

Reduced contribution from Na^+/H^+ exchange to acid extrusion during anoxia in adult rat hippocampal CA1 neurons

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Abstract

The effect of anoxia on Na^+/H^+ exchange activity was examined in acutely isolated adult rat hippocampal CA1 neurons loaded with the H^+ -sensitive fluorophore, BCECF. Five-minute anoxia imposed under nominally $\text{HCO}_3^-/\text{CO}_2$ -free conditions induced a fall in pH_i , the magnitude of which was smaller following prolonged exposure to medium in which *N*-methyl-D-glucamine (NMDG⁺) was employed as an extracellular Na^+ (Na_o^+) substitute. Also consistent with the possibility that Na^+/H^+ exchange becomes inhibited soon after the induction of anoxia, rates of Na_o^+ -dependent pH_i recovery from internal acid loads imposed during anoxia were slowed, compared to rates of Na_o^+ -dependent pH_i recovery observed prior to anoxia. At the time at which rates of pH_i recovery were reduced during anoxia, cellular adenosine triphosphate (ATP) levels had fallen to 35% of preanoxic levels, suggesting that

ATP depletion might contribute to the observed inhibition of Na^+/H^+ exchange. In support, incubation of neurons with 2-deoxyglucose and antimycin A under normoxic conditions induced a fall in cellular ATP levels that was also associated with reduced Na_o^+ -dependent rates of pH_i recovery from imposed acid loads; conversely, pre-treatment with 10 mM creatine attenuated the effects of anoxia to reduce both ATP levels and Na_o^+ -dependent rates of pH_i recovery from internal acid loads. Taken together, the results are consistent with the possibility that functional Na^+/H^+ exchange activity in adult rat CA1 neurons declines soon after the onset of anoxia, possibly as a result of anoxia-induced falls in intracellular ATP.

Keywords: ATP, anoxia, hippocampus, intracellular pH , Na^+/H^+ exchange.

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Early alterations in internal ionic homeostasis contribute to the pathogenesis of neuronal injury following O_2 deprivation (Erecińska and Silver 1994; Lipton 1999). Although changes in intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) and internal Ca^{2+} handling have long been recognized as important determinants of anoxia-induced neuronal injury, changes in intracellular pH (pH_i) and $[\text{Na}^+]_i$, which are linked through Na^+/H^+ exchange, may also be important (e.g. Mutch and Hansen 1984; Obrenovitch *et al.* 1990; Friedman and Haddad 1994; Siesjö *et al.* 1996; Vornov *et al.* 1996). Indeed, selective pharmacological inhibitors of Na^+/H^+ exchange are known to exert a protective effect in neurons in which the transport mechanism is sensitive to such compounds (Vornov *et al.* 1996; Phillis *et al.* 1999; Horikawa *et al.* 2001).

Recently, we have shown that Na^+/H^+ exchange activity in rat hippocampal neurons is strongly stimulated immediately after a transient period of anoxia and contributes to the increases in pH_i and $[\text{Na}^+]_i$ that occur at this time (Diarra *et al.* 1999; Sheldon *et al.* 2001; Sheldon and Church 2002; also see Jørgensen *et al.* 1999; Yao *et al.* 2001). However, it

remains unclear whether potentially detrimental changes in neuronal Na^+/H^+ exchange activity might also occur during anoxia itself (see Mutch and Hansen 1984; Obrenovitch *et al.* 1990; Taylor *et al.* 1996). The importance of this point is underscored by the fact that Na^+/H^+ exchange inhibitors would be more valuable in the clinical setting if their neuroprotective actions could be realized after an anoxic or ischemic insult. The purpose of the present study therefore was to examine whether Na^+/H^+ exchange in adult rat hippocampal CA1 neurons remains functional during anoxia.

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Abbreviations used: ATP, adenosine triphosphate; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentrations; 2-DG, 2-deoxyglucose; NMDG⁺, *N*-methyl-D-glucamine; Na_o^+ , extracellular Na^+ ; pH_i , intracellular pH ; NHE1, Na^+/H^+ exchanger isoform 1.

Our results suggest that observable Na⁺/H⁺ exchange activity in rat CA1 neurons declines during anoxia and that this reflects an inherent cellular response possibly related to reductions in internal adenosine triphosphate (ATP).

Materials and methods

Cell preparation

The procedures used to isolate hippocampal CA1 neurons from adult Wistar rats have been described in detail previously (Smith *et al.* 1998; Sheldon and Church 2002). In brief, transverse hippocampal slices (450 µm) were prepared and allowed to recover for 1 h prior to enzymatic digestion with protease type XIV (1.5 mg/mL; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The CA1 regions were then microdissected and triturated with fire-polished Pasteur pipettes of diminishing tip diameters. The triturated suspension was deposited onto a glass coverslip mounted in a perfusion chamber to form the floor of the chamber and neurons were allowed to adhere for 30 min, during which time they were loaded with the acetoxymethyl ester form of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, 2 µM; Molecular Probes, Eugene, OR, USA).

Experimental media

Neurons were superfused (2 mL/min) at 37°C with a nominally HCO₃⁻/CO₂-free medium containing: 136.5 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1.5 mM NaH₂PO₄, 1.5 mM MgSO₄, 17.5 mM or 5 mM D-glucose and 10 mM HEPES (pH 7.35 with 10 M NaOH); D-mannitol (12.5 mM) was added to 5 mM D-glucose-containing media to maintain osmolarity. Solutions containing 20 mM NH₄Cl or 17.5 mM 2-deoxyglucose (2-DG) were prepared by equimolar substitution for NaCl or D-glucose, respectively. When extracellular Na⁺ was reduced to 2–4 mM, N-methyl-D-glucamine (NMDG⁺) or Li⁺ were employed as substitutes and solutions were titrated to pH 7.35 (at 37°C) with 10 M HCl or 2 M LiOH, respectively. Anoxia was induced by the addition of 1–2 mM sodium dithionite, an O₂ scavenger, to the superfusing medium (Friedman and Haddad 1994; Diarra *et al.* 1999; Sheldon and Church 2002); during anoxia, the atmosphere in the recording chamber was switched from room air to 100% Ar. We have reported previously that media containing 1–2 mM Na₂S₂O₄ have PO₂ values < 1 mmHg (measured in the recording chamber) and that the pH_i changes evoked in rat hippocampal neurons by exposure to these media reflect reductions in PO₂ and are not secondary to any additional properties of the O₂ scavenger (Diarra *et al.* 1999; Sheldon and Church 2002).

