of transport will vary as a function of the membrane potential (V_m), and when V_m is depolarized above the equilibrium potential for the NBCe, HCO_3^- and Na^+ enter the cell and alkalinize intracellular pH (7). Since the reversal potential of NBCe is close to the resting V_m of astrocytes, relatively modest membrane depolarization can generate an inward movement in HCO_3^-, resulting in intracellular alkalosis, whereas hyperpolarization results in outward movement of HCO_3^- and an intracellular acidification (51). Given the exceedingly small volume of the extracellular space and the dependence of NBCe activity on the resting membrane potential, the activity of NBCe enables astrocytes to influence extracellular pH when the membrane potential of astrocytes shifts. A role for glial cells in the generation of activity-dependent acid shifts was suggested by observations in the developing optic nerve (11) and spinal cord (36). In these studies, interstitial pH was progressively acidified as animals matured. The progressive acid shift in the interstitial space was correlated with the

maturation and proliferation of the neuroglia. Early recordings of the intracellular pH of cortical astrocytes in the rat hinted that astrocytes may modify extracellular pH. During repetitive stimulation of the cortical surface, the intracellular pH of cortical astrocytes underwent a rapid alkalinization that paralleled the time course of membrane depolarization (depolarization-induced alkalosis; DIA) (8, 9). The time course of these alkaline shifts was rapid (within seconds), and the pH changes were large (~0.4 pH units) during repetitive electrical activity and nearly 1.0 pH unit during cortical spreading depression. Moreover, simultaneous extracellular measurements of cortical extracellular pH revealed that impairing the changes in astrocytic membrane potential reduced astrocytic DIA and the early, acid shifts in the interstitium. Grichtchenko and Chesler (27) generated brain slices devoid of neurons in the hippocampus and studied DIA and shift in extracellular pH in astrocytes located in these glial scars. Activation of NBCe alkalinized the intracellular space while inducing an extracellular acid shift, which was diminished in CO$_2$/HCO$_3^-$-free media and abolished in Na$^+$-free media (26).

The second pH-regulatory transport mechanism that we identified in astrocytes in the RTN and NTS was sodium hydrogen exchange (NHE). Removal of CO$_2$/HCO$_3^-$ or Na$^+$ from the perfusate acidified the glial cells, but the acidification after Na$^+$ removal was greater in the RTN than in the NTS. Treatment of the slice with 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) in saline containing CO$_2$/HCO$_3^-$ acidified the cells in both nuclei, but the acidification was greater in the NTS (implying that sodium/proton exchange was tonically active in both nuclei). The activity of NHE seemed be the inverse of NBCe activity; NHE activity was greater in the NTS than in the RTN, whereas NBCe activity was greater in the RTN than in the NTS, yet both of these sites are putative CO$_2$ chemosensory sites and both contain CO$_2$-sensitive neurons (18).

Sodium-bicarbonate cotransport may play a particularly interesting role in central chemosensitivity; increased neuronal activity associated with hypercapnia may increase extracellular potassium, depolarize astrocytes, activate NBCe, and enhance the fall in extracellular pH. There are two likely mechanisms in central chemosensory regions of the brain stem that may alter NBCe activity in glia by altering membrane potential. First, local changes in extracellular K$^+$ resulting from increased neuronal activity associated with elevated CO$_2$ could depolarize the glial membrane potential. Second, CO$_2$ may directly affect the glial membrane potential as it does in some medullary neurons by decreasing outward K$^+$ conductance. Thus the activity of NBCe might amplify the stimulus for CO$_2$ chemosensory cells, and astrocytic pH regulation may play an important role setting the stimulus in chemosensory regions of the brain by modifying one of the putative chemosensory stimuli, extracellular pH.

We examined intracellular pH regulation in astrocytes from the RTN and NTS to identify the proton transport processes that are active during steady-state, normocapnic conditions (19). We studied intracellular pH using fluorescence methods in brain stem slices that were enriched with astrocytes by pretreatment with kainic acid, which selectively kills neurons (46). We identified the two predominant proton transport processes in the NTS and RTN: NBCe and sodium-hydrogen exchange (NHE). We examined the activity of NBCe in the NTS and RTN by elevating extracellular K$^+$ ~3-fold to depo-

larize the membrane potential in glia ~20 to 40 mV (51). The baseline intracellular pH values did not differ significantly in the RTN and NTS; however, in the presence of elevated K$^+$, intracellular pH rose significantly in the RTN, but elevated extracellular K$^+$ had little effect on intracellular pH in the NTS. The alkalinization of intracellular pH that occurred in the RTN after depolarization of the cells was inhibited by DIDS, which suggests that NBCe was present in these astrocytes. In addition, intracellular Cl$^-$ depletion had little effect on intracellular pH (which it should not if the dominant pH-regulatory processes, NBCe and NHE, are not Cl$^-$ dependent); the intracellular pH values under Cl$^-$-free conditions were quite similar to intracellular pH values during perfusion with CO$_2$-containing artificial cerebrospinal fluid. Moreover, astrocytes in the RTN demonstrate DIA when depolarized with current injection (61), providing functional evidence that NBCe is present and active in the RTN.

