Influence of pH on Calcium Influx During Hypoxia in Rat Cortical Brain Slices

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Methods

Intracellular calcium ([Ca^{2+}]_i) and pH (pH_i) were measured fluorometrically with the dyes fura-2 and biscalcecarboxyethyl carboxyfluorescein, respectively, during two types of hypoxia: (1) slice perfused equilibrated with N_2/CO_2 at pH 6.6 or 6.2 ("gaseous hypoxia") or (2) perfuse equilibrated with 95% O_2/5% CO_2 plus 100 μmol/L NaCN at pH 7.3, 6.6, or 6.2 ("chemical hypoxia").

Results

Changes in perfuse pH under aerobic conditions did not change [Ca^{2+}]. However, influx of calcium caused by gaseous or chemical hypoxia increased significantly with decreasing perfuse pH. During chemical hypoxia, the elevation in [Ca^{2+}], at perfuse pH 6.2 was twice that at perfuse pH 7.3. Change in [Ca^{2+}] was correlated with perfuse pH but not pH_i.

Conclusions

These results, which differ from previous studies showing acid inhibition of calcium influx in isolated neurons, suggest that low extracellular pH may exacerbate cellular injury during severe hypoxia or ischemia in the intact brain.

Key Words • calcium • hypoxia • neuronal damage • rats

Materials and Methods

Preparation of Cortical Brain Slices

Intracellular calcium and pH were measured in cortical brain slices prepared from Sprague-Dawley rats aged 11 to 21 days.

Brain slices were prepared after decapitation and rapid removal of the cranium. A 5 × 5-mm portion of the temporal cerebral cortex was dissected free, briefly placed in 3 to 5°C artificial cerebrospinal fluid (aCSF [mmol/L]): NaCl 116, NaHCO_3 25, KCl 5.4, CaCl_2 1.8, MgCl_2 0.8, NaH_2PO_4 0.9, and glucose 10 bubbled with 5% O_2/95% O_2, pH 7.40 at 37°C, then gluined with cyanocrylate cement to a mounting block and resubmerged in the chilled aCSF. Slices 250-μm thick were prepared with a Campden Instruments (Cambridge, England) vibrating tissue slicer. The slices were then transferred to 29 to 31°C aCSF continuously bubbled with 95% O_2/5% CO_2, PCO_2 was 40±5 mm Hg.

Fura-2 acetoxymethyl ester and biscalcecarboxyethyl carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, Ore) was added (from 1-mmol/L stock solutions in dry dimethyl sulfoxide, final concentration 3 to 5 μmol/L) to
the aCSF containing the brain slices to permit measurements of $[Ca^{2+}]_i$ and pH. Satisfactory dye-loading, as indicated by fura-2 and BCECF signals at least 10 times background fluorescence, required approximately 1 hour. The slices were transferred to Petri dishes filled with dye-free aCSF and rinsed. The slices were mounted on a mesh baffle and fitted into a 3.0-mL fluorometer cuvette filled with $O_2/CO_2$-equilibrated aCSF. A gas manifold fitted with plastic (nylon) inlet and outlet tubing, was used to continuously perfuse the slice with gas-equilibrated aCSF at a rate of approximately 1 mL/min. A second inlet tubing dispensed equilibrating gas at the top of the cuvette to maintain desired gas tension. The cuvette was held in the cuvette holder of a Hitachi F-2000 fluorometer (Tokyo, Japan), and positioned so that excitation light (1x3 mm in area) fell within the confines of the slice. The cuvette holder and perfusing solutions were both temperature controlled to 37±0.5°C.

Fura-2 and BCECF signals remained stable (baseline drift less than 2% of total signal) for more than 1 hour after placing the slice in the study cuvette.

**Calibration of Fura-2 and BCECF**

In vivo calibration was used for fura-2 and BCECF. Calibration for fura-2 was done essentially as described by Jensen and Chiu. Slices were alternately excited with 340- and 380-nm wavelengths, and fluorescence intensity at 510 nm was recorded every 0.5 seconds with the fluorometer. Spectral analysis revealed an acceptable level of scattering of excitation light. $[Ca^{2+}]_i$ was calculated from fluorescent ratios (340:380) with the following equation:

$$[Ca^{2+}]_i = K_{eq}(R - R_{min})/(R_{max} - R)(F_0/F_1)$$

where $K_{eq}$ is 225 mmol/L and R is the ratio of fluorescence intensity excited by 340- and 380-nm wavelengths, and fluorescence intensity at 510 nm was recorded every 0.5 seconds with the fluorometer. The biochemical equivalent of hypoxia was produced by adding NaCN to oxygenated aCSF. Adjustment of pH in these solutions to 7.3, 6.6, and 6.2 was done by addition of 0.1N HC1 during bubbling with the 95% O2/5% CO2 mixture. To correct for this, undyed brain slices were exposed to either hypoxia or NaCN and the resulting raw fluorescence traces recorded. These background traces were made by duplicating the same types and timing of changes in perfusate used in dyed slices. The entire background trace was then computer subtracted from the raw data record from the fura- and BCECF-dyed slices.

