Role of Na\textsuperscript{+}/H\textsuperscript{+} exchanger during O\textsubscript{2} deprivation in mouse CA1 neurons

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Yao, Hang, Xiang-Qun Gu, Robert M. Douglas, and Gabriel G. Haddad. Role of Na\textsuperscript{+}/H\textsuperscript{+} exchanger during O\textsubscript{2} deprivation in mouse CA1 neurons. Am J Physiol Cell Physiol 281: C1205–C1210, 2001.—To determine the role of membrane transporters in intracellular pH (pHi) regulation under conditions of low microenvironmental O\textsubscript{2}, we monitored pHi in isolated single CA1 neurons using the fluorescent indicator carboxysemaphorin rhodfluor-1 and confocal microscopy. After total O\textsubscript{2} deprivation or anoxia (P O\textsubscript{2} = 0 Torr), a large increase in pHi was seen in CA1 neurons in HEPES buffer, but a drop in pHi, albeit small, was observed in the presence of HCO\textsubscript{3}\textsuperscript{-}. Ionic substitution and pharmacological experiments showed that the large anoxia-induced pHi increase in HEPES buffer was totally Na\textsuperscript{+} dependent and was blocked by HOE-694, strongly suggesting the activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE). Also, this pHi increase in HEPES buffer was significantly smaller in Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 1 (NHE1) null mutant CA1 neurons than in wild-type neurons, demonstrating that NHE1 is responsible for part of the pHi increase following anoxia. Both chelerythrine and H-89 partly blocked, and H-7 totally eliminated, this anoxia-induced pHi increase in the absence of HCO\textsubscript{3}\textsuperscript{-}. We conclude that J) O\textsubscript{2} deprivation activates Na\textsuperscript{+}/H\textsuperscript{+} exchange by enhancing protein kinase activity and 2) membrane proteins, such as NHE, actively participate in regulating pHi during low-O\textsubscript{2} states in neurons.

The regulation of intracellular pH (pHi) in neurons has been investigated fairly actively in the past several years, and it is clear now that this regulation is very complex. A number of membrane proteins that are relevant to this regulation are present and functional in neurons, and their role in various conditions is being delineated (3, 8, 20, 22–25, 27, 32).

Although we and others have been interested in understanding how neurons sense and respond to lack of oxygen, we still do not know how the various membrane proteins regulate pHi and how they participate in determining the pHi response to O\textsubscript{2} deprivation. For example, we do not know whether the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) is stimulated or inactivated during hypoxia. It can be argued that a drop in pHi during hypoxia can activate this exchanger (1). However, a drop in extracellular pH may inhibit it (33). There are also other factors in the microenvironment that may have major effects on membrane proteins and pH\textsubscript{i} change. These include extracellular ions and neurotransmitters released from adjacent neurons and glia (14, 29). Hypoxia may, therefore, change pH\textsubscript{i} by affecting the function of membrane transporters, intracellular metabolism, and the microenvironment around cells.

To examine some of these mechanisms during O\textsubscript{2} deprivation, we needed to simplify the system. In this work, we studied freshly dissociated single cells that were constantly perfused. We performed our experiments on CA1 neurons because we have considerable experience with them (3, 4, 11, 32). In addition, there are many studies in the literature using these neurons; hence, these studies could be helpful from a comparative point of view (12, 26). Our aim was then to investigate the role of neuronal membrane proteins involved in pH\textsubscript{i} regulation during O\textsubscript{2} deprivation. Our hypothesis was that neuronal membrane proteins, such as NHE, play a critical role in the regulation of pH\textsubscript{i} during low-O\textsubscript{2} states.

MATERIALS AND METHODS

Cell preparation. B6SJL,+/swe (slow-wave epilepsy) mice were obtained from Jackson Laboratories (9). These heterozygous mice (+/swe) were mated in our institution, and the resulting homozygous Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 1 (NHE1) mutant (25%) and wild-type (25%) F1 mice progeny were used at the age of 21–30 days. The mice genotypes were confirmed by a PCR-based test. Hippocampi were removed and sliced into transverse sections of 400 \( \mu \text{m} \) in thickness. The slices were immediately transferred to a container with 25 ml of fresh, oxygenated, and slightly stirred HEPES buffer at room temperature. After 30 min of trypsin (0.08%) and 20 min of protease (0.05%) digestion, the slices were washed and left in the oxygenated solution. The CA1 region was then dissected out and triturated in a small volume (0.25 ml) of HEPES buffer. When chelerythrine chloride, H-89, or H-7 was used, cells were incubated with the inhibitor for 1 h before pHi was measured (16). These studies have been approved by the Yale Animal Care and Use Committee.