Experimental procedures

Intracellular pH was measured by the dual-excitation ratio method, as described (Baxter and Church 1996; Smith *et al.* 1998; Sheldon and Church 2002). Fluorescence emissions at 520 nm were obtained from regions of interest placed on individual neuronal somata and raw intensity data at each excitation wavelength (488 and 452 nm) were corrected for background fluorescence prior to calculation of the ratio. Analysis was restricted to those neurons able to retain BCECF throughout the course of an experiment (see Bevensee *et al.* 1996; Sheldon and Church 2002). The one-point high-[K⁺]/nigericin technique was employed to convert background-corrected BCECF

emission intensity ratios (BI_{488}/BI_{452}) into pH_i values. To prevent cross-contamination with nigericin, perfusion lines were replaced and the perfusion chamber was decontaminated after each experiment, as described by Richmond and Vaughan-Jones (1997).

The effects of anoxia and other experimental maneuvers were examined on steady-state pH_i and rates of pH_i recovery from internal acid loads imposed by the NH₄⁺-pre-pulse technique. In experiments in which rates of pH_i recovery were examined, two or three consecutive intracellular acid loads were imposed, the first one (or two) being employed to calculate control rates of pH_i recovery for a given neuron and the second (or third) being performed under a test condition. Rates of pH_i recovery from imposed acid loads were determined by fitting the recovery portions of the experiment to a single exponential function; the first derivative of this function was then used to determine rates of pH_i change as a function of time (see Wu and Vaughan-Jones 1994; Baxter and Church 1996; Smith *et al.* 1998; Sheldon and Church 2002). Acid loads during anoxia were imposed such that the peak of the internal acidification occurred at approximately the same time at which steady-state pH_i during anoxia reached its minimum value (~2.5 min following the start of anoxia). Instantaneous rates of pH_i recovery were then determined at ~30 s after the peak acidification (i.e. at ~3 min after the start of anoxia) and compared statistically with control rates of pH_i recovery obtained at the same pH_i.

Cellular ATP content was determined from luciferin–luciferase luminescence, using the Molecular Probes ATP determination kit. Each sample, containing 5–6 CA1 principal cell layers microdissected from hippocampal slices (see above), was either exposed to anoxia or incubated under normoxic conditions with 5 µg/mL antimycin A and 17.5 mM 2-DG (to block oxidative phosphorylation and glycolysis, respectively; see Aharonovitz *et al.* 2000; Szabo *et al.* 2000) for the durations indicated in the Results; control samples were maintained in HEPES-buffered saline. Samples were then lysed by the addition of 0.1 M NaOH/1 mM EDTA and, after centrifugation, the supernatant was neutralized with 0.5 M perchloric acid (see Sheline *et al.* 2000). Ten-microlitre aliquots were then removed and sample bioluminescence was detected with a Berthold LB9507 Lumat luminometer (Fisher Scientific, Ottawa, ON, Canada). In all cases, measurements were made in triplicate. Protein content of the pellet was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories Inc., Mississauga, ON, Canada).

Data are reported as means ± SEM and, in the majority of experiments, the accompanying *n* value refers to the number of acutely isolated CA1 neurons from which data were obtained. In experiments in which internal ATP content was measured, *n* refers to the number of samples examined under a given experimental condition. Statistical analysis was performed with Student's two-tailed *t*-test, paired or unpaired as appropriate, with significance assumed at the 5% level.

Results

Steady-state pH_i

Consistent with previous reports from this laboratory (Smith *et al.* 1998; Sheldon and Church 2002) and others (Bevensee *et al.* 1996), steady-state pH_i under nominally HCO₃⁻/CO₂-free, HEPES-buffered conditions was

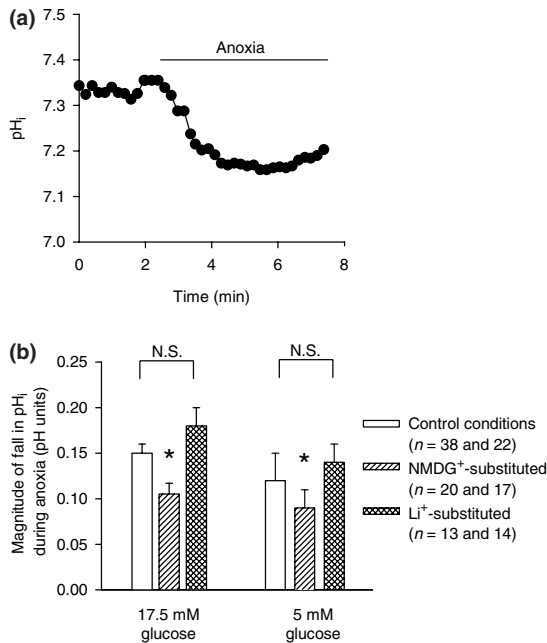


Fig. 1 Steady-state pH_i changes evoked by 5 min anoxia. (a) Under nominally $\text{HCO}_3^-/\text{CO}_2$ -free conditions, anoxia induced a fall in pH_i that in this example was maximal ~ 3 min after the start of anoxia. (b) The effects of external Na^+ substitutions, in the presence of 17.5 or 5 mM glucose, on the magnitudes of the reductions in pH_i observed during anoxia. Data were obtained under control conditions (normal Na_o^+ -containing; open bars); under reduced- Na_o^+ , NMDG⁺-substituted conditions (hatched bars); and under reduced- Na_o^+ , Li⁺-substituted conditions (cross-hatched bars). The magnitudes of the falls in pH_i were measured as the difference between the resting pH_i observed immediately prior to the induction of anoxia and the minimum pH_i obtained during anoxia. * $p < 0.05$ compared to control or Li⁺-substituted conditions at the same glucose concentration. NS, no significant difference ($p > 0.05$) between the falls in pH_i evoked by anoxia under normal Na_o^+ -containing compared to Li⁺-substituted conditions.

7.27 ± 0.02 ($n = 159$). Also consistent with previous reports in isolated rat hippocampal and mouse neocortical neurons (Diarra *et al.* 1999; Jørgensen *et al.* 1999; Sheldon and Church 2002), 5 min anoxia typically evoked a fall in pH_i , the maximum magnitude of which was 0.15 ± 0.01 pH units ($n = 38$) measured at 2.5 ± 0.1 min after the start of anoxia (Fig. 1a).