**Estimates of Extracellular pH in Brain Slices**

To define the relation of perfusate pH to actual pH, within the brain slice, we estimated intraslice pH with the non-cell-permeant form of the BCECF dye. Slices were incubated in medium containing 5 to 10 μmol/L BCECF acid for 10 to 15 minutes, then immersed into a cuvette containing dye-free perfusate. Apparent pH, was continuously determined fluorometrically and extrapolated to time zero to correct for diffusional loss of dye from slice extracellular fluid into the bulk perfusate. To determine how changes in perfusate pH related to actual pH during hypoxia, similar measurements were made with slices immersed into a cuvette containing dye-free perfusate plus 100 μmol/L NaCN.

**Experimental Protocol**

During study, slices were maintained at 37°C. To examine the effects of hypoxia on $[Ca^{2+}]_i$, aCSF equilibrated with nitrogen and CO2 mixtures was substituted for oxygenated aCSF. This replacement required 5 to 10 seconds at a perfusion rate of approximately 25 mL/min. The Po2 of the hypoxic aCSF was less than 5 mmHg as measured with an oxygen electrode. The Pco2 was carefully controlled to produce medium of pH 6.6 and 6.2 (to mimic changes in brain extracellular fluid pH known to occur during hypoxia or ischemia). In each case pH was continuously monitored in the perfusion reservoir and adjusted to within 0.05 units of target.

The biochemical equivalent of hypoxia was produced by adding NaCN to oxygenated solutions of aCSF. Adjustment of pH in these solutions to 7.3, 6.6, and 6.2 was done by addition of 0.1N HCl during bubbling with the 95% O2/5% CO2 mixture at 37°C. NaCN (to achieve a final concentration of 60 μmol/L) was introduced into the cuvette immediately after introduction of the test solution. Measurements of $[Ca^{2+}]_i$ were made during a stable 10-minute baseline period and then for 30 minutes after introduction of the test solution. Calibration followed. Only one intervention was studied in each brain slice.

**Statistical Methods**

Analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons was used to compare $[Ca^{2+}]_i$ between different treatment groups. Values in the text are reported as mean±SD, and $P<0.05$ was required for statistical significance. Linear regression was calculated by the methods of least squares; significance of regression was assessed according to Zar.

**Results**

Intracellular calcium in brain slices maintained in oxygenated aCSF was 165±63 nmol/L (n=96). Noise levels permitted detection of a change in $[Ca^{2+}]_i$ of approximately 10 nmol/L between 100 and 800 nmol/L and a change of roughly 25 nmol/L when $[Ca^{2+}]_i$ was greater than 750 nmol/L. Perfusion pH measurements were accurate to ±0.02 units. Noise level in measurements of fluorescence signals from BCECF permitted detection of changes in pH of approximately 0.03 to 0.05 units.
Hypoxia produced consistent elevations in [Ca\(^{2+}\)]\(_i\) in brain slices treated with chemical or gaseous hypoxia in medium of pH 7.3. [Ca\(^{2+}\)]\(_i\) began to increase almost immediately on initiation of hypoxia and reached a stable plateau in approximately 20 minutes. The final plateau levels reached in chemical hypoxia (256±45 nmol/L, Table) were not different from those in gaseous hypoxia (273±57). Whereas changes in [Ca\(^{2+}\)]\(_i\) developed gradually during hypoxia, pH\(_i\) decreased immediately on initiation of both types of hypoxia and reached a plateau within 2 to 5 minutes. Fig 1 shows typical traces of [Ca\(^{2+}\)]\(_i\) and pH\(_i\) during the transition from well-oxygenated medium (95% O\(_2\)/5% CO\(_2\)) to gaseous hypoxia.

