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EFFECTS OF ACUTE HYPOXIA ON INTRACELLULAR-pH REGULATION IN ASTROCYTES CULTURED FROM RAT HIPPOCAMPUS

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Abstract

We used the pH-sensitive dye BCECF to evaluate the effect of acute (5-10 min) hypoxia (~3% O_2) on the regulation of intracellular pH (pH_i) in astrocyte populations cultured from rat hippocampus. For cells in the nominal absence of CO_2/HCO_3^- at an extracellular pH of 7.40 (37°C), acute hypoxia caused a small (0.05) decrease in steady-state pH_i, but increased the pH_i-recovery rate from an acid load during all but the late phase of the pH_i recovery. During such pH_i recoveries, the total acid-extrusion rate (φ_E, the product of dpH_i/dt and proton buffering power) decreased with increasing pH_i. Hypoxia alkali shifted the plot of φ_E *vs.* pH_i; over the upper ~85% of the φ_E range, this shift was 0.15-0.30. Hypoxia also stimulated the pH_i-recovery rate from an alkali load. Under normoxic conditions, switching the extracellular buffer to 5% CO_2/22 mM HCO_3^- also alkali shifted the φ_E-pH_i plot (upper ~85%) by 0.4-0.5. Superimposing hypoxia on CO_2/HCO_3^- further alkali shifted the φ_E-pH_i plot (upper ~85% of the φ_E range) by 0.05-0.15. The SITS-insensitive component of φ_E was alkali shifted by 0.20-0.30, whereas the SITS-sensitive component of φ_E was depressed in the low pH_i range. Thus, in the nominal absence of CO_2/HCO_3^-, acute hypoxia has little effect on steady-state pH_i but stimulates acid extrusion and acid loading, whereas in the presence of CO_2/HCO_3^-, hypoxia stimulates the SITS-insensitive but inhibits the SITS-sensitive acid extrusion.

Keywords

Bicarbonate; Carbon dioxide; Glia; Membrane; Transporter

1. INTRODUCTION

The regulation of both intracellular pH (pH_i) and extracellular pH (pH_o) of brain cells is critically important because many cellular processes are sensitive to changes in pH_i and/or pH_o. These pH-sensitive processes include enzymes and transporters, as well as voltage- and ligand-gated channels that can influence neuronal activity (Roos and Boron, 1981; Tombaugh

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and Somjen, 1998; Traynelis, 1998; Chesler, 2003; McAlear and Bevensee, 2004). The pH sensitivity of glutamate-stimulated and voltage-gated Ca^{2+} channels is particularly relevant to ion disturbances associated with hypoxia and ischemia, when increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) can be neurotoxic (Yao and Haddad, 2004). Nearly all cells regulate pH_i by transporting acid-base equivalents such as H^+ and HCO_3^- across their plasma membranes. Throughout the body, the transport of acid-base equivalents across plasma membranes causes reciprocal changes in pH_i and pH_o. In the brain, however, the high surface-to-volume ratios and tortuosity of the extracellular space accentuate these effects.

Ischemia/hypoxia generally leads to decreases in both pH_i and pH_o in brain cells (Tombaugh and Sapolsky, 1993; Siesjö et al., 1996; Yao and Haddad, 2004), although the pattern and magnitude of the pH changes can vary among preparations (Yao and Haddad, 2004). If the primary insult were a fall in pH_i (i.e., at constant pH_o), then brain cells would attempt to regulate their pH_i by extruding acid, that is, transporting acid into or taking up HCO_3^- from the extracellular space. The resulting secondary decrease in pH_o could contribute to tissue damage. Conversely, if the primary insult were a fall in pH_o (i.e., at constant pH_i), then the resulting inhibition of acid-extruding processes and stimulation of acid-loading processes would lead to a secondary decrease in pH_i as well. Metabolic status also comes into play. Thus, preischemic hyperglycemia exacerbates the intra- and extracellular acidosis of brain caused by ischemia (see review by Katsura and Siesjö, 1998).

Glial cells play critical roles in neuronal development and the regulation of the ionic and chemical environment of brain cells (Ransom, 2000). Astrocytes in particular help regulate pH_o, as well as extracellular levels of K^+ and glutamate—parameters affected by ischemia/hypoxia (see Jϕrgensen et al., 1999). Plum proposed that ischemia-induced brain necrosis is the result of astrocytic death caused by lactic acidosis (Plum, 1983). Astrocytes in culture are quite resistant to isolated oxygen deprivation; surviving for 12-24 h after the onset of hypoxia (Yu et al., 1989; Kelleher et al., 1993; Sochocka et al., 1994). However, cultured astrocytes exposed transiently (15-40 min) to a hypoxic, acidic, ion-shifted Ringers solution that mimics an in-vivo ischemia/hypoxia condition will perish within 20 min after being re-exposed to a standard Ringers solution (Bondarenko and Chesler, 2001).

Key to understanding the above effects will be an elucidation of how ischemia/hypoxia affects specific acid-base transporters in astrocytes. However, less is known about the effect of O_2 deprivation on the activity of acid-base transporters in mammalian astrocytes than in neurons. In neurons, anoxia stimulates the activity of the acid extruding Na-H exchanger (NHE) in a subset of neocortical neurons from mouse (Jϕrgensen et al., 1999), as well as CA1 hippocampal neurons from both mouse (Yao et al., 2001) and rat (Diarra et al., 1999; Sheldon and Church, 2002). In the rat neurons, however, stimulation of the exchanger only occurred after the neurons were returned to normoxia. O_2 deprivation can also influence the activity of HCO_3^--dependent transporters. Yao et al. (2003) found that extant anoxia stimulates a stilbene-sensitive, Na^+- and HCO_3^--dependent transporter that is possibly electrogenic. Indeed, Na/HCO_3 cotransporters (NBCs) appear to be present in at least some neurons (Schmitt et al., 2000; Cooper at al., 2005; Majumdar et al., 2006; Rickmann et al., 2007).

In astrocytes, NHEs appear to play an important role in maintaining the health of astrocytes exposed to hypoxia and then returned to normoxia. For example, the NHE inhibitor HOE694 prevented cell death when rat cortical astrocytes—after being exposed 20-40 min to a hypoxia, acidic, ion-shifted Ringers solution—were returned to normoxia (Bondarenko et al., 2004). When mouse cortical astrocytes, after being deprived of oxygen and glucose for 2 h, were returned to an oxygen/glucose replete solution, they exhibited an ~80% increase in the pH_i-recovery rate from acid loads (Kintner et al., 2004). This increased pH_i recovery rate was due to stimulated NHE1 activity because the increased rate was absent in astrocytes treated with

HOE642 or astrocytes from NHE1 null mice. HOE642 also protected the energy state (i.e., [phosphocreatine]/[creatine] ratio) of glioma cells during two hours of hypoxia (Glunde et al., 2002). This last observation is consistent with the hypothesis that NHE activity rises even during the hypoxic stress. However, to our knowledge, this hypothesis remains untested. Similarly, the effects of acute extant hypoxia on the electrogenic Na/HCO_3 cotransporter, NBCe1—which is present (Bevensee et al., 2000) and makes a major contribution to pH_i regulation in astrocytes (Bevensee et al., 1997a, 1997b)—are unknown.

In the present study, we focused on the effect of acute (5-10 min) hypoxia on pH_i regulation in astrocyte populations cultured from the rat hippocampus. We chose astrocytes from the hippocampus because our group (Bevensee et al., 1997a,b), as well as other laboratories (see Rose and Ransom, 1998), have characterized in detail the mechanisms responsible for pH_i regulation of the cells. In addition, as described above, investigators have examined the effects of hypoxia/anoxia on the pH_i physiology of hippocampal neurons. Results from our astrocyte study thus complement and extend our understanding of hypoxia-induced changes in the acid-base status of the hippocampus.