Under $\text{HCO}_3^-/\text{CO}_2$ -free conditions, the dominant acid extrusion mechanism in rat CA1 neurons is Na^+/H^+ exchange which, unusually, is insensitive to known pharmacological inhibitors of the antiport in other cell types (see Raley-Susman *et al.* 1991; Schwiening and Boron 1994; Baxter and Church 1996; Bevensee *et al.* 1996; Smith *et al.* 1998). To inhibit Na^+/H^+ exchange therefore, neurons were perfused with reduced- Na_o^+ , NMDG⁺-substituted medium prior to the induction of anoxia. In agreement with previous reports in rat hippocampal neurons (Baxter and Church 1996; Bevensee *et al.* 1996; Smith *et al.* 1998), prolonged exposure to

reduced- Na_o^+ , NMDG⁺-substituted medium was marked by an initial intracellular acidification which then slowly recovered over the following 20–30 min to a new steady-state pH_i value of 7.35 ± 0.04 ($n = 20$); this pH_i value was not significantly different either to the pH_i value observed prior to the reduction in Na_o^+ (7.34 ± 0.03 ; $p > 0.05$) or to the pH_i value observed prior to the induction of anoxia in the presence of normal $[\text{Na}^+]_o$ (7.33 ± 0.03 ; $n = 38$; $p > 0.05$). The subsequent imposition of 5 min anoxia during continued perfusion with reduced- Na_o^+ , NMDG⁺-substituted medium evoked an internal acidification, the magnitude of which was significantly reduced compared to the fall in pH_i observed in the presence of normal Na_o^+ (Fig. 1b). In contrast to NMDG⁺, Li⁺ can act as a substrate for Na^+/H^+ exchange (see Aronson 1985; Jean *et al.* 1985). Consistent with previous reports in rat hippocampal neurons (Raley-Susman *et al.* 1991; Baxter and Church 1996; Smith *et al.* 1998), exposure to reduced- Na_o^+ , Li⁺-substituted medium caused a transient fall in steady-state pH_i which recovered within 5–10 min to a pH_i value (7.40 ± 0.02 ; $n = 13$) which was not significantly different either to the pH_i value observed prior to the reduction of Na_o^+ (7.38 ± 0.02) or to the pH_i value observed prior to the induction of anoxia in the presence of normal $[\text{Na}^+]_o$ (see above; $p > 0.05$ in each case). Under these conditions, the magnitude of the fall in pH_i induced by 5 min anoxia was not significantly different from that observed in the presence of normal Na_o^+ but was significantly greater than that observed after prolonged exposure to NMDG⁺-substituted medium (Fig. 1b). Similar results were obtained when anoxia was imposed in the presence of 5 mM, rather than 17.5 mM, glucose (Fig. 1b).

Taken together, these results suggest the possibility that Na^+/H^+ exchange in rat CA1 neurons becomes inhibited soon after the induction of anoxia. Under normoxic conditions, Na^+/H^+ exchange in rat hippocampal neurons is active at resting pH_i (see Raley-Susman *et al.* 1991; Schwiening and Boron 1994; Baxter and Church 1996; Bevensee *et al.* 1996) and, in the presence of normal Na_o^+ or under Li⁺-substituted conditions, reduced Na^+/H^+ exchange activity during anoxia would be expected to augment the internal acidosis produced at this time by, for example, increased metabolic acid production (see Chambers-Kersh *et al.* 2000). In contrast, under conditions where Na^+/H^+ exchange was blocked prior to the induction of anoxia by prolonged exposure to NMDG⁺-substituted medium, inhibition of Na^+/H^+ exchange activity by anoxia is precluded and will not contribute to the fall in pH_i during anoxia; thus, the observed reduction in the magnitude of the anoxia-induced acidification when NMDG⁺, as opposed to Li⁺, was employed as an external Na^+ substitute.

Recovery of pH_i from imposed internal acid loads

To further investigate the possibility that Na^+/H^+ exchange activity in rat CA1 neurons declines soon after the start of

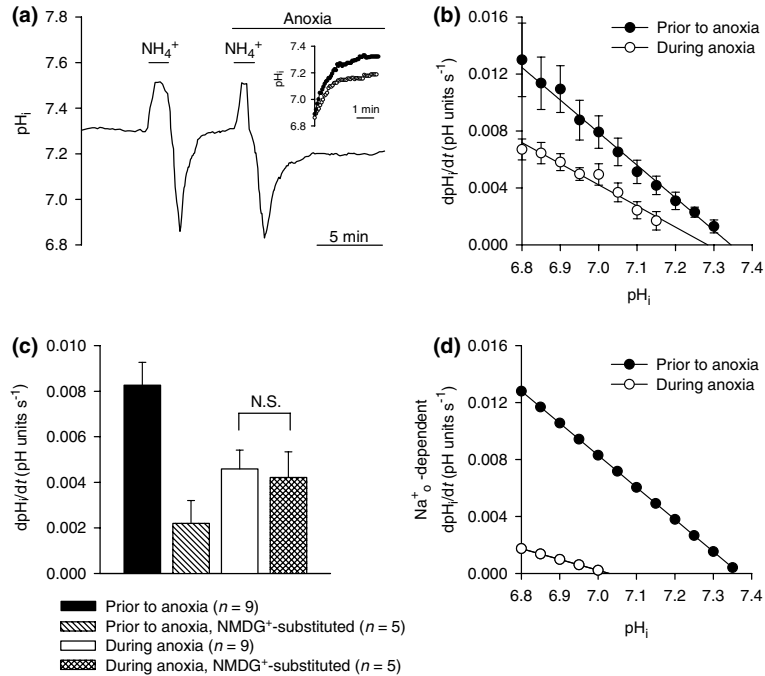


Fig. 2 Rates of pH_i recovery from internal acid loads are reduced during anoxia. (a) Following the first NH_4^+ -induced intracellular acid load, pH_i was allowed to recover. A second acid load was then imposed after the start of anoxia. (Inset) Superimposed records of the recoveries of pH_i from acid loads imposed prior to (●) and during (○) anoxia; the rate of recovery of pH_i was reduced during anoxia. (b) The pH_i dependencies of rates of pH_i recovery prior to (●) and during (○) anoxia under control conditions (pH_o 7.35, normal $[\text{Na}^+]_o$). Continuous lines represent the weighted non-linear regression fits to the data points indicated for each experimental condition ($n = 9$ in each case). (c) Rates of pH_i recovery from internal acid loads imposed prior to anoxia under normal Na_o^+ -containing conditions (black bar) were faster than those observed prior to anoxia under reduced- Na_o^+ , NMDG⁺-