The simplest hypothesis to explain these responses is that there was greater expression of NBCe in the RTN than in the NTS. We performed immunohistochemical studies in which we double-labeled cells with glial fibrillary acidic protein (GFAP) and NBC (22). The RTN is a more diffusely organized nucleus with many astrocytes intercalated between neurons compared with the more compact NTS, which has a higher density of closely packed neurons and a low density of astrocytes. Approximately 80% of the brain stem astrocytes are GFAP-positive, and GFAP staining was present in both nuclei at the blood-brain barrier (where one would expect it). There is, therefore, no evidence that we selectively missed GFAP-positive cells in the NTS. Moreover, many of the GFAP-positive cells in the RTN were also NBC-positive, but the small number of GFAP-positive cells in the NTS did not express NBC immunoreactivity. NBC immunoreactivity was present in NeuN-positive cells (NeuN stains neurons) in the NTS. The antibody we used does not differentiate between electroneutral (possibly expressed in NTS neurons) and electrogenic NBC (expressed in RTN astrocytes).

The foregoing information indicates that NBCe is well represented functionally and immunologically in astrocytes in the RTN but much less so (if at all) in astrocytes in the NTS. However, the apparent activity of NBCe may vary if the resting membrane potential of glia differs in the RTN and NTS. Astrocytes are usually hyperpolarized compared with neurons; the average resting membrane potential of astrocytes in the RTN is approximately −72 mV (61), and the reversal potential for NBCe is approximately −85 to −90 mV assuming 2:1 HCO$_3^-$-Na$^+$ cotransport. If the membrane potential were less negative in the RTN or if the intracellular Na$^+$ concentration were greater in the NTS than in the RTN, the apparent activity of NBCe might have been less in the NTS compared with the RTN even if the number of transport proteins were similar. A wide range of glial membrane potentials has been described in cultured rat hippocampal astrocytes (48), but we are not aware of any systematic differences in membrane potential based on the neuroanatomic location of the astrocytes. Whatever the explanation for the divergent intracellular pH responses to depolarization in the RTN and the NTS (differential expression of the NBCe, different electrochemical gradients, or different K$^+$ permeabilities in the two nuclei), the RTN behaves as if NBCe is more active. Thus any elevation in extracellular K$^+$ associated with neuronal activity will lead to greater alkalin-

ization of glial intracellular pH and a greater fall in extracellular pH in the RTN compared with the NTS even when the nominal chemosensory stimulus, CO_2, is equivalent.