Although hypoxia had little effect on the steady-state pH_i of hippocampal astrocytes in either the nominal absence or presence of CO_2/HCO_3^-, an exposure to ~3% O_2 stimulated acid extrusion during the pH_i recovery from an NH_4^+-induced acid load in the absence of the physiological buffer. Hypoxia also stimulated acid loading during the pH_i recovery from an alkali load induced by removing CO_2/HCO_3^-. In the presence of CO_2/HCO_3^-, hypoxia stimulated SITS-insensitive, but to a lesser extent inhibited SITS-sensitive acid extrusion. Thus, although acute hypoxia has little effect on steady-state pH_i, it enhances the dynamic recovery of pH_i from acid and alkali loads in cultured hippocampal astrocytes, which would presumably enhance the stability of pH_i.

Portions of this work have been published in preliminary form (Bevensee and Boron, 2000).

2. RESULTS

Effect of hypoxia on steady-state pH_i

Hippocampal astrocytes bathed in a nominally CO_2/HCO_3^--free, HEPES-buffered solution had a mean steady-state pH_i of 7.05 ± 0.05 (n=7). In the same seven experiments, subsequently exposing the astrocytes to the HEPES-buffered solution bubbled with N_2 (which reduced O_2 to ~3% in the recording chamber) caused pH_i to decrease slightly to a lower steady-state pH_i of 7.00 ± 0.04 (P = 0.008, paired t-test) in 4.8 ± 0.7 min. In additional studies conducted in 5% CO_2/22 mM HCO_3^-, we found that steady-state pH_i was indistinguishable under normoxic (7.23, n=3) vs. ~3% O_2 conditions (7.25, n=3). In summary, acute hypoxia (~3% O_2) has little effect on the resting pH_i of astrocytes either in the nominal absence or presence of CO_2/HCO_3^-.

Effect of hypoxia on acid-extrusion rates in the presence and absence of CO_2/HCO_3^-

To assess the effect of hypoxia on acid extrusion, we compared the pH_i recoveries from an intracellular acid load under normoxic and hypoxic conditions. As shown in Fig. 1A, astrocytes bathed in the HEPES-buffered solution had a steady-state pH_i of ~6.9 (prior to point *a*). We then acid loaded the cells using the NH_4^+-prepulse technique (Boron and De Weer, 1976). Applying 20 mM NH_3/NH_4^+ elicited an abrupt increase in pH_i (segment *ab* in Fig. 1A) due to NH_3 influx and protonation to form NH_4^+. In the continued presence of NH_3/NH_4^+, pH_i slowly decreased (*bc*) due to NH_4^+ influx and/or stimulation of acid-loading mechanisms. Removing NH_3/NH_4^+ caused pH_i to decrease sharply (*cd*) as the NH_4^+ that accumulated during segment

bc was converted to NH_3 (which diffused out of the cells) as well as H^+. Subsequently, pH_i recovered (*de*) to a value similar to that prevailing before the NH_4^+ prepulse. The segment-*de* pH_i recovery is primarily due to an amiloride-sensitive Na-H exchanger (Bevensee et al., 1997a), although a bafilomycin A1-sensitive H^+ pump may also make a small contribution (Pappas and Ransom, 1993).

When the cells were exposed to a HEPES-buffered solution equilibrated with ~3% O_2, pH_i decreased slightly (*ef*). After we acid loaded the now-hypoxic astrocytes a second time by applying and removing NH_3/NH_4^+ (*fghi*), the ensuing pH_i recovery exhibited a waveform (*ijk*) that was different from than seen under normoxic conditions (*de*). With hypoxia, the minimum pH_i attained after NH_3/NH_4^+ washout (point *i*) was consistently higher than with normoxia (*d*), consistent with stimulated acid extrusion. Moreover, with hypoxia, the rate of pH_i recovery was faster at the beginning (point *i*) and for most of the pH_i recovery (i.e., at relatively low pH_i values), but slower towards the end of the recovery (i.e., at relatively high pH_i values near point *j*).

In control normoxic experiments, pH_i-recovery rates from two sequential acid loads were very similar for astrocytes in the nominal absence or presence of CO_2/HCO_3^- (data not shown).

Using segment-*de* and -*ij* pH_i-recovery rates in experiments similar to that shown in Fig. 1, we calculated the pH_i dependence of total acid extrusion ($dpH_i/dt \times \beta_{Total}$) for 20% O_2 (open circles in Fig. 2) and ~3% O_2 (filled circles). As previously reported for single cultured rat hippocampal astrocytes (Bevensee et al., 1997a), total acid extrusion decreased at progressively higher pH_i. However, hypoxia alkali shifted the plot of φ_E *vs.* pH_i by 0.15-0.30 in the pH_i range 6.2-6.6, and also caused it to become almost linear.

We also performed experiments similar to that shown in Fig. 1, but on astrocytes bathed in 5% CO_2/22 mM HCO_3^-. The squares in Fig. 2 summarize the φ_E *vs.* pH_i data. As previously reported for single astrocytes under normoxic conditions (Bevensee et al., 1997a), total acid extrusion was higher at all pH_i values in the presence of CO_2/HCO_3^- (open squares) compared to the nominal absence of the physiological buffer (open circles), predominantly reflecting the activity of an electrogenic Na/HCO_3 cotransporter. For astrocytes in the presence of CO_2/HCO_3^-, hypoxia (filled squares) alkali shifted the plot of φ_E *vs.* pH_i compared to normoxia (open squares). The plots are better fit ($P<0.0001$) with a model of 2 separate cubic polynomials than a simpler model of 1 cubic polynomial indicating that the data sets are distinct. The effects in the presence of CO_2/HCO_3^- (squares) were not as pronounced as seen in the nominal absence of CO_2/HCO_3^- (circles). For example, hypoxia alkali shifted the plot of φ_E *vs.* pH_i by only 0.05-0.15 (pH_i range: 6.8-7.0) for cells bathed in CO_2/HCO_3^- (compared to 0.15-0.3 for cells in the HEPES-buffered solution).

Effect of hypoxia on stilbene-sensitive acid-extrusion

An electrogenic Na/HCO_3 cotransporter (NBC) inhibited by stilbene derivatives is the predominant HCO_3^--dependent acid extrusion mechanism in cultured hippocampal astrocytes from rat (Bevensee et al., 1997a,1997b). To characterize the effect of hypoxia on HCO_3^--dependent acid extrusion further, we used the stilbene derivative SITS to inhibit NBC, and computed the pH_i dependencies of SITS-sensitive and -insensitive acid extrusion under both normoxic and hypoxic conditions. As previously reported, pretreating hippocampal astrocytes for ~6 h in tissue-culture media containing 400 μM SITS irreversibly reduces φ_E (computed from the pH_i recovery from a CO_2/HCO_3^--induced acid load) by ~65% (Bevensee et al., 1997a). Because other transporters likely contributed to the pH_i recovery, the SITS inhibition was probably substantially more than 65%. Irreversibly inhibiting NBC activity with SITS

prior to experiments avoids the necessity of using stilbenes during experiments, which would interfere with the BCECF fluorescence signal. In the present study, we inhibited NBC activity by pretreating with 500 μM SITS in the media for 6-8.3 h. In principle, the prolonged incubation in SITS could have induced compensatory changes in the pH physiology of the cells. On the other hand, we previously found that stilbene inhibition of the CO_2/HCO_3^--stimulated pH_i increase was similar for hippocampal astrocytes incubated in SITS for ~6 h or exposed only briefly to DIDS (Bevensee et al., 1997a).