substituted conditions (hatched bar) and during anoxia, under both normal Na_o^+ -containing (open bar) and reduced- Na_o^+ , NMDG⁺-substituted conditions (cross-hatched bar) ($p < 0.05$ in each case). There was no significant difference (NS, $p > 0.05$) between rates of pH_i recovery from acid loads imposed during anoxia under normal Na_o^+ -containing and reduced- Na_o^+ , NMDG⁺-substituted conditions. Rates of pH_i recovery shown were determined at a common test pH_i of 7.00. (d) The Na_o^+ -dependent component of pH_i recovery prior to (●) and during (○) anoxia, revealed by plotting the differences between the regression fits obtained under normal Na_o^+ -containing conditions and reduced- Na_o^+ , NMDG⁺-substituted conditions (see Sheldon and Church 2002).

anoxia, we compared rates of pH_i recovery from intracellular acid loads imposed prior to and during anoxia.

As illustrated in Fig. 2(a), under control conditions pH_i recovery from an acid load imposed during anoxia was slowed, compared to that observed prior to anoxia in the same cell. Examined in a total of nine neurons, instantaneous rates of pH_i recovery were reduced significantly during anoxia at all absolute values of pH_i (Fig. 2b); at a common test pH_i of 7.00, for example, there was a 44% decrease in the rate of pH_i recovery during anoxia (Fig. 2c). The increases in pH_i evoked by NH_4^+ (quantified by taking the difference between the steady-state pH_i immediately prior to the application of NH_4^+ and the maximum pH_i observed during its application; see Smith *et al.* 1998; Sheldon and Church 2002) were similar prior to and during anoxia (0.25 ± 0.02 and 0.21 ± 0.04 pH units, respectively; $n = 9$ in each case; $p > 0.05$), suggesting that marked alterations in intracellular buffering power are unlikely to contribute to the reduction in rates of pH_i recovery observed during anoxia.

Next, internal acid loads were imposed prior to and during anoxia under reduced- $[\text{Na}^+]_o$, NMDG⁺-substituted conditions (Na^+/H^+ exchange blocked). Consistent with previous reports in rat hippocampal neurons (Schwiening and Boron 1994; Baxter and Church 1996; Bevensee *et al.* 1996; Sheldon and Church 2002), rates of pH_i recovery prior to anoxia were significantly reduced, compared to rates of pH_i recovery established in the presence of normal Na_o^+ (Fig. 2c). In contrast, rates of pH_i recovery during anoxia were not significantly different from those established during anoxia in the presence of normal Na_o^+ (Fig. 2c). Also consistent with the possibility that functional Na^+/H^+ exchange activity is reduced during anoxia, plots of the differences between rates of pH_i recovery under normal Na_o^+ -containing and reduced- Na_o^+ , NMDG⁺-substituted conditions both prior to and during anoxia (Fig. 2d) revealed a reduced contribution from Na_o^+ -dependent mechanism(s) to pH_i recovery from acid loads during anoxia. We have shown previously that the residual Na_o^+ -independent recovery of pH_i observed during anoxia in

rat hippocampal neurons is inhibited by Zn^{2+} and Cd^{2+} , consistent with its mediation by a H^+ -conductive pathway (see Diarra *et al.* 1999; Sheldon and Church 2002).

In light of the fact that Na^+/H^+ exchangers possess internal H^+ modifier site(s) that modulate transport activity (see Wakabayashi *et al.* 1997), the observed functional reduction in the contribution of Na^+/H^+ exchange to pH_i recovery from acid loads imposed during anoxia may simply reflect the relatively high pH_i values that pertain at pH_o 7.35. Therefore, internal acid loads were imposed prior to anoxia at pH_o 7.35 and then during anoxia at pH_o 6.60, conditions that mimic the changes in pH_o that occur in response to anoxia *in vivo* ($n = 8$; Fig. 3a). Although the minimum pH_i values imposed by NH_4^+ pre-pulses during anoxia at pH_o 6.60 were lower than those observed at pH_o 7.35 ($pH_i \sim 6.10$ and ~ 6.80 , respectively; also see Vornov *et al.* 1996), rates of pH_i recovery during anoxia at pH_o 6.60 were further reduced ($p < 0.05$), rather than increased, from those observed at pH_o 7.35 (Figs 3b and c). This result is consistent with the

possibility that Na^+/H^+ exchange continues to be inhibited during anoxia at low pH_i values; however, the fact that rates of pH_i recovery during anoxia at pH_o 6.60 were slower than those observed at pH_o 7.35 suggests the possibility that low pH conditions might be affecting the activity of an additional mechanism that participates in acid extrusion during anoxia in rat CA1 neurons (e.g. the Na_o^+ -independent H^+ -conductive pathway referred to above, the activity of which is known to be inhibited at low pH_o ; see DeCoursey and Cherny 2000; Sheldon and Church 2002). Therefore, to more rigorously assess the effects of anoxia on Na^+/H^+ exchange activity at low pH_o/pH_i , the Na_o^+ -dependent component of pH_i recovery from internal acid loads was assessed by imposing acid loads prior to and during anoxia at pH_o 6.60, under both normal Na_o^+ -containing ($n = 9$) and reduced- Na_o^+ , NMDG $^+$ -substituted ($n = 10$) conditions. As illustrated in Figs 3(b and c), rates of pH_i recovery prior to anoxia at pH_o 6.60 were significantly reduced, compared to those observed at pH_o 7.35, consistent with the known effect of falls in pH_o to