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Solutions. The HEPES-buffered solution contained (in mM) 125 NaCl, 3 KCl, 1.2 CaCl\(_2\), 1.2 MgSO\(_4\), 1.25 NaH\(_2\)PO\(_4\), 30 HEPES, and 10 glucose. This solution was titrated to pH 7.38 at 35°C with NaOH. Na\(^+\) was removed from the solution by replacing NaCl and NaH\(_2\)PO\(_4\) with N-methyl-D-glucamine (NMDG) and KH\(_2\)PO\(_4\), respectively. Only 1.5 mM Na\(^+\) remained in the anoxia solution since dithionite is in the form of sodium salt. For the CO\(_2\)/HCO\(_3\)\(^-\) solution, HEPES was replaced by 22 mM NaHCO\(_3\) and bubbled with 5% CO\(_2\) and 95% O\(_2\). The nigericin calibration solution contained (in mM) 105 KCl, 50 NMDG, 5 MgSO\(_4\), 10 glucose, and 30 HEPES. TNE buffer contained (in mM) 10 Tris (pH 7.5), 400 NaCl, 100 EDTA, and 0.6% SDS. TE buffer contained (in mM) 10 Tris (pH 8.0) and 10 EDTA. Cell-Tak was purchased from Collaborative Research (Bedford, MA), and carboxyseminalphathorhodafluor-1 (SNARF-1) was obtained from Molecular Probes (Eugene, OR). Nigericin, chelerythrine chloride, H-89, and H-7 were purchased from Sigma. HOE-694 was obtained as a gift from Dr. Hans-J. Lang (HMR/Hoechst Marion Roussel Chemical Research, Frankfurt, Germany). Dithionite was obtained from Marion Roussel Chemical Research, Frankfurt, Germany). Nigericin, chelerythrine chloride, H-89, and H-7 were purchased from Sigma. SNARF-1 (prepared in dimethyl sulfoxide) for

Statistics. Data are presented as means ± SD. Levels of significance were assessed using paired and unpaired forms of the Student’s t-test. Differences in means were considered significant when \(P = 0.05\).

RESULTS

This study is based on pH measurements in 152 CA1 neurons, which fulfilled our study criteria (32). In HEPES buffer, the mean steady-state pH\(_i\) was 7.22 ± 0.24 (n = 66), and this increased to 7.39 ± 0.20 (n = 51) in HCO\(_3\)\(^-\). Anoxia induces pH\(_i\) changes in CA1 neurons. The pH\(_i\) of each CA1 neuron was monitored before, during, and after applying anoxia. In the presence of CO\(_2\)/HCO\(_3\)\(^-\), cells responded to anoxia with a slow and relatively small drop in pH\(_i\). However, in the absence of CO\(_2\)/HCO\(_3\)\(^-\), cells responded with a dramatic pH\(_i\) increase after the initiation of anoxia (Fig. 1A). The average decrease of pH\(_i\) over 5 min of anoxia in CA1 neurons was 0.06 ± 0.11 pH units (n = 10) in the presence of CO\(_2\)/HCO\(_3\)\(^-\), but the mean increase in HEPES buffer was 0.46 ± 0.13 pH units (n = 13, P < 0.001; Fig. 1B). Hypoxia (without the addition of dithionite) also caused an alkalization in CA1 neurons in HEPES buffer. Although this alkalization was significantly smaller (0.12 ± 0.07 pH units, n = 8, P < 0.05) than the alkalization induced by anoxia, both hypoxia and anoxia (with dithionite) induced an increase in pH\(_i\). It would seem, then, that the increase in pH\(_i\) did not result from a nonspecific action of dithionite.