Most of the change in cytosolic calcium observed during hypoxia was due to uptake of calcium from the perfusate. In perfusate devoid of calcium and containing 1 mmol/L EGTA, the increase in [Ca\(^{2+}\)]\(_i\), was only 5% to 10% (range; mean, 6.8%; n=4) of that seen in perfusate containing 1.8 mmol/L Ca\(^{2+}\). This suggests that only a small amount of the elevation in cytosolic calcium seen during hypoxia was due to release from intracellular organelles or other binding sites.

**Effects of Perfusate pH on Intracellular Calcium During Hypoxia**

The acid-base balance of the aCSF used to produce chemical or gaseous hypoxia significantly influenced pH\(_i\). Chemical hypoxia, in which pH of aCSF was adjusted with HCl, resulted in less intracellular acidification than did gaseous hypoxia, which involved pH adjustments with CO\(_2\). Fig 2 shows pH\(_i\) changes during the different experimental regimens; the actual values before and after anoxic treatment are given in the Table. In all cases, pH\(_i\) decreased 0.2 to 0.6 units during hypoxia. The changes in intracellular acid-base balance observed during hypoxia developed within 2 to 3 minutes and remained stable during the subsequent 20 to 30 minutes.

**Effects of pH Changes on Intracellular Calcium During Normoxia**

There were no significant changes in resting [Ca\(^{2+}\)]\(_i\) produced by changing perfusate pH from 7.35 to 6.6 or 6.2 (ANOVA, P>0.5) in normoxic slices. However, the acid-base balance of the perfusate significantly influenced the total change in cytosolic calcium observed during hypoxia. With lower perfusate pH, greater uptake of calcium was observed (Fig 3). This was true irrespective of whether the acid-base balance of the anoxic perfusate was controlled with CO\(_2\) or with HCl. The magnitude of the effect of acidity on calcium uptake was large: calcium uptake in gaseous hypoxia
The elevations in \( \text{[Ca}^{2+}] \) observed during hypoxia in the 11- to 21-day-old rats in this study are smaller than those seen in rats aged older than 30 days. This is because the magnitude of the elevation in \( \text{[Ca}^{2+}] \) during hypoxia increases with development (P.E.B., unpublished data, 1992). Age effects within the 11- to 21-day range could not affect our conclusions because the age distribution of rats in the various treatment groups was similar, and because the majority (80%) of the rats were aged 18 to 21 days.

Measurements of changes in \( \text{[Ca}^{2+}] \) during hypoxia made with fura-2 are complicated by the fact that hypoxia increases fluorescence from NADH (Sick and Rosenthal\textsuperscript{14} discuss this problem with the dye Indo-1). Although fura-2 emits at a relatively long wavelength (510 nm), up to 30% of the fluorescence signal can come from NADH rather than calcium-bound fura-2. To avoid this error, we subtracted this NADH artifact from the total fluorescence signal. Changes in NADH occurred in a stepwise fashion immediately on initiation of hypoxia; no further changes were seen. If corrections were in error, only the absolute level of \( \text{[Ca}^{2+}] \) would be affected, and changes in calcium developing over the course of minutes would not. Measurements of changes in \( \text{[Ca}^{2+}] \), with fluorescent indicators are limited by the relatively small dynamic range of the calcium-fluorescence relation.\textsuperscript{18} Microelectrode measurements of calcium concentrations are less limited by this constraint and typically show larger elevations in \( \text{[Ca}^{2+}] \), during anoxia (eg, Reference 19).

Estimates of brain slice pH, based on perfusate pH may be problematic. Undoubtedly, pH varies within the slice environment in relation to depth and metabolic activity. These relations may be substantially altered during hypoxia, when cell swelling may further decrease diffusion of acids into the perfusate. We believe, however, that perfusate pH closely approximated slice pH\textsubscript{e} in our study because measurements of extracellular fluid pH with the non-cell-permeant form of the BCECF dye were within 0.1 pH unit of perfusate. Even if slice pH\textsubscript{e} was significantly different from perfusate, this error should be similar at different perfusate pH levels and would not alter the conclusion that low pH\textsubscript{e} augments calcium influx during hypoxia.

Another variable of importance in measurements of \( \text{[Ca}^{2+}] \), with fura-2 is pH. As shown by Ganz et al,\textsuperscript{20} alkalization of pH (over the range 7.1 to 8.0) is associated with increases in apparent \( \text{[Ca}^{2+}] \), as mea-

**Figure 2.** Plot shows change in intracellular pH (pH\textsubscript{i}) resulting from anoxic treatments. Points represent mean±SD, with 10 to 15 slices per point. Gaseous hypoxia was created by perfusion with artificial cerebrospinal fluid of pH 6.2 or 6.6 equilibrated with CO\textsubscript{2} and N\textsubscript{2} mixtures. Chemical hypoxia was created by the cessation of perfusion and the addition of NaCN (final concentration in the cuvette to 60 μmol/L).

was approximately 50% greater at pH 6.2 compared with pH 7.35.