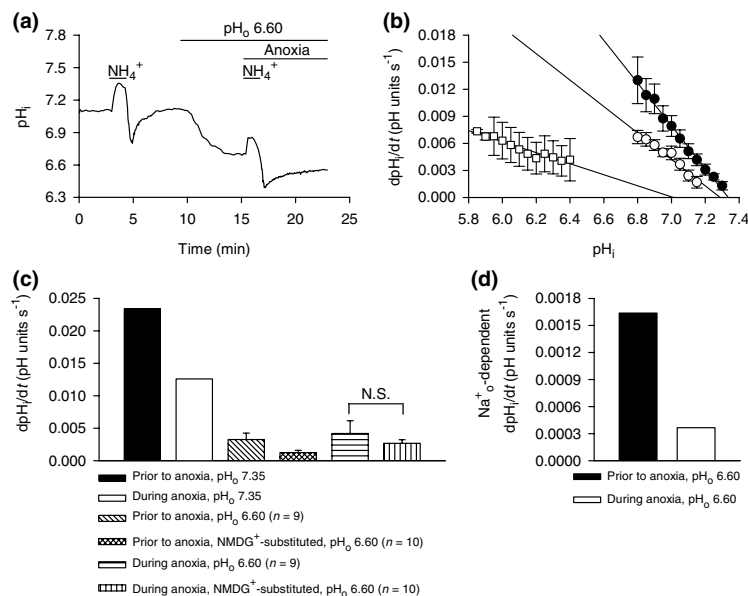


Fig. 3 pH_i recovery from acid loads imposed prior to and during anoxia under reduced pH_o conditions. (a) An initial acid load was imposed prior to anoxia at pH_o 7.35. After the recovery of pH_i , pH_o was reduced to 6.60 and, when pH_i had stabilized at a new resting level (pH 6.80 ± 0.05 , $n = 9$), a second acid load was imposed during anoxia. (b) The pH_i dependency of rates of pH_i recovery from internal acid loads imposed during anoxia under normal Na_o^+ -containing conditions at pH_o 6.60 (\square). Also illustrated are the pH_i dependencies of rates of pH_i recovery from internal acid loads imposed prior to (\bullet) and during (\circ) anoxia under normal Na_o^+ -containing conditions at pH_o 7.35 (see Fig. 2). A comparison for overall coincidence of the regression fits representing the pH_i dependencies of rates of pH_i recovery under pH_o 7.35 and pH_o 6.60 conditions indicated that the rate of pH_i recovery from acid loads imposed during anoxia at pH_o 6.60 was significantly slower ($p < 0.05$) than the rate established during anoxia at pH_o 7.35. (c) Rates of pH_i

recovery from internal acid loads imposed prior to and during anoxia under the conditions shown on the figure, measured at a common test pH_i of 6.40. Rates of pH_i recovery from acid loads imposed prior to (black bar) and during (open bar) anoxia under normal Na_o^+ -containing conditions at pH_o 7.35 were estimated by extrapolating the weighted non-linear regression fits relating absolute pH_i values to the rates of pH_i recovery obtained under each experimental condition (see b). At pH_o 6.60, there was no significant difference (NS, $p > 0.05$) between rates of pH_i recovery from internal acid loads imposed during anoxia under Na_o^+ -containing or reduced- Na_o^+ , NMDG $^+$ -substituted conditions. (d) The Na_o^+ -dependent component of pH_i recovery prior to and during anoxia at pH_o 6.60, revealed by plotting the difference between the regression fits obtained under normal Na_o^+ -containing conditions and reduced- Na_o^+ , NMDG $^+$ -substituted conditions (note the change in scale of the y-axis from c). Rates were measured at a common test pH_i of 6.40.

inhibit the activities of Na⁺/H⁺ exchangers (e.g. Jean *et al.* 1985; Wu and Vaughan-Jones 1997; Sheldon and Church 2002). However, rates of pHi recovery during anoxia at pH_o 6.60 were not significantly different ($p > 0.05$) to rates observed prior to anoxia at pH_o 6.60. In addition, although rates of pHi recovery during anoxia at pH_o 6.60 were not significantly different under normal Na_o⁺-containing versus reduced-Na_o⁺, NMDG⁺-substituted conditions (Fig. 3c), plots of the differences between rates of pHi recovery under Na_o⁺-containing and NMDG⁺-substituted conditions both prior to and during anoxia revealed a reduced contribution from Na_o⁺-dependent mechanism(s) to pHi recovery from acid loads during anoxia at pH_o 6.60 (Fig. 3d). The reduced rate of Na_o⁺-dependent pHi recovery during anoxia at pH_o 6.6 compared to pH_o 7.35 (compare Figs 2d and 3d) is consistent with a low pH_o-induced inhibition of residual Na⁺/H⁺ exchange activity during anoxia.

Role of internal ATP depletion

In all cell types studied to date, optimal Na⁺/H⁺ exchange activity requires the presence of normal physiological levels of intracellular ATP (Demaurex and Grinstein 1994; Wu and Vaughan-Jones 1994; Demareux *et al.* 1997; Wakabayashi *et al.* 1997; Szabó *et al.* 2000). This raises the possibility that an anoxia-induced fall in internal ATP levels (see Erecińska and Silver 1994) might contribute to the anoxia-evoked decline in Na⁺/H⁺ exchange activity. This was examined using a number of different approaches.

First, to assess whether rates of pHi recovery from acid loads imposed in rat CA1 neurons in the nominal absence of HCO₃⁻ are sensitive to internal ATP depletion, microdissected CA1 regions were incubated with 2-DG and antimycin A under glucose-free, normoxic conditions. Consistent with previous reports (e.g. Kass and Lipton 1982; Obrenovitch *et al.* 1990; Carter *et al.* 1995), resting ATP levels were 10.6 ± 3.5 μmol/g protein ($n = 6$), equivalent to ~4.4 mM assuming a cytosolic volume of 2.4 μL/mg protein (see Chinopoulos *et al.* 2000). After 10 min treatment with 2-DG and antimycin A, there was an $80 \pm 13\%$ fall in internal ATP levels to a value below the K_{0.5} of Na⁺/H⁺ exchange for ATP (see Discussion). At the time that ATP levels were reduced by 2-DG and antimycin A, rates of pHi recovery from imposed acid loads were slowed, compared with rates measured prior to ATP depletion in the same neurons (Fig. 4a). At a common test pHi of 7.00, for example, there was a 53% decrease in the rate of pHi recovery ($p < 0.05$), which was not further slowed when the experiments were repeated under reduced-Na_o⁺, NMDG⁺-substituted conditions (Fig. 4b). However, plotting the difference between rates of pHi recovery under normal Na_o⁺-containing and reduced-Na_o⁺, NMDG⁺-substituted conditions prior to and following treatment with 2-DG and antimycin A revealed a reduced contribution from Na_o⁺-dependent mechanism(s) to pHi