The experimental protocol was similar to that shown in Fig. 1, except the astrocytes in Fig. 3 were continually exposed to 5% CO_2/22 mM HCO_3^-. We acid loaded the cells both before (*abcd*) and after (*fghi*) acute hypoxia, and either with or without a ~7-h pretreatment with 500 μM SITS. For clarity, we show only the pH_i recoveries (*d'e'* and *i'j'*) from the SITS experiment. SITS pretreatment reduced the pH_i-recovery rate for both normoxia (compare segments *de* and *d'e'*) and hypoxia (segments *ij* and *i'j'*)—data consistent with reduced NBC activity in the two conditions. Note that in SITS-pretreated cells, hypoxia increased the rate of pH_i recovery (compare segments *i'j'* and *d'e'*)—data consistent with hypoxia-induced stimulation of HCO_3^--independent acid extrusion (as shown in Fig. 1).

From experiments similar to those shown in Fig. 3, we computed the pH_i dependence of φ_E under the different conditions, using the same approach as described for Fig. 2. From data comparable to the segment-*de* and -*ij* pH_i recoveries in Fig. 3, we computed total φ_E *vs.* pH_i for normoxia (Fig. 4A, open squares) and hypoxia (filled squares). For this batch of astrocytes exposed to CO_2/HCO_3^-, hypoxia only elicited a slight alkali shift of the total φ_E *vs.* pH_i plot. Nevertheless, the data sets are better fit ($P<0.0001$) with a model of 2 separate cubic polynomials than a simpler model of 1 cubic polynomial.

We next used the segment-*d'e'* and -*i'j'* pH_i recoveries to compute the SITS-insensitive φ_E *vs.* pH_i in normoxia (Fig. 4B, open triangles) and hypoxia (closed triangles). As expected from the Fig. 1 data, hypoxia alkali shifted the SITS-insensitive φ_E *vs.* pH_i plot.

We obtained the SITS-sensitive φ_E vs. pH_i for normoxia (open diamonds) and hypoxia (closed diamonds) by subtracting each SITS-insensitive φ_E *vs.* pH_i plot (that yielded Fig. 4B) from the corresponding mean total φ_E *vs.* pH_i plot (Fig. 4A). Note that at pH_i 6.4, for example, the SITS-sensitive φ_E is about twice as great as the SITS-insensitive φ_E; that is, NBC accounts for about 2/3 of the pH_i recovery (assuming that SITS produces a 100% blockade of NBC) from an acid load at pH_i 6.4. This "2/3" figure underestimates the true NBC flux to the extent that SITS fails to block NBC. Under normoxic conditions, the SITS-sensitive φ_E (Fig. 4C, open diamonds) gradually decreased at progressively higher pH_i values (from 6.3 to 6.85)—a finding similar to previous observations (Bevensee et al., 1997a). We observed the opposite relationship under hypoxic conditions. In summary, hypoxia increases the SITS-insensitive φ_E, but decreases SITS-sensitive φ_E (particularly at a low pH of 6.4) for astrocytes exposed to CO_2/HCO_3^-.

Effect of hypoxia on acid loading

To examine the effect of hypoxia on acid loading, we monitored the rate of pH_i recovery in astrocytes that we alkali loaded by applying and removing 5% CO_2/22 mM HCO_3^- (see Bevensee and Boron, 1995). For the experiment represented in Fig. 5A, normoxic astrocytes bathed in a HEPES-buffered solution had a steady-state pH_i of ~7.3 (prior to point *a*). Applying CO_2/HCO_3^- elicited an abrupt decrease in pH_i (*ab*), due to CO_2 influx and formation of H^+ and HCO_3^-. In the continued presence of the physiologic buffer, pH_i increased (*bc*) due to acid-extruding mechanisms such as electrogenic Na/HCO_3 cotransport, and to a lesser extent, Na-H exchange (Bevensee et al., 1997a, 1997b). Removing CO_2/HCO_3^- caused pH_i first to increase rapidly (*cd*), greatly overshooting the initial pH_i (*d* vs *a*), due to the efflux of CO_2 and the

subsequent consumption of H^+ and HCO_3^- to replace the lost CO_2 (Boron and De Weer, 1976). Only nominal levels of intracellular HCO_3^- remain at the peak of the pH_i increase (point *d*). Subsequently, the cells recovered (*de*) to a pH_i similar to that at the beginning of the experiment. The mechanism responsible for the pH_i recovery is not known. We then exposed the astrocytes to ~3% O_2 and imposed another alkali load (*fgh*). (Note that, in this experiment, the initial CO_2-induced pH_i decrease is hidden by the overwhelming HCO_3^--dependent acid extrusion.) The subsequent pH_i recovery (*hi*) from the alkali load was faster than the segment-*de* recovery.

We used the segment-*de* and -*hi* pH_i recoveries in Fig. 5A to plot linear fits to the pH_i dependence of the pH_i-recovery rate (dpH_i/dt) in normoxia (Fig. 5B, open circles) and hypoxia (closed circles). We did not compute acid-loading rates (i.e., φ_L) because H^+ buffering power measurements at high pH_i obtained during alkali loads are not available. Buffering power measurements —particularly at high pH_i— are difficult to obtain in the astrocytes because of pronounced acid loading, even in the presence of inhibitors and ion substitutions that minimize acid-base transporter activity (Bevensee et al., 1997a). Hypoxia increased the pH_i-recovery rate at all pH_i values during the recovery and steepened the plot of dpH_i/dt *vs.* pH_i. We obtained similar results in two additional experiments. Overall, hypoxia steepened the plot of dpH_i/dt *vs.* pH_i during recoveries from alkali loads by ~50% ($P = 0.04$) from a mean of $44 \pm 5 \times 10^{-4}$ s^{-1} to $65 \pm 2 \times 10^{-4}$ s^{-1}.

3. DISCUSSION

The goal of the present study was to characterize the effect of hypoxia (~3% O_2) on rates of acid extrusion and acid loading in cultured hippocampal astrocytes. We made the following three key observations. First, although hypoxia has little effect on steady-state pH_i, hypoxia stimulates net acid extrusion during the pH_i recovery from an acid load in the nominal absence of CO_2/HCO_3^-. Second, although hypoxia has little effect on total acid extrusion during the pH_i recovery from an acid load in the presence of CO_2/HCO_3^-, hypoxia stimulates SITS-insensitive acid extrusion and inhibits SITS-sensitive acid extrusion. Third, hypoxia stimulates net acid loading during the pH_i recovery from an alkali load elicited by removing CO_2/HCO_3^-. In summary, the small effect of hypoxia on steady-state pH_i belies the more complex stimulatory/inhibitor effect of hypoxia on at least three mechanisms of pH_i regulation.

Hypoxia and steady-state pH_i

It is well established that hypoxia/ischemia leads to a decrease in both pH_i and pH_o *in vivo* (Tombaugh and Sapolsky, 1993). At least in some brain-cell preparations, however, the effect of hypoxia/anoxia alone on steady-state pH_i is variable. Acute chemical anoxia (10 mM azide) of mouse neocortical neurons induces an abrupt decrease in the pH_i, which is then followed by either a further decrease, no change, or a marked increase in pH_i (Jørgensen et al., 1999). According to another study on acutely isolated CA1 neurons from the adult rat, acute anoxia elicits a transient decrease in pH_i ($-\Delta pH_i$: 0.08-0.17) followed by a slower pH_i increase (ΔpH_i: 0.06-0.11) in the continued absence of O_2, and then a larger pH_i increase (ΔpH_i: 0.15-0.27) upon return to normoxia (Sheldon and Church, 2002). The slow pH_i increase during anoxia is due to a Zn^{2+}-sensitive acid extrusion mechanism; possibly a H^+ conductance, whereas the faster pH_i increase upon return to normoxia is due to a Na-dependent process; presumably a Na-H exchanger. Similar results were obtained on cultured postnatal rat hippocampal neurons (Diarra et al., 1999).