**Perfusate Versus Intracellular pH Effects on Calcium Uptake During Hypoxia**

Fig 4 shows the relation between change in perfusate pH and pH\textsubscript{i} on \( \text{[Ca}^{2+}] \), during hypoxia. As can be seen, there is no relation between pH\textsubscript{i} and calcium uptake (P>0.05), whereas there is a highly significant relation between perfusate pH and calcium change (P<0.001). Regressions of transcellular pH gradients (ie, perfusate pH-pH\textsubscript{i}) versus calcium change were significant if comparisons were restricted to cases when pH\textsubscript{i} was less than perfusate pH and vice versa, but not when the absolute value of the pH difference was compared with changes in \( \text{[Ca}^{2+}] \).

**Discussion**

**Critique of Methods**

This study involved severe insults (hypoxia or NaCN) that result in essentially complete inhibition of aerobic metabolism and depletion of adenosine triphosphate within 10 minutes.\textsuperscript{2} During severe hypoxia, multiple avenues of cytosolic calcium elevation exist, including uptake via NMDA and non-NMDA receptor ion channels, voltage-gated calcium channels, and release from intracellular stores.\textsuperscript{17} Even so, the insults used in this study may not be as severe in some respects as those expected to occur in severe hypoxia/ischemia in vivo, both because they do not involve significant extracellular lactic acidosis and because they do not involve substrate depletion.

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**Figure 3.** Plot shows effects of acid-base conditions on elevation in intracellular calcium (\( \text{[Ca}^{2+}] \)) during gaseous and chemical hypoxia. Points represent mean±SD. *Significant difference from change in \( \text{[Ca}^{2+}] \), in medium of pH 7.3.
Effects of pH on Calcium Influx in Hypoxic Brain Slices

O'Donnell and Bickler

FIG 4. Scatterplot shows relation between intracellular pH (pH,) during hypoxia and change in intracellular calcium ([Ca2+]j) during hypoxia (panel A). Data points represent values from either chemical hypoxia (95% O2/5% CO2 plus 100 μmol/L NaCN) or gaseous hypoxia (CO2 and N2 mixtures). In panel B, extracellular pH during the anoxic insult is plotted as a function of change in [Ca2+]j.

**Effects of Acidity on Ion Channels Associated With Calcium Uptake**

Our results stand in contrast to several other studies that have examined the effects of acidity on calcium uptake during hypoxia. We suggest that this is due to the fact that previous studies have measured cell survival or calcium accumulation in cultured neurons, whereas we studied brain slices. Acid inactivation of NMDA receptors, demonstrated in isolated cells or cultures (eg, References 12 and 22), while perhaps limiting calcium entry via NMDA receptors, would of course not affect calcium entry via other mechanisms. During prolonged severe hypoxia or exposure to NaCN, the contribution of NMDA receptor–linked calcium entry to overall cytosolic calcium levels in our brain slice model is relatively small (P.E.B., unpublished data, 1992) and does not account for all calcium uptake in the intact brain either. In addition, other factors occurring in the intact brain may increase calcium uptake into neurons. For example, glycine released by brain tissue may play a role in increasing calcium flux during hypoxia by blocking acid-inactivation of NMDA receptors. The non-NMDA glutamate receptors (ie, kainate and quisqualate) and γ-aminobutyric acid receptors may also be activated during hypoxia, not as sensitive to pH decreases as the NMDA receptor, and may contribute to elevations in cytosolic calcium. It is therefore not surprising that our results differ from those of Takadera et al or Giffard et al, who examined only the effects of pH on NMDA-mediated processes.

Several very different types of insults may occur during ischemia in the intact brain, and acidity may affect each differently. In the center of an infarct, oxygen and substrate depletion may be severe, but in the surrounding penumbra region, hypoxia and ischemia may be minimal. The chief insult in the penumbra may be accumulation of cytotoxic levels of excitatory amino acid neurotransmitters (for review, see References 5 and 7). It is conceivable that acidity potentiates calcium uptake only in the severely hypoxic core of an infarct and has an opposite effect in the penumbra. This proposal would explain the difference between our results and those showing NMDA receptor inactivation by acidity.