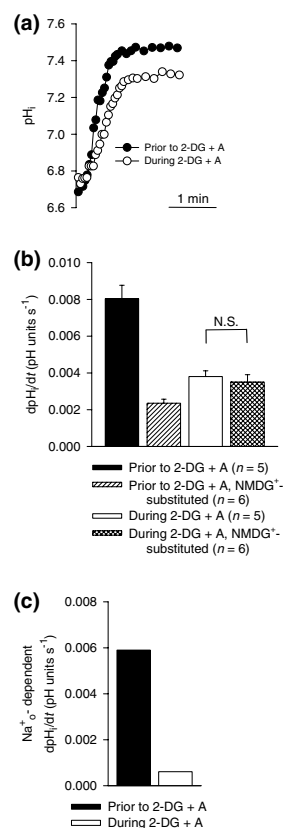


Fig. 4 Treatment with 2-DG and antimycin A slows rates of pHi recovery from internal acid loads. (a) Superimposed records of the recoveries of pHi from acid loads imposed in a CA1 neuron prior to and following 10 min incubation with 2-DG and antimycin A (2-DG + A). The rate of recovery of pHi was reduced following ATP depletion. (b) Rates of pHi recovery following 10 min incubation with 2-DG + A (open bar) were significantly slower than those observed in the same neurons prior to ATP depletion (black bar). No significant difference (NS, $p > 0.05$) was observed between rates of pHi recovery from acid loads imposed following exposure to 2-DG + A under normal Na_o⁺-containing and reduced-Na_o⁺, NMDG⁺-substituted conditions. (c) The Na_o⁺-dependent component of pHi recovery prior to (black bar) and following (open bar) 10 min exposure to 2-DG + A, revealed by plotting the difference between the regression fits obtained under normal Na_o⁺-containing conditions and reduced-Na_o⁺, NMDG⁺-substituted conditions. In (b) and (c), rates of pHi recovery were determined at a common test pHi of 7.00.

recovery from imposed acid loads in the presence of 2-DG and antimycin A (Fig. 4c).

Next, we examined whether anoxia imposed under our experimental conditions results in intracellular ATP depletion. Consistent with previous reports (e.g. Obrenovitch *et al.* 1990; Erecińska and Silver 1994; Lipton 1999), after 3 min anoxia there was a $65 \pm 4\%$ ($n = 3$) fall in internal ATP levels, which declined further to $76 \pm 4\%$ ($n = 2$) after 5 min anoxia. Thus, pHi recovery from acid loads imposed during anoxia was slowed at a time when cellular ATP was depleted.

Pre-treatment of hippocampal slices with 10 mM creatine for ≥ 2 h has been shown to increase intracellular phosphocreatine levels in hippocampal neurons and delay the depletion of internal ATP during O_2 deprivation (e.g. Kass and Lipton 1982; Carter *et al.* 1995; Balestrino *et al.* 1999). Therefore, in the third series of experiments, we examined whether this maneuver could preserve internal ATP levels and concomitantly attenuate the anoxia-induced decline in rates of pH_i recovery from acid loads observed in untreated neurons. In creatine-treated slices, 3 min anoxia caused a $38 \pm 6\%$ ($n = 4$) fall in ATP levels, a reduction significantly less ($p < 0.05$) than that observed in untreated slices. In neurons isolated from creatine-treated slices, rates of pH_i recovery from acid loads imposed during anoxia were not significantly different from rates of pH_i recovery established in the same neurons prior to anoxia (Figs 5a and b). Intracellular acid loads were then imposed in neurons isolated from creatine-treated slices both prior to and during anoxia under reduced- Na_o^+ , NMDG $^+$ -substituted conditions. At a common test pH_i of 7.00, rates of pH_i recovery from acid loads imposed under NMDG $^+$ -substituted conditions both prior to and during anoxia were slowed by $\sim 60\%$, compared with rates of pH_i recovery observed in the presence of normal Na_o^+ (Fig. 5b). Thus, Na_o^+ -dependent acid extrusion mechanism(s) remain functional during anoxia in neurons isolated from creatine-treated slices. Indeed, plotting the difference between rates of pH_i recovery measured under normal Na_o^+ -containing and reduced- Na_o^+ , NMDG $^+$ -substituted conditions prior to and during anoxia revealed that, in contrast to slices that had not been treated with creatine (see Fig. 2d), the contribution of Na_o^+ -dependent mechanism(s) to pH_i recovery from acid loads during anoxia is preserved in neurons isolated from creatine-treated slices (Fig. 5c).

Discussion

Anoxia induces a marked decline in HCO_3^- -independent, Na_o^+ -dependent acid extrusion from adult rat hippocampal CA1 neurons. The only established HCO_3^- -independent, Na_o^+ -dependent acid extrusion mechanism that supports Na^+ and Li^+ , but not NMDG $^+$, transport in this cell type is Na^+/H^+ exchange. As such, the results of the present study are consistent with the possibility that Na^+/H^+ exchange activity in adult rat CA1 neurons declines soon after the onset of anoxia. In support of this possibility, we have shown previously that the internal alkalization sometimes observed following the initial anoxia-induced fall in pH_i in rat hippocampal neurons is mediated, not by Na^+/H^+ exchange but by a Zn^{2+} -sensitive acid extrusion mechanism that possesses many of the characteristics of a H^+ -conductive pathway (Diarra *et al.* 1999; Sheldon and Church 2002). In addition, we have failed to uncover any contribution from Na^+/H^+ exchange to the increase in $[Na^+]_i$ that occurs during