DIRECT EFFECTS OF CO_2/pH ON ASTROCYTES

The simple presence of NBCe is not sufficient to implicate it in central chemosensitivity; one needs to know the circumstances under which it might be active. Using sharp electrodes to record from CO_2-sensitive cells in brain slices, Fukuda et al. (23, 24) made the first electrophysiological recordings from astrocytes in the ventral medulla (many of the cells were probably in the RTN although that nomenclature was not then in use). The cells from which they recorded were electrically silent (no action potentials despite depolarization) and had biophysical properties consistent with astrocytes. About half of the cells studied were depolarized when extracellular pH was reduced (23, 24), presumably reflecting hypercapnic inhibition of pH-sensitive potassium channels in astrocytes. We have seen a similar distribution of CO_2-sensitive astrocytes in the RTN: about half of the astrocytes depolarized in brain stem slices containing the RTN when CO_2 in the perfusate was increased from 5% to 10% CO_2 (22). More recently, we studied astrocytes in the RTN using both traditional electrophysiological methods and voltage-sensitive dyes to measure membrane potential and pH (61). All of the cells that we patched lacked the ability to generate action potentials, had a relatively hyperpolarized V_m, and exhibited depolarization-induced alkalinization, thereby indicating that they were likely to be astrocytes. In young animals (P0–P17), hypercapnic acidosis resulted in a modest depolarization ($\sim$5 mV), but there was no apparent regulation of intracellular pH. This was a surprise, which we attributed to the early developmental state of these astrocytes. In contrast, measurements from adult animals ($>$P32 days) showed that approximately half of the astrocytes depolarized $\sim$10 mV during hypercapnic acidosis whereas the remaining astrocytes were unaffected by this stimulus (22). There are two points to be made from this. First, some of the observed increase in CO_2 sensitivity in rodents as they mature may be attributed to the developmental emergence of astrocytic processes in the first postnatal weeks (55). Second, there is a bimodal distribution of V_m responses to hypercapnic acidosis in the RTN in adult rodent astrocytes, (much as Fukuda et al. described years earlier). If NBCe is important in defending intracellular pH in the RTN, then one would expect that those astrocytes that are depolarized in response to hypercapnia should also show intracellular pH recovery. Our preliminary studies show a similar bimodal response of intracellular pH: $\sim$60% of adult astrocytes showed rapid recovery of intracellular pH during hypercapnic acidosis whereas 40% did not. In those cells that show intracellular pH recovery, DIDS abolished intracellular pH recovery during hypercapnic acidosis, and chloride depletion slowed recovery (implying that Cl^-/HCO_3^- exchange was active during hypercapnia and responsible for some of the intracellular pH regulation). On the other hand, inhibition of sodium-hydrogen exchange had little effect. Both DIDS and NHE inhibition resulted in an acid shift in baseline intracellular pH in those astrocytes that did not regulate pH during hypercapnia. A tentative interpretation of these data is that two types of astrocytes within the RTN express different sets of pH-regulatory processes and contribute differently to the ventilatory response to hypercapnia. The regulation of intracellular pH during hypercapnia in some astrocytes suggests that the resulting transmembrane movement of protons into the extracellular space could increase the level of acidification in the ECF surrounding chemosensory neurons and thereby increase the stimulus and neural output of these cells for any level of hypercapnia.

The differential distribution of pH-regulatory mechanisms in astrocytes between the RTN and NTS may have implications for the larger organization of chemosensory responses among the widely distributed chemosensory nuclei in the brain stem. If control of ECF pH differs among chemosensory sites, then the sites will not respond equally to equivalent levels of hypercapnia [and measurements of ECF pH in anesthetized animals reveal surprisingly disparate pH values under conditions of constant CO_2 (4)]. The presence of NBCe in astrocytes in the RTN and the hypercapnia-induced depolarization of approximately half the astrocytes in the RTN means that the fall in ECF pH will be greater for any level of hypercapnia in the RTN (and any other nucleus with a similar distribution of astrocytes) than in the NTS. The RTN appears to posses a local amplification process reflecting the detailed cellular environment within the RTN (the level of ECF K^+ and CO_2 and the number and distribution of pH-regulatory mechanisms of astrocytes). On the other hand, local astrocytic processes will play a smaller role in the NTS (there are fewer astrocytes and those that are present do not seem to express NBCe). Perhaps it is appropriate that the NTS, which integrates information from a variety of distant visceral sensors, would be relatively unaffected by local astrocytic events. The NTS may sacrifice some CO_2 sensitivity compared with the RTN, which seems to reflect more local events within the brain tissue, in exchange for an integrative function more influenced by distant sensory events, but less susceptible to modification by local tissue metabolic events.

THE LACTATE SHUTTLE AND CENTRAL CHEMOSENSITIVITY

Oxygen and glucose use of the brain accounts for $\sim$20% of the total resting metabolic rate even though the brain represents less than 2% of the body mass (42). Neuronal activity and glucose metabolism are tightly linked in vivo, but the cellular mechanisms involved in this metabolic coupling remain the subject of debate. Glucose uptake by glia in vitro is similar (80) or greater than glucose uptake of neurons (73), and metabolic intermediates are shuttled from glial cells to axons. Lactate produced from glucose by astrocytes is preferentially oxidized by neurons in coculture experiments (75). The baseline extracellular tissue lactate concentrations are $\sim$1–3 mM during resting conditions in vivo but can exceed 10 mM during shock, seizures, cerebral ischemia, and trauma (25, 34). These data have given rise to the hypothesis that there normally is large flux of lactate between cellular compartments in the brain, which can have important metabolic ramifications but also may have profound effects on extracellular pH.