CO_2/HCO_3^- can also influence the effect of anoxia/hypoxia on steady-state pH_i. For instance, acute anoxia elicits a small pH_i decrease of ~0.06 in mouse CA1 hippocampal neurons bathed in CO_2/HCO_3^-, but a considerably larger pH_i increase of 0.46 in the neurons bathed in the

nominal absence of CO_2/HCO_3^- (Yao et al., 2001). The pH_i increase is due to anoxia-induced stimulation of the NHE because the increase can be blocked by removing external Na^+ or applying the inhibitor HOE694.

In our study, we found that ~3% O_2 elicited only a small pH_i decrease of 0.05 in hippocampal astrocytes exposed to a nominally CO_2/HCO_3^--free solution. Hypoxia also had little effect on pH_i of astrocytes in CO_2/HCO_3^-. In a similar fashion, acute hypoxia of cultured cortical and acutely isolated hippocampal rat astrocytes elicited a relatively small, gradual pH_i decrease compared to a more pronounced pH_i decrease when the hypoxia was combined with a low pH_o and/or an ion-shifted Ringers solution (Bondarenko and Chesler, 2001).

It is important to recognize that a change, or lack thereof, in steady-state pH_i in response to hypoxia (or any other stimulus) provides little information on the activity of specific acid-base transporters. As described in Methods, pH_i is in a steady state when the rates of acid extrusion (φ_E) and acid loading (φ_L)—both of which vary with pH_i—are equal. Steady-state pH_i will increase if φ_E (e.g., due to NHE activity) rises, if φ_L (e.g., due to Cl-HCO_3 exchanger activity) falls, or both (Bevensee and Boron, 2008). This line of reasoning is important for the present study, where it is clear that hypoxia has little effect on steady-state pH_i, but certainly has differential effects on the activity of acid-base transporters (see below).

Effects of hypoxia on acid extrusion

HCO_3^--independent—For astrocytes in the nominal absence of CO_2/HCO_3^-, hypoxia stimulated the pH_i recovery from an acid load (Fig. 1). Interestingly, hypoxia stimulated the early part of the pH_i recovery, but actually inhibited the recovery as the astrocytes approached steady-state pH_i. Consequently, hypoxia altered the pH_i dependence of total acid extrusion, φ_E (Fig. 2) in two ways. First, hypoxia alkali shifted the plot of φ_E *vs.* pH_i by 0.15-0.30 pH units. This hypoxia-induced alkali shift is modest compared to an alkali shift of ~0.7 pH units seen with cell shrinkage of mesangial cells (Bevensee et al., 1999). Second, hypoxia altered the shape of the φ_E *vs.* pH_i relationship, such that the decrease in φ_E with pH_i was more linear than exponential. This shape change reflects the greater hypoxia-stimulated increase in the dpH_i/dt during the early vs. the late part of the pH_i recovery.

As we have previously reported for rat hippocampal astrocytes bathed in the nominal absence of CO_2/HCO_3^-, an amiloride-sensitive NHE is the predominant acid-base transporter responsible for the pH_i recovery from an acid load (Bevensee et al., 1997a). The amiloride-sensitive NHE in these astrocytes is likely NHE1 based on immunoblot data (Pizzonia et al., 1996). Thus, the hypoxia-induced increase in acid extrusion for astrocytes bathed in the nominal absence of CO_2/HCO_3^- is likely due at least in part to stimulation of the NHE. Increased activity of the NHE during the pH_i recovery from an acid load has been reported for reoxygenated rat hippocampal neurons after acute anoxia (Diarra et al., 1999; Sheldon and Church, 2002), as well as reoxygenated mouse cortical astrocytes after 2 h of oxygen and glucose deprivation (Kintner et al., 2004). The anoxia/ischemia-induced stimulation of NHE activity can involve a number of kinases, including ERK1/2 and protein kinases A and C (Sheldon and Church, 2002; Yao et al., 2001; Kintner et al., 2005).

It is important to note that other acid-extrusion mechanisms in astrocytes such as an H^+ pump (Pappas and Ransom, 1993) and an H-Lactate cotransporter (Wuttke and Walz, 1990) may also contribute to the hypoxia-induced increase in acid extrusion. The high glucose concentrations of our cultured medium (i.e., 28 mM) and experimental solutions (e.g., ~10 mM) likely promotes an elevated glycogen content and increased lactate release from our cells (Abe et al., 2006). Furthermore, 5% O_2 for 1 day stimulates both lactate efflux and monocarboxylate transporter 1 (MCT1) expression in rat primary cortical astrocytes (Véga et al., 2006).

HCO_3^-**-dependent—**Although hypoxia did not have any appreciable effect on the total pH_i recovery rate of astrocytes acid loaded in CO_2/HCO_3^- (Fig. 3), it is clear from our analysis with SITS that hypoxia enhanced SITS-insensitive acid extrusion, but depressed SITS-sensitive acid extrusion (Fig. 4). The two predominant acid-extrusion mechanisms in rat hippocampal astrocytes are a SITS-insensitive NHE and a SITS-sensitive NBC. Therefore, it is not surprising that the SITS-insensitive φ_E *vs.* pH_i plots shown in Fig. 4B look similar to the HEPES-buffered pair in Fig. 2. Hypoxia expectedly right shifted and increased the slope of the SITS-insensitive φ_E *vs.* pH_i plot. The SITS-sensitive φ_E *vs.* pH_i plot shown in Fig. 4C predominantly represents the pH_i dependence of NBC activity. Interestingly, hypoxia eliminated the pH_i dependence of NBC activity by lowering SITS-sensitive acid extrusion at low pH_i values. The SITS-sensitive NBC in cultured rat hippocampal astrocytes is likely NBCe1-B, based on RT-PCR analysis and immunoblot labeling with an antibody that recognizes the carboxy terminus of NBCe1-A and -B (data not shown).

Effects of hypoxia on acid loading

Less is known about the mechanisms by which brain cells recover from alkali loads, and even less is known about their sensitivity to hypoxia. In hippocampal neurons, removing extracellular Cl^- reveals the functional presence of the Cl-HCO_3 exchanger AE3, which most likely functions as an acid loader (Hentschke et al., 2006). As shown in Fig. 5, astrocyte pH_i recovers rapidly from an alkali load imposed by removing CO_2/HCO_3^-, and the pH_i recovery is considerably faster when the astrocytes are exposed to hypoxia. The pH_i recovery could be due to any number of HCO_3^--independent acid loading mechanisms, including a Ca-H pump or a Cl-OH exchanger. A Cl-HCO_3 exchanger could also be involved. According to preliminary data (Bevensee & Boron, unpublished), the recovery is inhibited by phenylpropyltetraethylammonium (PPTEA), consistent with a contribution from a K/HCO_3 cotransporter (KBC) that has previously been identified in the squid giant axon (Hogan et al., 1995a, 1995b; Zhao et al., 1995; Davis et al., 2001). If one of the aforementioned HCO_3^- transporters were responsible, then the transporter would have to have a very high affinity for intracellular HCO_3^-, which presumably would have a low intracellular concentration in the nominal absence of CO_2. Nevertheless, it is possible that metabolically produced HCO_3^- could have provided sufficient substrate for such a HCO_3^--efflux mechanism. Additional studies are required to identify the acid-loading mechanisms in the astrocytes, and to determine which are stimulated by hypoxia.