![Image](https://i.imgur.com/3QX.png)

**Fig. 1.** Anoxia-induced intracellular pH (pH\(_i\)) change in CA1 neurons in the presence and absence of CO\(_2\)/HCO\(_3\), A: 2 neurons with similar initial pH\(_i\) are shown; one perfused with CO\(_2\)/HCO\(_3\) solution showed a slight decrease in pH\(_i\) when exposed to 5 min of anoxia, and the other was perfused with HEPES buffer and responded to the same anoxic stimulation with a dramatic increase in pH\(_i\). B: bar graph showing mean change of pH\(_i\) induced by 5 min of anoxia in both the presence and absence of CO\(_2\)/HCO\(_3\). Means are significantly different from each other.
The effect of Na\(^+\) removal on the anoxia-induced pH\(_i\) increase in HEPES. NHE has been considered to play a very important role in the pH\(_i\) regulation of central nervous system (CNS) neurons. To determine whether the increase in pH\(_i\) with anoxia is dependent on Na\(^+\)/H\(^+\) exchange, we first studied neurons in HEPES buffer in the presence or absence of Na\(^+\). The removal of Na\(^+\) caused an acidification in normoxic conditions (Fig. 2A), probably because of the inhibition of Na\(^+\)-dependent acid extruders (such as NHE) or reversal of their activity. This acidification partly recovered, and this is most likely due to the activation of H\(^+\)-ATPases on the cell membrane. After the pH\(_i\) had reached a plateau in the absence of Na\(^+\), cells were exposed to anoxia. As seen in Fig. 2A, pH\(_i\) did not increase, rather, it actually decreased. The mean pH\(_i\) drop was 0.10 ± 0.10 (n = 7; Fig. 2, A and B), which contrasts with the response of cells bathed with HEPES containing Na\(^+\), demonstrating a major increase in pH\(_i\) (0.46 ± 0.13, n = 13, P < 0.001). Therefore, in the absence of HCO\(_3^-\), the anoxia-activated acid extrusion and increase in pH\(_i\) was totally dependent on Na\(^+\), and the most likely candidate responsible for this acid extrusion is the NHE.

The effect of HOE-694 on anoxia-induced pH\(_i\) change. To be able to dissect out the role of the transporters that regulate pH\(_i\) during O\(_2\) deprivation, we further examined the effect of the neuronal NHE blocker HOE-694 (32) on the anoxia-induced pH\(_i\) changes in CA1 neurons in HEPES buffer. The anoxia-induced alkalinization was almost totally eliminated by 100 \(\mu\)M HOE-694 in HEPES buffer (Fig. 3A), and the pH\(_i\) change was 0.02 ± 0.13 units (n = 7, P < 0.001 vs. control group; Fig. 3B, left).

NHE1 is involved in the anoxia-activated acid-extrusion process. Because HOE-694 is a relatively nonspecific NHE blocker and since NHE1 is the most ubiquitous isoform in the CNS (18, 19), we took advantage of the NHE1 null mutant mouse to examine whether the anoxia-induced alkalinization in HEPES is the result of overactivation of NHE1. Figure 4A illustrates the pattern and amplitude of pH\(_i\) changes following anoxia in neurons isolated from both wild-type and NHE1 mutant mice in HEPES solution. Compared with the response in the wild-type neuron, the mutant neuron showed a significantly slower and smaller pH\(_i\) change following anoxia. The average anoxia-induced pH\(_i\) increase in mutant neurons was 0.30 ± 0.13 (n = 10), and this was significantly smaller than that in wild-type neurons (0.46 ± 0.13 pH units, n = 13, P < 0.05; Fig. 4B). This result suggested that NHE1 is activated during anoxia and is only partly responsible for the anoxia-induced alkalinization seen in the absence of HCO\(_3^-\). Presumably, other isoforms, such as NHE2, NHE4, and NHE5, might have also been activated. Another possible mechanism for the alkalinization is the activation of H\(^+\)-ATPases.

Kinase inhibition markedly attenuates the anoxia-activated acid extrusion. To understand how anoxia activates the NHE, we first pretreated cells with the rather nonspecific protein kinase inhibitor H-7 (60 \(\mu\)M) to determine whether reducing kinase activity can affect the anoxia-induced alkalinization. Figure 5A shows one example from these experiments. In HEPES...
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Fig. 4. Anoxia-induced pH\(_i\) increase was smaller in the Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) null mutant neurons. A: 2 neurons [one was a wild-type (WT) and the other a NHE1 mutant] with similar initial pH\(_i\). Both neurons were perfused with HEPES buffer and subjected to 5 min of anoxia. Although both neurons had a similar pattern in pH\(_i\) change in response to anoxia, a smaller increase was seen in the mutant neuron. B: bar graph showing mean change of pH\(_i\) induced by 5 min of anoxia in both NHE1 mutant and wild-type neurons. Means are significantly different from each other.

solution, the H-7-pretreated cell responded to anoxia very little, if at all (0.006 ± 0.0324 pH units, n = 8; Fig. 5D). These data demonstrated that kinase activity was enhanced during anoxia, which, in turn, increased the activity of the NHE.