Lactate is an anion at physiological pH, and it cannot cross cell membranes easily by diffusion; it requires a specific transport mechanism, which is provided by proton-linked monocarboxylate transporters (MCTs). Three members of a family of MCTs (MCT1, MCT2, and MCT3) have been iden-

tified in the central nervous system. MCT1 and MCT4 are expressed predominantly in the astrocytes, and MCT2 is the predominant neuronal transporter (14). MCT2 has a lower K_m for lactate than MCT1 or MCT4 for lactate, and the asymmetrical distribution of MCT1 and MCT2 between astrocytes and neurons can support unidirectional transport of lactate from astrocytes (low-affinity transporters) to neurons (high-affinity transporters), which constitutes one arm of the astrocyte-neuron lactate shuttle hypothesis (ANLS) (43). The ANLS hypothesis states that the sodium-coupled reuptake of glutamate by astrocytes and the ensuing activation of the Na-K-ATPase triggers glucose uptake by astrocytes and rapid formation and release of lactate via MCT1 and MCT4. Lactate released by astrocytes can then be transported into neurons by MCT2 and used to fuel activity-dependent energy demands in neurons.

An important consequence of the ANLS hypothesis is that a robust lactate shuttle will change extracellular pH. Alterations in pH are not only a by-product of cellular activity but also modulate neuronal activity in the brain (57). Protons can modulate the conductance of at least eight classes of ion channels in the brain including Kir, K_{Ca2+}, TASK, high-voltage-activated Ca^{2+} channels (L and N), low-voltage-activated Ca^{2+} channels, and acid-sensitive ion channels. Monocarboxylate transport can be inhibited by 4-hydroxycinnamate (4-CIN). The IC_{50} of MCT2 for 4-CIN is ~20=fold less than the IC_{50} for MCT1 and 40-fold less than the $K_{0.5}$ for MCT 4. In a recent study, we examined the ventilatory effects of focally inhibiting MCT2 in the RTN in vivo using focally injected 4-CIN (20). We also studied the intracellular accumulation and uptake of lactate in astrocytes and neurons during both steady-state conditions and during conditions of impaired lactate transport in brain slices containing the RTN to confirm that changes in extracellular and intracellular pH in astrocytes and neurons were consistent with a model of lactate transport from astrocytes to neurons.

Microinjection of 4-CIN into the RTN rapidly acidified the extracellular space. The degree of acidification in the RTN was nearly identical to levels seen after increasing the fractional inspired CO_2 ($F_{I_{CO_2}}$) to 5%. These findings are consistent with previous work in the RTN examining the effects of acidic stimuli on tidal volume and breathing frequency in vivo (1, 20). 4-CIN treatment reduced astrocytic intracellular pH slightly but actually alkalinized intracellular pH in neurons. Our interpretation of these results is that intracellular pH in neurons fell because lactate was no longer transported into the neuron when MCT2 was inhibited by 4-CIN, and lactate transport out of astrocytes was inhibited slightly by the accumulation of lactate in the extracellular space so that astrocytic intracellular pH fell. The 4-CIN analog increased uptake of a fluorescent 2-deoxy-D-glucose analog in neurons (presumably to compensate for the loss of lactate uptake) but did not alter the uptake rate of this 2-deoxy-D-glucose analog in astrocytes. Thus shuttling lactate from astrocytes to neurons supports oxidative metabolism in neurons in the RTN, but the lactate is also part of the central chemosensory stimulus for ventilation in the RTN.

CONCLUSION

We discussed the influence of astrocytes on respiratory function, particularly central CO_2 chemosensitivity. The FC

studies in intact animals provide incontrovertible evidence that disrupting astrocytic function can influence CO_2 chemosensitivity and ventilation. The evidence that gap junctions and K^+ siphoning and Kir channels alter respiratory control is inferential and will require further study to confirm. The role of NBCe and heterogeneity of astrocytic function is also indirect. Further studies in intact animals are needed, but the lack of a specific NBCe inhibitor certainly limits what can be done at this time. The lactate shuttle hypothesis seems to be active and important in setting the chemosensory stimulus in the RTN (and possibly other chemosensory nuclei). Acid-base regulation, K^+ homeostasis, and energy metabolism are not new topics and not particularly glamorous, given the current scientific focus on genetic manipulations in reduced preparations. But the evidence we reviewed suggests that these work-a-day processes, nonetheless, profoundly influence central CO_2 chemosensitivity and respiratory control. Astrocytes and neurons involved in the regulation of respiration seem to participate in an exchange of information (in which the astrocytes and neurons are equal partners) necessary to regulate ECF homeostasis throughout the central nervous system.

DISCLOSURES

No conflicts of interest are declared by the authors.

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