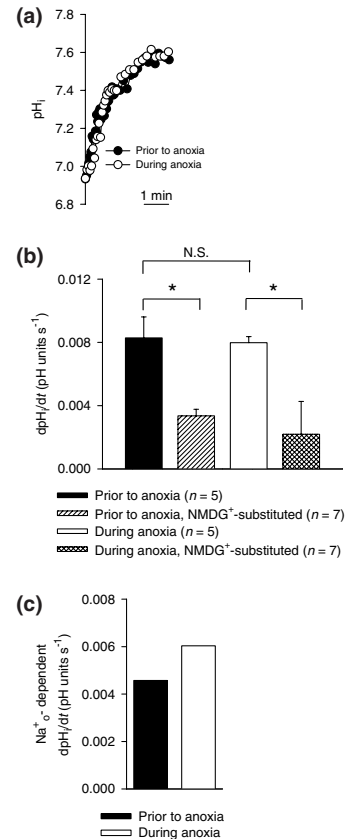


Fig. 5 pH_i recovery from internal acid loads in creatine-pretreated neurons. (a) Superimposed records of the recoveries of pH_i from acid loads imposed prior to and during anoxia in a CA1 pyramidal neuron isolated from a hippocampal slice pre-treated for 2 h with 10 mM creatine. In contrast to untreated neurons (see Fig. 2a), the rate of pH_i recovery from the acid load imposed during anoxia was not slowed. (b) In neurons pre-treated with 10 mM creatine, rates of pH_i recovery measured at ~ 3 min after the start of anoxia under normal Na_o^+ -containing conditions (open bar) were not significantly different to those observed in the same neurons prior to anoxia (black bar; NS, $p > 0.05$). Reducing Na_o^+ (NMDG $^+$ -substitution) slowed rates of pH_i recovery from acid loads imposed both prior to and during anoxia (hatched and cross-hatched bars, respectively; * $p < 0.05$ in each case). (c) The Na_o^+ -dependent component of pH_i recovery prior to (black bar) and during (open bar) anoxia in neurons isolated from creatine-treated slices, revealed by plotting the difference between the regression fits obtained under normal Na_o^+ -containing conditions and reduced- Na_o^+ , NMDG $^+$ -substituted conditions. In (b) and (c), rates of pH_i recovery were determined at a common test pH_i of 7.00.

anoxia in rat hippocampal neurons (Sheldon *et al.* 2001; also see Chen *et al.* 1999). The present finding is consistent with extensive studies in cardiac myocytes (e.g. Bond *et al.* 1993; Park *et al.* 1999; Satoh *et al.* 2001) and supports previous suggestions, made largely on the basis of pH_o measurements *in vivo* and in slice preparations *in vitro*, that Na^+/H^+ exchange activity in brain tissue is compromised during anoxia (Pirttilä and Kauppinen 1992, 1994; Taylor *et al.*

1996; Chambers-Kersh *et al.* 2000; but see Yao *et al.* 2001). However, it contrasts with the fact that Na⁺/H⁺ exchange is the primary mechanism whereby p*H*_i recovers from the internal acidosis imposed by the application of excitotoxins under normoxic conditions (e.g. Hartley and Dubinsky 1993), highlighting a further difference between the effects of excitotoxins and anoxia on central neuronal function (see Chow and Haddad 1998).

Given that the majority of the experiments in the present study were performed under constant extracellular conditions, the reduction in observable Na⁺/H⁺ exchange activity during anoxia is unlikely to be secondary to anoxia-evoked changes in the composition of the microenvironment. In particular, although reductions in p*H*_o (as occur during anoxia *in vivo* and in slices *in vitro*; Mutch and Hansen 1984; Obrenovitch *et al.* 1990; Erecińska and Silver 1994) are known to inhibit Na⁺/H⁺ exchange activity (e.g. Jean *et al.* 1985; Wakabayashi *et al.* 1997; Wu and Vaughan-Jones 1997), the present results indicate that a fall in p*H*_o is not an absolute requirement for reduced antiport activity during anoxia in rat CA1 neurons. In addition, although anoxia-evoked increases in [Na⁺]_i (Chen *et al.* 1999; Diarra *et al.* 2001) would act to reduce the thermodynamic driving force for Na⁺/H⁺ exchange (see Wu and Vaughan-Jones 1997), calculations indicate that the quotient [Na⁺]_o/[Na⁺]_i remains greater than [H⁺]_o/[H⁺]_i during anoxia at either p*H*_o 7.35 or p*H*_o 6.60, thereby favoring net H⁺ efflux. This is in agreement with studies in guinea pig neocortical slices (Pirttilä and Kauppinen 1994) as well as cardiac myocytes (e.g. Park *et al.* 1999; Moor *et al.* 2001) and indicates that factor(s) other than changes in transmembrane H⁺ and/or Na⁺ gradients must contribute to the lack of observable Na⁺/H⁺ exchange activity in rat CA1 neurons during anoxia. Rather, our results are consistent with the possibility that the decline in Na⁺/H⁺ exchange activity during anoxia might at least in part be consequent upon the fall in internal ATP levels which occurs rapidly after the induction of anoxia in adult rat CA1 neurons (present study; also see Obrenovitch *et al.* 1990; Lipton 1999).

In all cases studied to date, physiological levels of internal ATP are required for optimal Na⁺/H⁺ exchange activity (Demaurex and Grinstein 1994; Wakabayashi *et al.* 1997; Szabó *et al.* 2000). In AP-1 cells transfected with Na⁺/H⁺ exchanger isoform 1 (NHE1), for example, half-maximal activation of the antiporter occurs at ~5 mM ATP (Demaurex *et al.* 1997). In the present study, rates of p*H*_i recovery from acid loads imposed during anoxia were slowed at a time when internal ATP levels were reduced from ~4.4 mM under resting conditions to ~1.5 mM, consistent with the established ATP dependence of not only NHE1 but also NHE5 (a candidate for the amiloride-insensitive Na⁺/H⁺ exchanger found in rat CA1 neurons; Szabó *et al.* 2000). Both the reduced slope of the rate of Na_o⁺-dependent p*H*_i recovery versus p*H*_i relationship and the

acidic shift in the p*H*_i dependence of the rate of Na_o⁺-dependent p*H*_i recovery from acid loads observed during anoxia (Fig. 2d) are also consistent with previous findings that internal ATP depletion decreases the affinities of Na⁺/H⁺ exchangers for internal protons and lowers their maximum transport velocities (see Demaurex and Grinstein 1994; Wakabayashi *et al.* 1997; Szabó *et al.* 2000). The involvement of internal ATP depletion in the decline in Na⁺/H⁺ exchange activity during anoxia is also suggested by the present findings that: (i) incubation with 2-DG and antimycin A under normoxic conditions produced not only a similar fall in internal ATP levels to that observed during anoxia but also reduced rates of Na_o⁺-dependent p*H*_i recovery from internal acid loads to a similar extent; and (ii) creatine pre-treatment not only limited anoxia-evoked reductions in ATP levels but also attenuated anoxia-induced reductions in rates of Na_o⁺-dependent p*H*_i recovery from imposed acid loads. The apparent relationship between internal ATP levels and Na⁺/H⁺ exchange activity would act to link the activity of the exchanger with the metabolic state of the cell. A reduction in antiport activity during a period of metabolic stress may, for example, limit its contribution to potentially detrimental elevations in [Na⁺]_i and (via reverse Na⁺/Ca²⁺ exchange) [Ca²⁺]_i, albeit at the expense of a reduced rate of acid extrusion.