To investigate the possible role of protein kinase C (PKC) or protein kinase A (PKA) in the modulation of pH\(_i\) by NHE during anoxia, we pretreated neurons with either chelerythrine (1.5 μM), a PKC inhibitor, or H-89 (30 μM), a PKA inhibitor, and examined their response to anoxia. Figure 5B shows an example of the pH\(_i\) measurement of chelerythrine-pretreated CA1 neurons. In HEPES solution and in the presence of this blocker, although pH\(_i\) increased (0.12 ± 0.20 pH units, n = 6), the change was significantly smaller than for the untreated group (0.46 ± 0.13 pH units, n = 13, P < 0.001; Fig. 5D). These results suggested that the activation of PKC was at least partly responsible for the anoxia-induced pH\(_i\) increase. Figure 5C also shows only a slight pH\(_i\) increase in another experiment of a neuron pretreated with H-89 and followed by anoxia. The mean anoxia-induced pH\(_i\) change was very small (0.05 ± 0.06 pH units, n = 6) compared with the control group (0.46 ± 0.13 pH units, n = 13, P < 0.001; Fig. 5D).

DISCUSSION

Because it has been well demonstrated that pH\(_i\) is reduced in cells during hypoxia/ischemia in vivo (12, 26), it would seem reasonable to assume that anaerobic metabolism plays an important role in lowering pH\(_i\). However, the role of various neuronal exchangers and transporters in pH\(_i\) regulation during low-O\(_2\) conditions has not been well studied. One major reason for trying to understand the role of such membrane proteins during hypoxia is that there is already evidence from work in heart muscles and from our previous work on neurons that Na\(^+\)/H\(^+\) exchange and Na\(^+\)\(^+\) loading play an important role in the pathogenesis of hypoxic or ischemic neuronal injury (7, 8, 15, 17). It is important to mention here that our current studies showed that pH\(_i\) had a seemingly paradoxical change during anoxia, i.e., an increase in pH\(_i\) during anoxia in HEPES solution in the absence of CO\(_2\)/HCO\(_3\)\(_2\). Clearly, however, we do not suggest that pH\(_i\) increases during anoxia in vivo. We should highlight two issues in this regard: 1) the studies in the literature that showed a pH\(_i\) decrease during anoxia or hypoxia were done in the presence of HCO\(_3\), unlike our experiments in which we used both solutions containing or lacking CO\(_2\)/HCO\(_3\), and 2) by studying the effect of anoxia in HEPES as well as in HCO\(_3\), we have been able to uncover the response of some membrane transporters in low-O\(_2\) environments.

Effect of anoxia on the regulation of pH\(_i\). One of our major findings in this paper is that O\(_2\) deprivation
induced an alkalinization in the absence of CO₂/HCO₃⁻. To ascertain that this increase in pHᵢ during anoxia is not a nonspecific effect of the O₂ scavenger that we used (dithionite), we performed two types of experiments. In the first, we exposed neurons to a hypoxic solution, a solution that was bubbled with nitrogen only, with no dithionite. Although the cells responded with a smaller pHᵢ increase in this hypoxic solution than in anoxia, this increase in pHᵢ in the absence of dithionite supports the idea that the increase in pHᵢ is not related to the O₂ scavenger per se but to the lowering of P₀₂. In the second type of experiment, we used another pH-sensitive dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), with the same cells and obtained similar data (data not shown), indicating that the increase in pHᵢ in anoxia is not dependent on interactions between SNARF-1 and dithionite.