Summary and significance

Although acute hypoxia has little effect on steady-state pH_i of rat hippocampal astrocytes, hypoxia stimulates HCO_3^--independent acid extrusion (e.g., NHE activity) and inhibits HCO_3^--dependent acid extrusion (e.g., NBC activity). Hypoxia also stimulates one or more acid loading mechanisms. Apparently, a low O_2 level is a signal that alters the activity of at least three pH_i-regulating mechanisms within a short timeframe of ~10 min. It is worth noting that the effects of hypoxia on astrocyte pH_i homeostasis are reminiscent of those of growth factors on renal mesangial cells (Boyarsky et al., 1990). In quiescent mesangial cells studied in the presence of CO_2/HCO_3^-, applying any of a wide range of growth factors has little effect on steady-state pH_i but stimulates the recovery of pH_i from both acid and alkali loads—changes that would enhance the stability of pH_i in the face of acute acid and alkali loads.

In astrocytes, the differential effects of hypoxia on various acid-base transporters may help optimize the overall pH-regulatory ability of the astrocytes exposed to different forms of hypoxia/ischemia (see Lascola and Kraig, 1998). The stimulation of NHE activity by hypoxia would not be surprising based on a similar finding in neurons (see Introduction). Such stimulation would certainly help astrocytes regulate their pH_i when faced with intracellular

acidosis caused by low-grade, or normoglycemic ischemia. Because this form of ischemia can also lead to a depolarization-induced astrocytic alkalosis, the stimulation of acid loading mechanisms would also be beneficial. However, during hyperglycemic and complete ischemia, astrocytes undergo compartmentalized acidosis and membranes become less permeable to HCO_3^-. Hypoxia-induced inhibition of NBC activity would contribute to the reduced HCO_3^- permeability of the astrocytes. Further studies are required to examine the effects of hypoxia on pH_i/pH_o regulation in the brain *in-vivo*, where normal oxygen tensions are likely to be less than the 20% used in the present *in-vitro* study (Ndubuizu and LaManna, 2007). Such future studies will be complicated however because *in-vivo* oxygen tensions are dynamic and display spatial and temporal heterogeneity.

In summary, the O_2 level can be included in the list of signals such as changes in pH_i, pH_o, and ion concentrations that can regulate acid-base transporter activity of astrocytes during ischemia/hypoxia. The extent to which each of these signals contributes to altered transporter activity will depend on the type and severity of the ischemia/hypoxia.

4. EXPERIMENTAL PROCEDURE

Culturing astrocytes

Astrocytes from the hippocampi of Sprague-Dawley rats were harvested and cultured as previously described (Bevensee et al., 1997a). Briefly, hippocampi from 0-3 day-old rats were harvested and placed in ice-cold PBS supplemented with 33 mM glucose. The tissues were subjected to mechanical digestion with pipettes of decreasing pore diameter, and then enzymatic digestion with ~0.1% trypsin (GIBCO BRL, Life Technologies Inc.) for 1 min. The cell suspension was subjected to several rounds of centrifugation (400 × *g*) followed by cell resuspension; twice with PBS + glucose, and twice with the tissue culture media consisting of minimum essential medium (MEM) supplemented with 28 mM glucose, 2 mM L-glutamine, 100 units ml^{-1} penicillin/streptomycin (GIBCO BRL, Life Technologies Inc.), and 10% fetal calf serum (Gemini Bioproducts, Inc. or GIBCO BRL, Life Technologies Inc.). After the final centrifugation, the cell suspension was divided into two 25-cm^2 tissue-culture flasks. On day 4 after plating, the flasks were agitated at ~100 RPM on an American Rotator V (Model R4140) shaker for approximately two hours to dislodge contaminating oligodendrocytes. 10-12 days after the initial plating, the flasks were again agitated for at least 12 hours before the cells were trypsinized and replated. From two preparations, astrocytes passaged three or four times were plated onto 1-cm^2 glass coverslips for pH_i measurements. Prior to cell plating, coverslips were sequentially washed with concentrated RadiacWash® (Biodex Medical Systems, Shirley, NY), 70-100% ethanol, and deionized water. Experiments were performed on the astrocytes 1-4 weeks after being plated onto coverslips.

Measuring pH_i

The pH_i of astrocyte populations was measured using the pH-sensitive dye BCECF and a SPEX Fluorolog-2 dual-beam spectrofluorometer (model CM1T10E; SPEX Industries, Inc., Edison, NJ) as described by Bevensee et al. (1999). Prior to an experiment, astrocytes on a coverslip were loaded with BCECF by incubating the coverslip in the standard HEPES-buffered solution containing 10 μM of the cell permeant acetoxymethylester form of the dye, BCECF-AM. The coverslip was then secured in a custom-designed flow-through cuvette, which fit into a temperature-controlled cuvette holder that was mounted in the excitation light path of the spectrofluorometer. The dye was alternately excited with 502-nm and 440-nm light every 3 s, and the emitted light at 530 nm from each excitation was captured and integrated for 1 s. The emitted light from 502-nm excitation (I_{502}) is pH sensitive, whereas that from 440-nm excitation (I_{440}) is relatively pH insensitive. Thus, the fluorescence-excitation ratio (I_{502}/I_{440}) is predominately a function of pH. Normalized ratios were converted to pH_i values using

the high-K^+/nigericin technique (Thomas et al., 1979), as modified for a single-point calibration (Boyarsky et al., 1988). Background I_{502} and I_{440} signals from cells without dye were subtracted from total I_{502} and I_{440} signals. All experiments were performed at ~37°C.

As described by Bevensee and Boron (1995), the rate of acid extrusion (φ_E) or acid loading (φ_L) is the product of the rate of change in pH_i (dpH_i/dt) and total intracellular H^+ buffering power (β_{Total})[1]. φ_E and φ_L are "pseudofluxes" with the units of moles per unit cell volume per unit time (i.e., $\mu M\ s^{-1}$). β_{Total} is the sum of the theoretically computed HCO_3^- buffering power ($\beta_{HCO3} = 2.3 \times [HCO_3^-]_i$) and the intrinsic buffering power (β_i). The pH_i dependence of β_i for cultured rat hippocampal astrocytes has previously been described by the linear relationship $\beta_i = -9.98 \times pH_i + 80.5$ (Bevensee et al., 1997a). The pH_i dependence of β_i for astrocytes cultured from mouse cortex is unaffected by 2 h of oxygen and glucose deprivation, followed by 1 h of reoxygenation (Kintner et al., 2005).

Solutions

The standard HEPES-buffered solution contained (in mM): 125 NaCl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 32 HEPES, 10.5 glucose, and NaOH to pH 7.4 at 37°C. NH_3/NH_4^+-containing solutions were made by replacing 20 mM NaCl with an equimolar amount of NH_4Cl. For the 10-μM nigericin solution, Na^+ was replaced with 105 mM K^+ and $NMDG^+$. For the 5% CO_2/22 mM HCO_3^- solution (pH 7.4), 32 mM HEPES was replaced with 22 mM $NaHCO_3$ plus 3.4 mM NaCl to maintain a constant ionic strength, and the solution was equilibrated with either 5% CO_2/balance air or 5% CO_2/balance N_2 in hypoxia experiments. For hypoxia experiments performed in the nominal absence of CO_2/HCO_3^-, solutions were equilibrated with 100% N_2. P_{O2} of the superfusate in the cuvette was ~23 torr (~3%) as measured with a platinum wire electrode (Croning and Haddad, 1998; Yao et al., 2001).

