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

Brendan R. O'Donnell, MS; Philip E. Bickler, MD, PhD

Background and Purpose: Acidity of brain intracellular and extracellular fluids appears to increase brain injury from stroke, but low extracellular pH decreases the activity of N-methyl-d-aspartate receptor ion channels and decreases calcium influx into isolated neurons. To further investigate the role of acid-base balance in hypoxic brain injury, we studied the influences of intracellular and extracellular pH on calcium influx in cortical brain slices during hypoxia.

Methods: Intracellular calcium ([Ca\(^{2+}\)]) and pH (pHi) were measured fluorometrically with the dyes fura-2 and biscarboxyethyl carboxyfluorescein, respectively, during two types of hypoxia: (1) slice perfusate equilibrated with N\(_2\)/CO\(_2\) at pH 6.6 or 6.2 (“gaseous hypoxia”) or (2) perfusate equilibrated with 95% O\(_2\)/5% CO\(_2\) plus 100 \(\mu\)mol/L NaCN at pH 7.3, 6.6, or 6.2 (“chemical hypoxia”).

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

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

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calcium during hypoxia in brain slices. Because changes in pH in hypoxic brain are due both to hypercarbia and metabolic acidosis (ie, from accumulation of protons and lactate generated from anaerobic glycolysis), we separately examined the effects of acidity caused by high CO\(_2\) and by addition of mineral acid to the perfusate during hypoxia. Because CO\(_2\) acidifies both intracellular and extracellular fluids, whereas exogenously added mineral acids have minimal direct effects on pHi, we have been able to study the relative roles of changes of pHi and pHe on changes in [Ca\(^{2+}\)], during hypoxia.

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 x 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\(_2\)PO\(_4\), 0.9, and glucose 10 bubbled with 5% CO\(_2\)/95% O\(_2\), pH 7.40 at 37°C, then gassed with cyanocrylate cement to a mounting block and resubmerged in the chilled aCSF. Slices 250-\(\mu\)m 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\(_2\), PC\(_2\)= 40±5 mm Hg.

Fura-2 acetoxyethyl ester and biscarboxyethyl carboxyfluorescein acetoxyethyl ester (BCECF-AM; Molecular Probes, Eugene, Ore) were added (from 1-mmol/L stock solutions in dry dimethyl sulfoxide, final concentration 3 to 5 \(\mu\)mol/L) to
the aCSF containing the brain slices to permit measurements of [Ca\textsuperscript{2+}] 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 malleable base and fitted into a 3.0-mL fluorometer cuvette filled with O\textsubscript{2}/CO\textsubscript{2}-equilibrated aCSF. A cuvette 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\textsuperscript{2+}] (nanomolar) was calculated from fluorescent ratios (340:380) with the following equation:

\[ [Ca^{2+}]_j = K_0 \frac{(R - R_{min})/(R_{max} - R)}{(F_0/F_1)} \]

where \( K_0 \) is 225 mmol/L and R is the ratio of the fluorescence intensity excited by 340:380 nm, after first correcting for background fluorescence. Background fluorescence was measured at the end of each study by applying calcium-free Earle’s buffer solution containing 2 mmol/L ethylene glycol-bis(\( \beta \)-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 5 mmol/L MnCl\textsubscript{2}, and 10 mmol/L imidazole. F\textsubscript{0}/F\textsubscript{1} is the ratio of the 380-nm excitation intensity at zero and saturating Ca\textsuperscript{2+}, levels. R\textsubscript{min} and R\textsubscript{max} were recorded by exposing the slice to Earle’s salt solution containing 1.5 mmol/L Ca\textsuperscript{2+} and imidazolinc (saturating the cytoplasmic fura with Ca\textsuperscript{2+}), then to one with 2 mmol/L EGTA without Ca\textsuperscript{2+}.

Measurement of pH was done simultaneously with that of [Ca\textsuperscript{2+}] by interleaving excitation at wavelengths of 440 and 495 nm with those for fura-2. Emission intensity for BCECF was measured at the 380-nm excitation intensity at zero and saturating Ca\textsuperscript{2+}, levels. The temporal course of [Ca\textsuperscript{2+}] was tracked to 37±0.5°C. NaCN (to achieve a final concentration of 60 \( \mu \)mol/L) was introduced into the cuvette immediately after introduction of the test solution. Measurements of [Ca\textsuperscript{2+}] 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.

**Biological Control of pH**

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 HC\textsubscript{1} during bubbling with the 95% O\textsubscript{2}/5% CO\textsubscript{2} mixture at 37°C. NaCN (to achieve a final concentration of 60 \( \mu \)mol/L) was introduced into the cuvette immediately after introduction of the test solution. Measurements of [Ca\textsuperscript{2+}] 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\textsuperscript{2+}] 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\textsuperscript{2+}] of approximately 10 nmol/L between 100 and 800 nmol/L and a change of roughly 25 nmol/L when [Ca\textsuperscript{2+}] 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.
Intracellular Calcium and pH in Brain Slices Treated With Gaseous Hypoxia (N₂ and CO₂ to Produce Indicated pH) or Chemical Hypoxia (95% O₂/5% CO₂ Plus 100 μmol/L NaN₃)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>Hypoxia</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH, [Ca²⁺]</td>
<td>pH, [Ca²⁺]</td>
</tr>
<tr>
<td>Gaseous hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.6</td>
<td>7.10±0.11 (5)†</td>
<td>6.57±0.13* (5)</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>7.07±0.09 (4)†</td>
<td>6.50±0.09* (4)</td>
</tr>
<tr>
<td>Chemical hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>6.97±0.04 (5)†</td>
<td>6.95±0.05 (5)</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>6.88±0.09 (17)</td>
<td>6.72±0.11* (17)</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>6.86±0.05 (5)†</td>
<td>6.67±0.10* (5)</td>
</tr>
</tbody>
</table>