It is reasonable to assume that the changes in pHᵢ that are observed in vivo during low-O₂ states at any one time are the net result of the simultaneous activation (or inactivation) of a variety of cellular processes. Because physiological solutions are often used, i.e., CO₂/HCO₃⁻-containing solutions, the role of certain exchangers, such as the NHE, may not be readily observed. Similarly, the differences in the response of CA1 cells when CO₂/HCO₃⁻ solution is used or is omitted provided us with the idea that HCO₃⁻-dependent acid loaders and acid extruders might be involved. Another potential reason for the differences between in vivo and in vitro situations is related to the control over the microenvironment. The same consideration may apply to cultured preparations. For example, the accumulation of H⁺ in the extracellular space might affect the activity of NHE (33) in vivo or cultured neurons more than in our freshly dissociated neurons, since we control the composition of the perfusate throughout the experiments, such as the ion concentration and extracellular pH, to tease apart some of the mechanisms that are operative. Indeed, in their recent work, Diarra et al. (10) have shown an anoxia-induced acidification in HEPES buffer in cultured neurons, although a dramatic alkalinization was seen after the reapplication of O₂. The major difference between our preparation and theirs may be related to the presence of neuronal connectivity and synaptic activity among cultured neurons that may constitute a different microenvironment.

Na⁺/H⁺ exchange is activated during anoxia. In the mammalian CNS, the NHE is the major HCO₃⁻-independent acid extruder and, so far, six isoforms of its gene family have been identified. However, it has previously been shown that NHE1 is the most ubiquitously expressed (2, 19, 21) in the CNS. Although hippocampal neurons do not have the full complement of NHE isoforms (19), our current data demonstrate that the anoxia-induced pHᵢ increase could be caused by the activation of NHE. Although NHE1 is a major membrane protein in the CNS, other isoforms of NHE could also be involved. HOE-694 eliminated the anoxia-induced pHᵢ increase completely, but the fact that we still had a substantial rise in pHᵢ in the NHE1 mutant during anoxia argues for the lack of specificity of HOE-694 for NHE1 activity. This lack of specificity of HOE-694 is not unlike what was found in the pancreatic duct (18). Indeed, the NHE1 mutant cells that we used in this study had a smaller increase in pHᵢ during anoxia than wild-type cells. Because the anoxia-induced alkalinization in HEPES buffer is totally Na⁺ dependent, we believe that other isoforms of NHE, such as isoforms 2, 4, and 5 (2, 5, 9), could have been activated in the mutant cells and could have been responsible for the pHᵢ increase in NHE1 mutant neurons.

How NHE activity increases during hypoxia has not been totally delineated. Sheldon and Church (28) have reported the involvement of PKA in anoxia-induced pHᵢ change in CA1 neurons. In this paper, we have evidence that NHEs are activated during anoxia, at least partly, because of the upstream activation of PKA and PKC. The interesting finding in our system is that anoxia seems to activate both kinases, A and C, since H-7 and both H-89 and chelerythrine block, one at a time, the major increase in pHᵢ in HEPES solutions. We do not know, however, how the lack of O₂ activates protein kinases. There are a number of possible mediators, with one being an increase in cytosolic Ca²⁺ concentration, which can lead to increased kinase activity and activation of NHE (31).

Physiological significance. We believe that our observations in this paper put into perspective the physiological importance of membrane proteins in regulating pHᵢ during low-O₂ states. Indeed, the increased levels of protons inside neurons during these states are a result of many cellular processes besides anaerobic metabolism, which had been thought to be the major or sole source of intracellular acidosis. This acidosis is most likely due to the net result of intracellular buffering capacity (which may be different during anoxia), intracellular Ca²⁺ levels, the activity and the level of firing of neurons, which certainly changes during hypoxia (13), how disturbed the ionic homeostasis is (25), and the initial pHᵢ of these neurons, which determines the activity of membrane proteins at the outset. Last, as we have found in this work, the activity of membrane acid loaders or extruders will be important in determining the pHᵢ level. For example, the NHEs are incriminated in our studies during anoxia. How they get activated is at present unknown but may be related, as we have shown, to kinase activation. Indeed, through manipulations of various solutions and blockers, we have been able to demonstrate that this particular exchanger is important during O₂ deprivation.

In summary, we show in this work that the major pH regulators in hippocampal neurons, the NHEs, play a key role in keeping the homeostasis of intracellular acid base balanced in neurons after O₂ deprivation. Our study demonstrates the active role of membrane transporters that regulate pHᵢ in neurons as a function of O₂ level in their microenvironment.

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