For stock solutions, BCECF-AM was prepared in dimethylsulfoxide (DMSO) and nigericin in 100% ethanol. BCECF-AM was obtained from Molecular Probes, Inc. SITS, nigericin, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Data are reported as mean ± standard error of the mean (SEM). Levels of significance were assessed using either the paired or unpaired Student's *t*-test, and $P < 0.05$ was considered significant. dpH_i/dt *vs.* pH_i during recoveries were fit with either third-order polynomials (following acid loads) or lines (following alkali loads) using a least-squares method. F tests were performed on cubic polynomial fits to plots of acid extrusion vs. pH_i using GraphPad Prism 5 (GraphPad Software, Inc.) to determine if data sets were distinct. Each pair of data sets shown in Figs. 2 and 4 was fit significantly better ($P<0.05$) with a model of 2 separate cubic polynomials than a simpler model of 1 cubic polynomial indicating that the data sets were distinct. Reported *n* values represent number of experiments, even from a single batch of cells.

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[1]The effect of total acid extrusion (φ_E) and total acid loading (φ_L) on steady-state pH_i can be described by the fundamental law of pH_i regulation: $dpH_i/dt = (\varphi_E - \varphi_L)/\beta_{Total}$, where β_{Total} is defined as the total intracellular H^+ buffering power.

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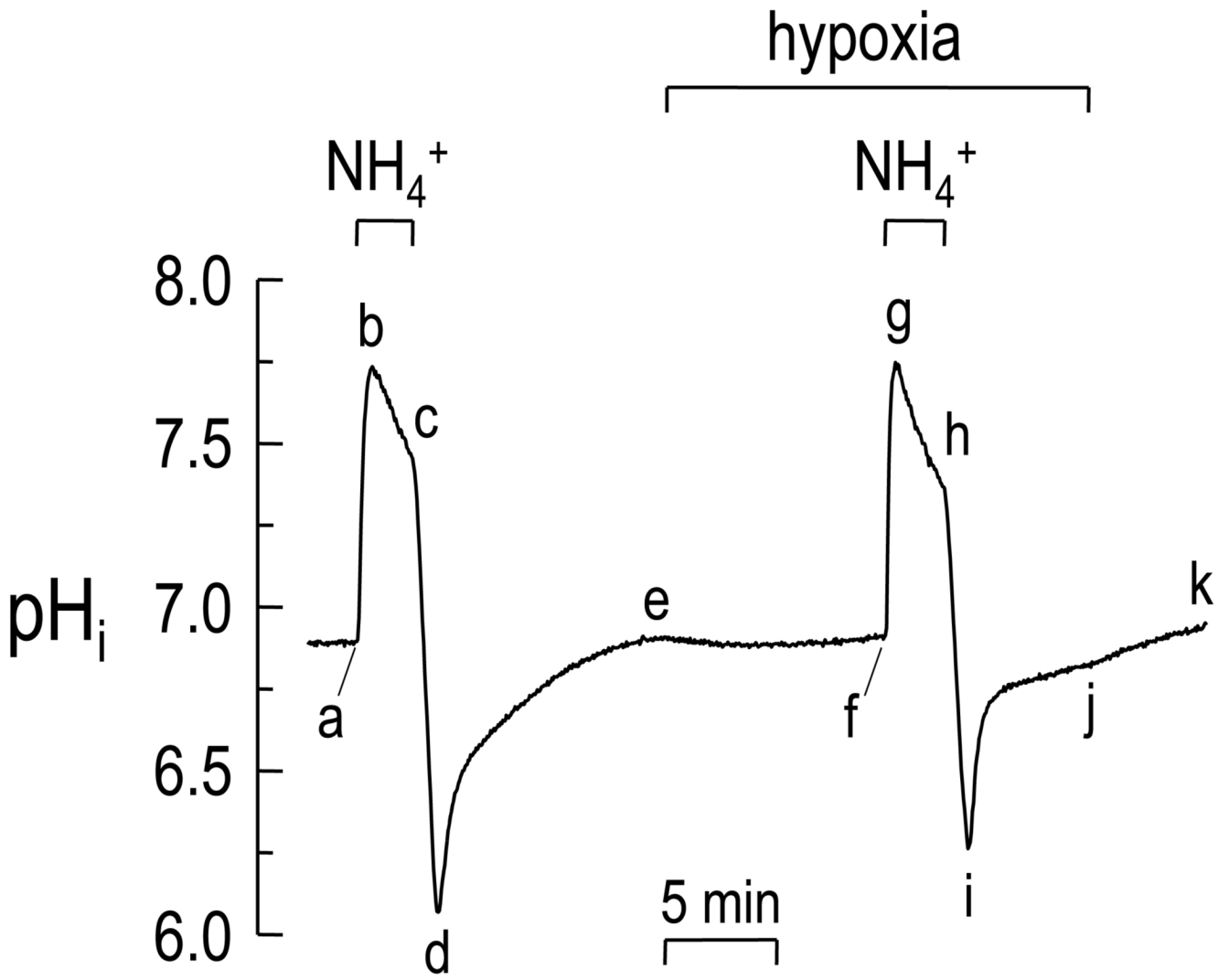

Fig. 1.

Hypoxia-induced stimulation of the pH_i recovery from an acid load in the absence of HCO_3^-. Astrocytes were exposed to hypoxia during segment *ej*. The cells were acid loaded by applying and removing 20 mM NH_3/NH_4^+ both before (*abcd*) and after (*fghi*) exposing the cells to acute hypoxia (~3% O_2). dpH_i/dt during most of the pH_i recovery was faster with hypoxia (*ij*) than normoxia (*de*).

