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\(^{2+}\)]\(_i\)) and pH (pHi) were measured fluorometrically with the dyes fura-2 and biscalboxyethyl 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. (Stroke. 1994;25:171-177.)

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 pH,\(^3\) we have been able to study the relative roles of changes of pHi and pH\(_e\) 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 × 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, 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 glued with cyanoacrylate 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\(_2/5\% CO_2\), P\(_{CO_2}\) was 40±5 mm Hg.

Fura-2 acetoxymethyl ester and biscalboxyethyl 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 \(\mu\)mol/L) to
the aCSF containing the brain slices to permit measurements of [Ca\(^{2+}\)] 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 mesh baffle and fitted into a 3.0-mL fluorometer cuvette filled with O\(_2\)/CO\(_2\)-equilibrated aCSF. A second inlet 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. \(^{13}\) Slices were alternatively 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. \([Ca^{2+}]\), (nanomolar) was calculated from fluorescence ratios (340:380) with the following equation:

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

where \(K_o\) is 225 nmol/L and \(R\) is the ratio of fluorescence intensity excited by 340:380 nm. Since the effect of acute changes in \(P_{CO_2}\) on pH is known, \(^{3}\) changing \(P_{CO_2}\) can be used as a method of pH calibration. A \(P_{CO_2}\) 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.

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where \(K_o\) is 225 nmol/L and \(R\) is the ratio of 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(β-aminooxyethoxy) N,N,N',N'-tetraacetic acid (EGTA), 5 mmol/L MnCl\(_2\), and 10 mmol/L iodonium. \(F_0\) is the ratio of the 380-nm excitation intensity at zero and saturating \([Ca^{2+}]\), levels. \(R_{max}\) and \(R_{min}\) were recorded by exposing the slice to Earle’s salt solution containing 1.5 mmol/L Ca\(^{2+}\) and iodonium (saturating the cytoplasmic fura with \([Ca^{2+}]\)), then to one with 2 mmol/L EGTA without \([Ca^{2+}]\).

Measurement of pH was done simultaneously with that of \([Ca^{2+}]\), by interleaving excitation at wavelengths of 440 and 495 nm with those for fura-2. Excitation intensity for BCECF was recorded every 0.5 seconds at 530 nm. Excitation at 495 nm (instead of the usual 500 nm) was used with BCECF because this greatly reduced the intensity of scattered light detected at 530 nm and improved sensitivity to pH changes. Calibration of BCECF was done by two methods. First, equilibration of pH and \([Ca^{2+}]\) was done using the \(K^+\)-H+ exchanger nigericin. At the end of some of the studies, the cells were perfused with a high \(K^+\) Earle’s solution containing 100 mmol/L KCl and 10 mmol/L nigericin. \(F_1\) was calculated from the equation presented by Eisner et al. \(^{14}\)

\[
\text{pH} = pK + \log(R - R_{min})/(R_{max} - R) + \log(F_{0}/F_{1})
\]

where \(R_{min}\) is the ratio at extreme acid pH and \(R_{max}\) at extreme alkaline pH. These values are taken to establish the range of fluorescence ratios possible in the physiological pH range. The \(n\)igericin- and \(P_{CO_2}\) methods were used together.

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 \([Ca^{2+}]\) of approximately 10 nmol/L between 100 and 800 nmol/L and a change of roughly 25 nmol/L when \([Ca^{2+}]\) was greater than 750 nmol/L. Perfusate 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.
Effects of pH on Calcium Influx in Hypoxic Brain Slices

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 NaCN)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline pH</th>
<th>Baseline [Ca²⁺]</th>
<th>Hypoxia pH</th>
<th>Hypoxia [Ca²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaseous hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.6</td>
<td>7.10±0.11</td>
<td>135±48</td>
<td>6.57±0.13</td>
<td>273±57</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>7.07±0.09</td>
<td>157±54</td>
<td>6.50±0.09</td>
<td>366±70</td>
</tr>
<tr>
<td>Chemical hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>6.97±0.04</td>
<td>129±43</td>
<td>6.95±0.05</td>
<td>163±23</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>6.88±0.09</td>
<td>155±36</td>
<td>6.72±0.11</td>
<td>256±45</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>6.86±0.05</td>
<td>180±36</td>
<td>6.67±0.10</td>
<td>333±53</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 \([\text{Ca}^{2+}]_f\) during those seen in rats aged older than 30 days. This is established data, 1992). Age effects within the 11- to 21-day 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 expected to occur in severe hypoxia/ischemia in vivo, ever, that perfusate pH closely approximated slice pH,. BCECF dye were within 0.1 pH unit of perfusate. 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.

The elevations in \([\text{Ca}^{2+}]_f\) 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 \([\text{Ca}^{2+}]_f\) 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 \([\text{Ca}^{2+}]_f\) during hypoxia made with fura-2 are complicated by the fact that hypoxia increases fluorescence from NADH (Sick and Rosenthal15 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 \([\text{Ca}^{2+}]_f\) would be affected, and changes in calcium developing over the course of minutes would not. Measurements of changes in \([\text{Ca}^{2+}]_f\), with fluorescent indicators are limited by the relatively small dynamic range of the calcium-fluorescence relation.18 Microelectrode measurements of calcium concentrations are less limited by this constraint and typically show larger elevations in \([\text{Ca}^{2+}]_f\), 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, 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, was significantly different from perfusate, this error should be similar at different perfusate pH levels and would not alter the conclusion that low pH, augments calcium influx during hypoxia.

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

Acknowledgments

This study was supported by a grant from the Foundation for Anesthesia Education and Research to Dr. Bickler. This work represents a portion of a Master of Arts thesis submitted to San Francisco State University by Mr. O'Donnell.

References

The results reported by O'Donnell and Bickler question the postulate that a reduction of extracellular pH (pHe) ameliorates neuronal damage due to glutamate exposure or anoxia by blocking calcium influx through NMDA-gated channels. Thus, the authors found 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+]i). This effect is opposite to that reported in cultured neurons. The data 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 intact and isolated tissues or by the fact that influx through channels other than the NMDA-gated ones is not blocked by low pH.

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 or pHe. It is also unknown whether the [Ca2+]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.
Influence of pH on calcium influx during hypoxia in rat cortical brain slices.
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Stroke. 1994;25:171-177
doi: 10.1161/01.STR.25.1.171

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