The role of acid-base disturbances in the pathophysiology of hypoxia and ischemia is controversial. Although a number of studies have shown that low pH and pHj during hypoxia and ischemia are associated with adverse changes (reviewed in References 3 and 4) and that pHj values as low as 5.3 are directly cytotoxic, low pH per se may not be. For example, Litt et al showed that supercarbia (pHj reduced 0.63 units below control) with adequate oxygenation did not lead to adverse neurological outcome or change in cellular ATP. In cortical brain slices, only very low pHj (=6.3) with adequate oxygenation was associated with energy depletion. Apparently, acid-base disturbances are injurious only in the context of the pathophysiological changes that occur during hypoxia and ischemia.

We have not determined what mechanisms account for greater cellular uptake of calcium in brain slices during hypoxia under acidic conditions. Increased release of excitatory neurotransmitters, enhanced Na+-Ca2+ exchange, or effects on voltage-gated calcium channels are possibilities. An additional possibility is that calcium release from intracellular stores during hypoxia is pH dependent. Because cytosolic calcium elevation was more than 90% inhibited by calcium-free perfusate, this effect is likely to be relatively small. In addition, there was no statistically significant correlation observed between [Ca2+]j and change in pHj.

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**References**

The results reported by O'Donnell and Bickler question the postulate that a reduction of extracellular pH (pH₆) ameliorates neuronal damage due to glutamate exposure or anoxia by blocking calcium influx through NMDA-gated channels. Thus, the authors find that in slices made anoxic by removal of O₂ supply or addition of NaCN, low pH enhances rather than attenuates the effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. 

Nonetheless, the present data are important in that they highlight a controversy in the field. Thus, whereas acidosis in vitro blocks NMDA receptor–coupled Ca²⁺ currents, reduces ⁴⁰Ca²⁺ influx, and ameliorates neuronal damage due to glutamate and anoxia,¹³ acidosis in vivo exaggerates damage due to dense, transient ischemia.⁴ What adds to the complexity of the problem is that cell calcium accumulation in rat cortical cultures, due to glutamate and anoxia, is much the same way as it is retarded by the NMDA antagonist dizocilpine.⁶ This suggests that hyperglycemia and hypercapnia reduce Ca²⁺ influx by lowering pH and that the effect is exerted on NMDA-gated Ca²⁺ currents. Why then are preischemic hyperglycemia or by excessive hypercapnia in much the same way as it is retarded by the NMDA antagonist dizocilpine maleate?⁵ This suggests that hyperglycemia and hypercapnia reduce Ca²⁺ influx by lowering pH and that the effect is exerted on NMDA-gated Ca²⁺ currents. Why then are preischemic hyperglycemia and/or excessive hypercapnia associated with exaggerated ischemic brain damage? The discrepancy in results cannot be explained by a lack of effect of acidosis on voltage-gated calcium channels, since acidosis also blocks calcium influx through such channels (O.Y. YiBing, T. Kristian, P. Mellerård, and B.K. Siesjö, unpublished observations); besides, the in vitro blockade of calcium influx into cells is sufficient to retard net calcium influx and to lower Ca²⁺ concentrations in the hippocampal slice: complications of labile NADH fluorescence. J Neurosci Methods. 1989;28:129-132. 


Editorial Comment

The results reported by O'Donnell and Bickler question the postulate that a reduction of extracellular pH (pH₆) ameliorates neuronal damage due to glutamate exposure or anoxia by blocking calcium influx through NMDA-gated channels. Thus, the authors find that in slices made anoxic by removal of O₂ supply or addition of NaCN, low pH enhances rather than attenuates the rise in the free cytosolic calcium concentration (Ca²⁺). This effect is opposite that expected from previous results obtained on cultured neurons. The results were the same whether "gaseous" or "chemical" hypoxia was used. As suggested by O'Donnell and Bickler, the discrepancy could be explained by the difference in response between isolated and composite tissues or by the fact that influx through channels other than the NMDA-gated ones is not blocked by low pH₆.

The data reported by O'Donnell and Bickler are really interesting, but it is not clear that they relate to the effect of acidosis on ischemic damage. One reason for this is that we lack information on how anoxia damage in brain slices is influenced by changes in pH₆ or pH. It is also unknown what constitutes the [Ca²⁺] signal in brain slices, i.e., whether the signal derives predominantly from neurons or glial cells. In theory, therefore, it is possible that the signal reflects changes in glial cells and that acidosis reduces calcium influx into neurons.
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