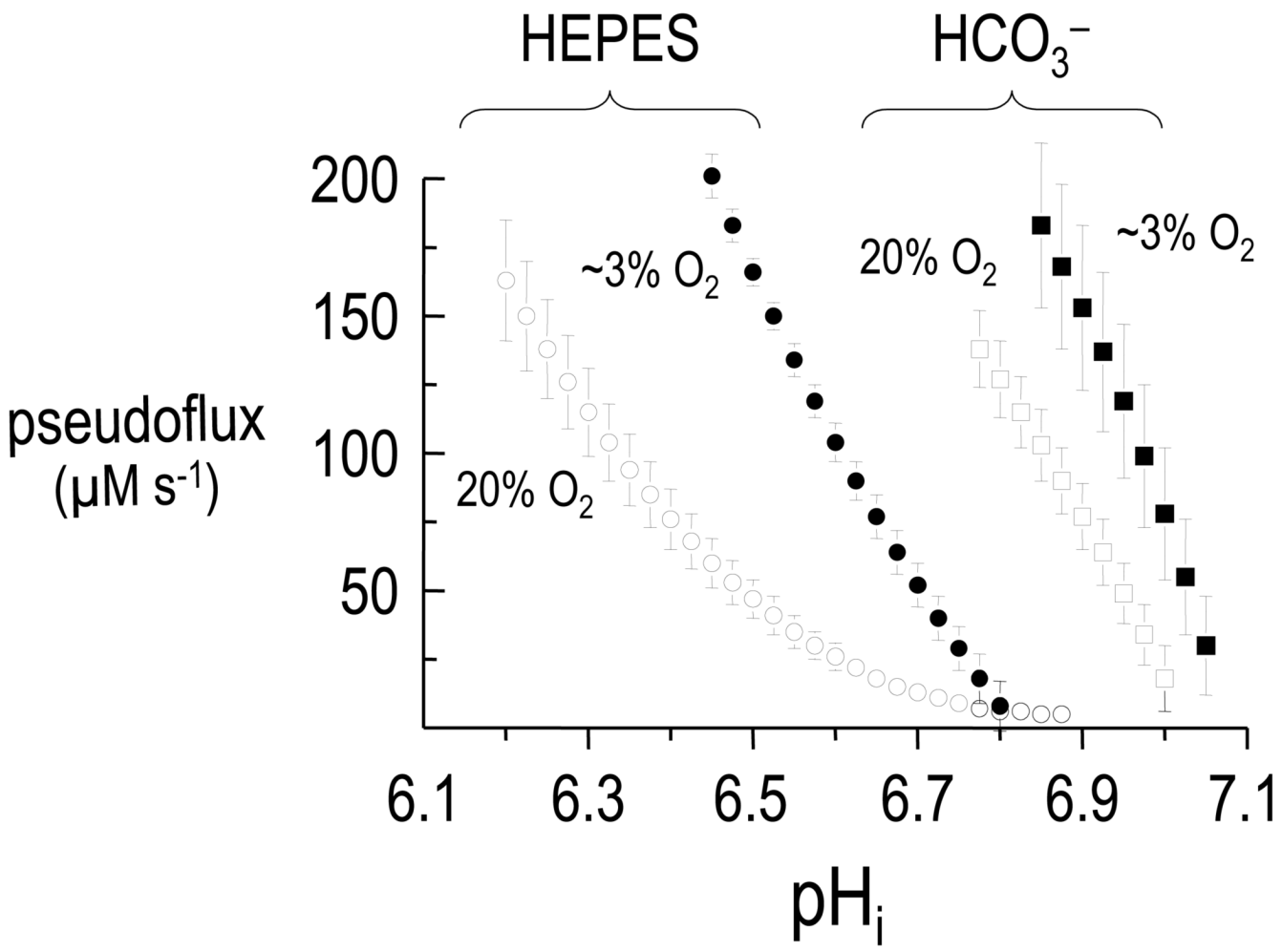

Fig. 2.

Hypoxia-induced stimulation of total acid extrusion in the absence or presence of HCO_3^-. For astrocytes bathed in the nominal absence of CO_2/HCO_3^- (circles), the pH_i dependence of total acid extrusion with normoxia (open circles) or hypoxia (filled circles) was computed from segments *de* and *ij* shown in Fig. 1. In a similar fashion, the pH_i dependence of total acid extrusion with normoxia (open squares) or hypoxia (filled squares) was computed for astrocytes bathed in 5% CO_2/22 mM HCO_3^-. n = 3 for each data point.

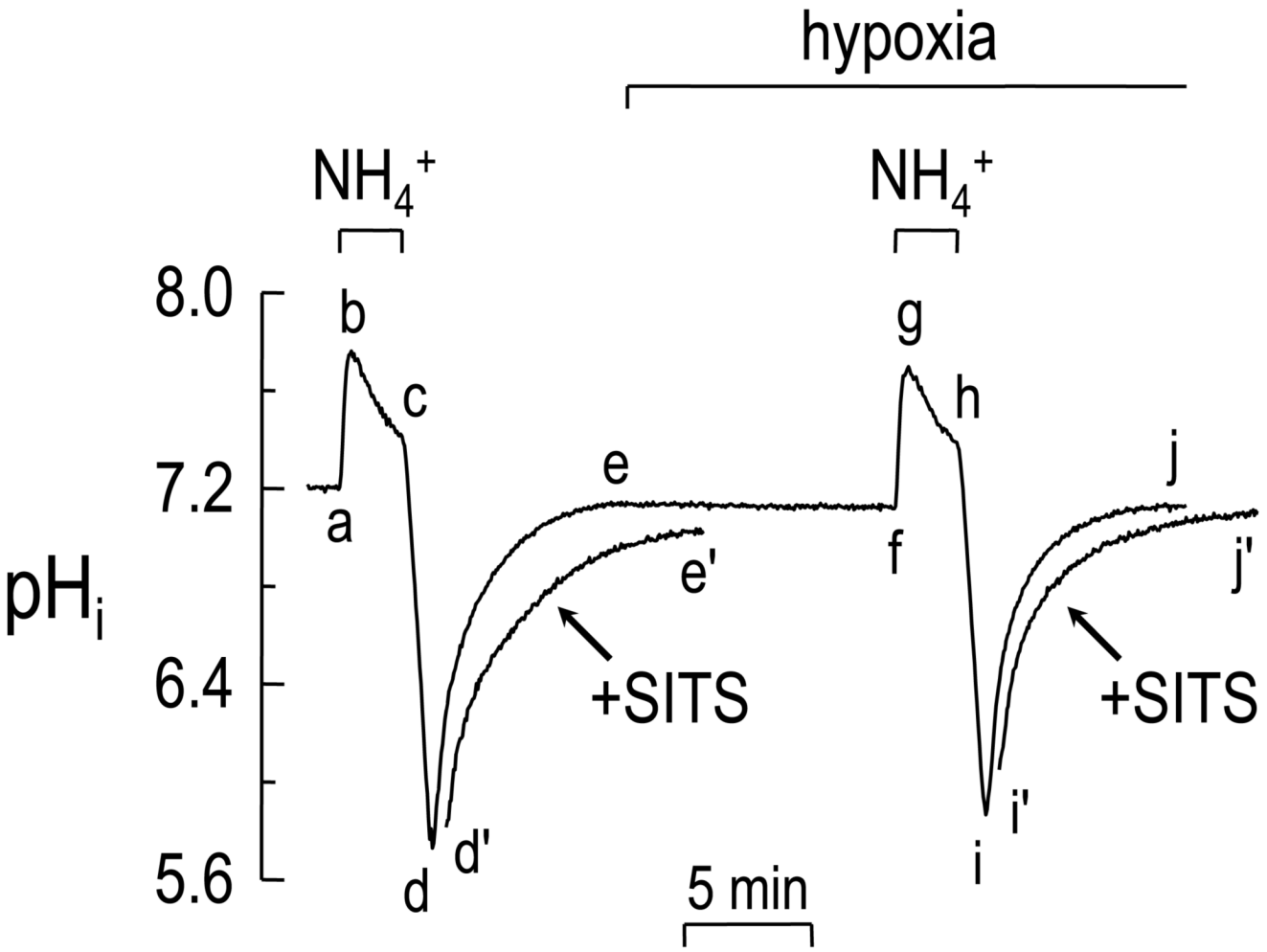

Fig. 3.

Hypoxia-induced effects on acid extrusion in the presence of HCO_3^- with and without SITS. Astrocytes were either untreated (full trace) or pretreated (partial trace) for 7 h with 500 μM SITS. Astrocytes were exposed to hypoxia during segment *ej*. The cells were acid loaded by applying and removing 20 mM NH_3/NH_4^+ both before (*abcd*) and after (*fghi*) exposing the cells to acute hypoxia. In the absence of SITS pretreatment, the pH_i recovery rates were similar under conditions of hypoxia (*ij*) and normoxia (*de*). However, with SITS pretreatment, the rates were faster with hypoxia (*i'j'*) than with normoxia (*d'e'*).