Values are mean±SD, with n in parentheses. pH indicates intracellular pH; [Ca²⁺], intracellular calcium. Baseline values before gaseous hypoxia obtained during incubation in perfusate equilibrated with 95% O₂/5% CO₂ at extracellular pH 7.30; baseline data for chemical hypoxia obtained in 95% O₂/5% CO₂ at indicated pH. *Significant difference from baseline value (P<.05, analysis of variance). †Values measured at pH 7.3 with 95% O₂ and 5% CO₂.

Apparent slice pH, determined with non-cell-permeant BCECF dye closely approximated perfusate pH in five slices maintained in perfusate of pH 6.6 to 7.4. In all cases, slice pH, was between 0.03 and 0.07 pH units more acid than perfusate. Chemical anoxia with or without a decrease in perfusate pH caused a further acidification of apparent pH, by an additional 0.01 to 0.03 units compared with perfusate (n=4).

Changes in Intracellular Calcium and pH During Gaseous or Chemical Hypoxia

Hypoxia produced consistent elevations in [Ca²⁺], in brain slices treated with chemical or gaseous hypoxia in medium of pH 7.30. [Ca²⁺], 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 [Ca²⁺], developed gradually during hypoxia, pH, decreased immediately on initiation of both types of hypoxia and reached a plateau within 2 to 5 minutes. Fig 1 shows typical traces of [Ca²⁺], and pH, during the transition from well-oxygenated medium (95% O₂/5% CO₂) 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 [Ca²⁺], was only 5% to 10% (range; mean, 6.8%; n=4) of that seen in perfusate containing 1.8 mmol/L Ca²+. 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 pH During Hypoxia

The acid-base balance of the aCSF used to produce chemical or gaseous hypoxia significantly influenced pH, 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 CO₂. Fig 2 shows pH, changes during the different experimental regimens; the actual values before and after anoxic treatment are given in the Table. In all cases, pH, 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 [Ca²⁺], produced by changing perfusate pH from 7.35 to 6.6 or 6.2 (ANOVA, P>.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 CO₂ or with HCl. The magnitude of the effect of acidity on calcium uptake was large: calcium uptake in gaseous hypoxia
range could not affect our conclusions because the magnitude of the elevation in \([\text{Ca}^{2+}]_i\) during hypoxia increases with development (P.E.B., unpublished data, 1992). Age effects within 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 \([\text{Ca}^{2+}]_i\) during hypoxia increases with development.

Another variable of importance in measurements of \([\text{Ca}^{2+}]_i\), with fura-2 is \(pH_i\). As shown by Ganz et al, alkalinization of \(pH_i\) (over the range 7.1 to 8.0) is associated with increases in apparent \([\text{Ca}^{2+}]_i\), as mea-
Effects of pH on Calcium Influx in Hypoxic Brain Slices

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Fig 4. Scatterplot shows relation between intracellular pH (pHi) during hypoxia and change in intracellular calcium ([Ca^{2+}]) during hypoxia (panel A). Data points represent values from either chemical hypoxia (95% O_{2}/5% CO_{2} plus 100 μmol/L NaCN) or gaseous hypoxia (CO_{2} and N_{2} mixtures). In panel B, extracellular pH during the anoxic insult is plotted as a function of [Ca^{2+}].

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 binding by fura-2 over the pH range 6.75 to 7.05, which encompasses most of our data.

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, are 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 pH, during hypoxia and ischemia are associated with adverse changes (reviewed in References 3 and 4) and that pH, values as low as 5.3 are directly cytotoxic, low pH per se may not be. For example, Litt et al showed that supercarbia (pH, 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 pH, (=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^{+}-Ca^{2+} 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 [Ca^{2+}] and change in pH.

Acknowledgments

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References

The results reported by O'Donnell and Bickler question the postulate that a reduction of extracellular pH (pH$_e$) 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 O$_2$ supply or addition of NaCN, low pH$_e$ enhances rather than attenuates the rise in the free cytosolic calcium concentration (Ca$^{2+}$). 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 pH$_e$. 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 anoxic damage in brain slices is influenced by changes in pH$_e$ or pH$_i$. It is also unknown what constitutes the [Ca$^{2+}$]$_i$ signal in brain slices, ie, 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. Nonetheless, the present data are important in that they highlight a controversy in the field. Thus, whereas acidosis in vitro blocks NMDA receptor-coupled Ca$^{2+}$ currents, reduces 4Ca$^{2+}$ influx, and ameliorates neuronal damage due to glutamate and anoxia,1,2 acidosis in vivo exaggerates damage due to dense, transient ischemia.3,4 What adds to the complexity of the problem is that cell calcium related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. J Cereb Blood Flow Metab. 1989;9:127-140.


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