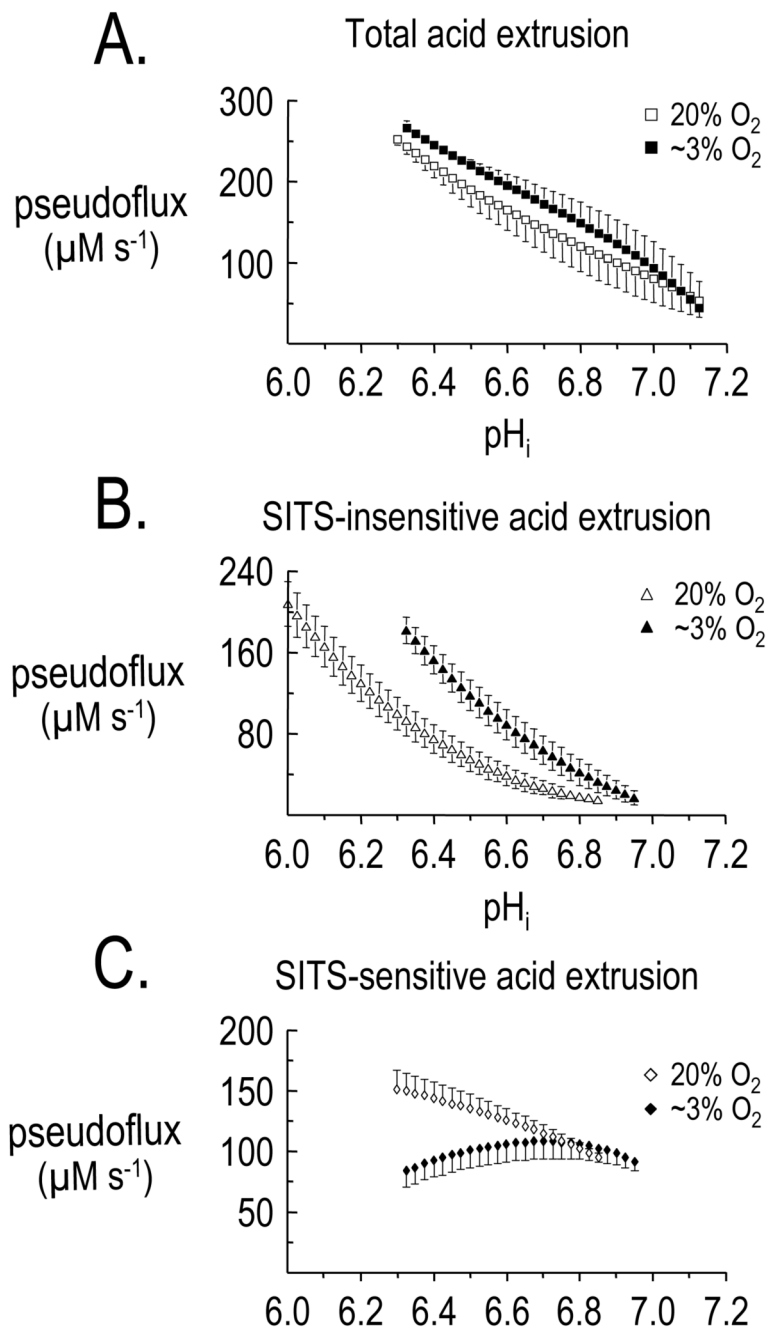

Fig. 4.

Hypoxia-induced stimulation of SITS-insensitive acid extrusion and inhibition of SITS-sensitive acid extrusion. A: The pH_i dependence of total acid extrusion with normoxia (open squares) or hypoxia (filled squares) was computed from segments *de* and *ij* shown in Fig. 3. B: The pH_i dependence of SITS-insensitive acid extrusion with normoxia (open triangles) or hypoxia (filled triangles) was computed from segments *d'e'* and *i'j'* shown in Fig. 3. C: The pH_i dependence of the SITS-sensitive acid extrusion with normoxia (open diamonds) or hypoxia (filled diamonds) was computed by subtracting the SITS-insensitive plot from each experiment (panel B) from the mean total acid extrusion plot (panel A). $n = 3$ for each data point in all panels.

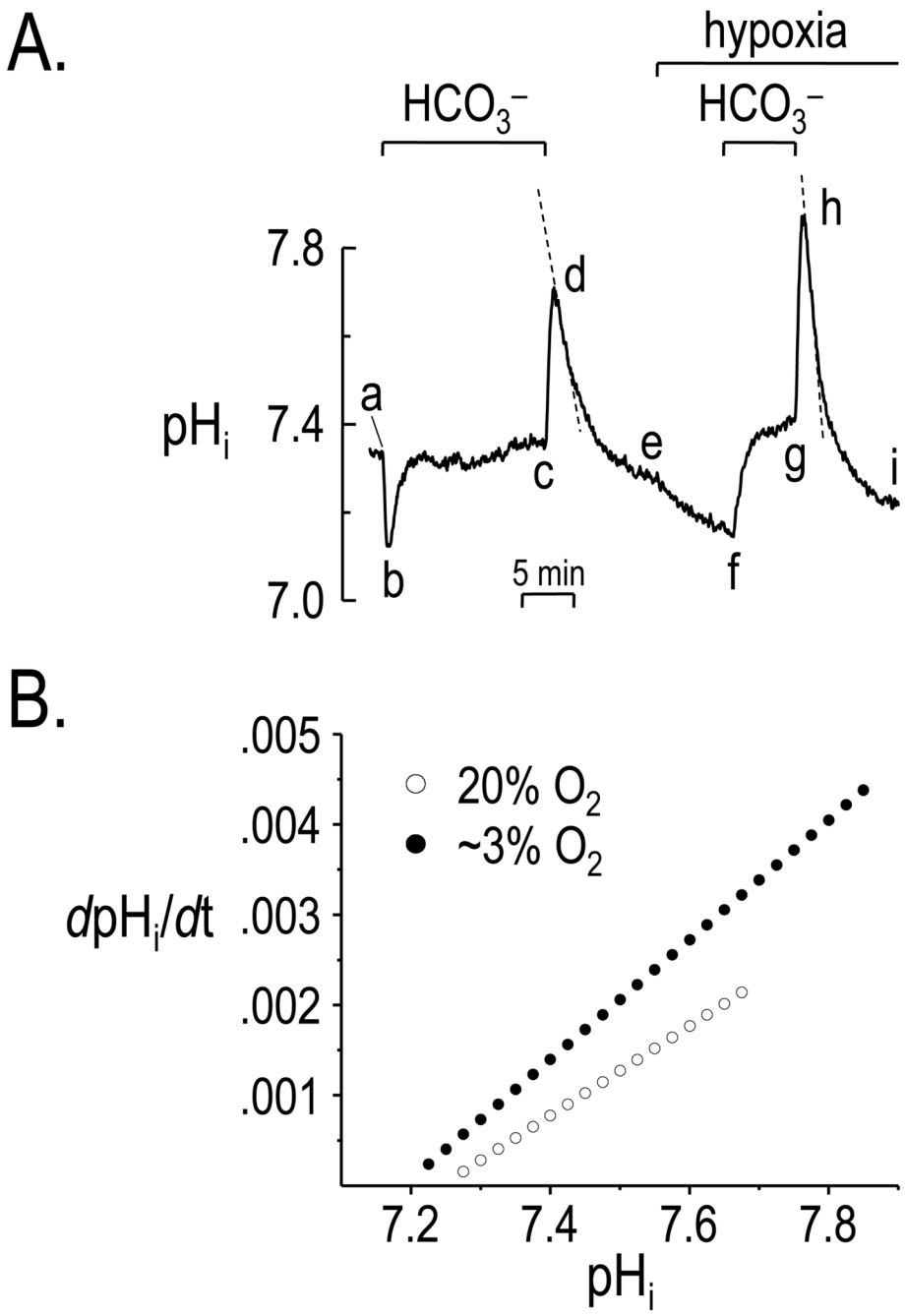

Fig. 5.

Hypoxia-induced stimulation of the pH_i recovery from an alkali load in the absence of HCO_3^-. A: Astrocytes were exposed to hypoxia during segment *ei*. The cells were alkali loaded by applying and removing 5% CO_2/22 mM HCO_3^- both before (*abcd*) and after (*fgh*) exposing the cells to acute hypoxia. As evident from the slopes of the dashed lines, the pH_i recovery from the alkali load was faster with hypoxia (*hi*) than normoxia (*de*). B: Linear fits to pH_i recovery rates vs. pH_i with normoxia (open circles) or hypoxia (filled circles) were obtained from segment-*de* and -*hi* pH_i recoveries shown in panel A.