Although cellular depletion of ATP reduces the activities of all known Na⁺/H⁺ exchanger isoforms, it is also apparent that Na⁺/H⁺ exchange transport activity is not necessarily dependent on the direct hydrolysis of ATP (reviewed by Demaurex and Grinstein 1994; Wakabayashi *et al.* 1997). In this regard, recent evidence indicates that the effect of acute ATP depletion to decrease NHE1 transport activity is in large part consequent upon the depletion of plasmalemmal PIP₂, rapid reductions in which are known to occur not only following chemical ATP depletion (Aharonovitz *et al.* 2000) but also in response to short (e.g. 3 min) periods of cerebral ischemia (Sun and Hsu 1996). Indeed, in initial experiments, we have found that pre-treatment with neomycin, which acts to reduce the availability of PIP₂ (see Aharonovitz *et al.* 2000), reduces rates of p*H*_i recovery from internal acid loads imposed under normoxic conditions (C. Sheldon and J. Church, unpublished observations).

Despite the marked reduction in Na⁺/H⁺ exchange activity induced by anoxia, p*H*_i was still able to recover from internal acid loads imposed during anoxia, albeit slowly (see Fig. 2d). Under the HCO₃⁻-free conditions employed in the present experiments, this slow p*H*_i recovery is likely mediated by residual Na⁺/H⁺ exchange activity and the aforementioned Na_o⁺- and HCO₃⁻-independent, Zn²⁺-sensitive H⁺-conductive pathway which is activated during anoxic depolarization in rat CA1 neurons (Diarra *et al.* 1999; Sheldon and Church 2002). The latter pathway may be particularly important for the alleviation of anoxia-induced internal acidifications because, in contrast to cardiac

myocytes (see Lamers 2001), there are no indications to date that HCO_3^- -dependent acid-extruding mechanisms are activated during anoxia in mammalian central neurons (e.g. Pirttilä and Kauppinen 1994). Rather, in mouse (Yao *et al.* 2001, 2003) and, possibly, rat (Sheldon and Church 2002) CA1 neurons, HCO_3^- -dependent acid-loading mechanism(s) become active during anoxia; in mouse CA1 neurons, the HCO_3^- -dependent mechanism may be an electrogenic $\text{Na}^+/\text{HCO}_3^-$ -co-transporter (Yao *et al.* 2003) although we (Baxter and Church 1996) and others (Schwiening and Boron 1994) have failed to uncover a contribution from $\text{Na}^+/\text{HCO}_3^-$ -co-transport to the regulation of pH_i in rat CA1 neuron somata under normoxic conditions (also see Schmitt *et al.* 2000). Nonetheless, it is becoming increasingly apparent that the complement of mechanisms which contribute to pH_i regulation during anoxia or ischemia in a given type of mammalian central neuron is not necessarily identical to that which operates under normoxic conditions.

In conclusion, the present study suggests that Na^+/H^+ exchange activity in adult rat hippocampal CA1 neurons is reduced during anoxia. The decline in observable Na^+/H^+ exchange activity during anoxia contrasts with the sudden activation of Na^+/H^+ exchange that occurs in this cell type immediately upon reoxygenation (Diarra *et al.* 1999; Sheldon and Church 2002; also see Obrenovitch *et al.* 1990; Pirttilä and Kauppinen 1992). The latter findings, together with those of the present study, suggest that, as in cardiac myocytes (Bond *et al.* 1993; Park *et al.* 1999), the neuroprotective effects of selective Na^+/H^+ exchange inhibitors (Vornov *et al.* 1996; Phillis *et al.* 1999; Horikawa *et al.* 2001) may be exerted in rat CA1 neurons immediately after anoxia.

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References

- Aharonovitz O., Zaun H. C., Balla T., York J. D., Orłowski J. and Grinstein S. (2000) Intracellular pH regulation by Na^+/H^+ exchange requires phosphatidylinositol 4,5-bisphosphate. *J. Cell Biol.* **150**, 213–224.
- Aronson P. S. (1985) Kinetic properties of the plasma membrane Na^+/H^+ exchanger. *Annu. Rev. Physiol.* **47**, 545–560.
- Balestrino M., Rebaudo R. and Lunardi G. (1999) Exogenous creatine delays anoxic depolarization and protects from hypoxic damage: dose–effect relationship. *Brain Res.* **816**, 124–130.
- Baxter K. A. and Church J. (1996) Characterization of acid extrusion mechanisms in cultured fetal rat hippocampal neurons. *J. Physiol.* **493**, 457–470.
- Bevensee M. O., Cummins T. R., Haddad G. G., Boron W. F. and Boyarsky G. (1996) pH regulation in single CA1 neurons acutely isolated from the hippocampi of immature and mature rats. *J. Physiol.* **494**, 315–328.
- Bond J. M., Chacon E., Herman B. and Lemasters J. J. (1993) Intracellular pH and Ca^{2+} homeostasis in the pH paradox of reperfusion injury to neonatal rat cardiac myocytes. *Am. J. Physiol.* **265**, C129–C137.
- Carter A. J., Muller R. E., Pschorn U. and Stransky W. (1995) Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampal slices. *J. Neurochem.* **64**, 2691–2699.
- Chambers-Kersh L., Ritucci N. A., Dean J. B. and Putnam R. W. (2000) Response of intracellular pH to acute anoxia in individual neurons from chemosensitive and non-chemosensitive regions of the medulla. *Adv. Exp. Med. Biol.* **475**, 453–464.
- Chen W.-H., Chu K.-C., Wu S.-J., Wu J.-C., Shui H.-A. and Wu M.-L. (1999) Early metabolic inhibition-induced intracellular sodium and calcium increase in rat cerebellar granule cells. *J. Physiol.* **515**, 133–146.
- Chinopoulos C., Tretter L., Rozsa A. and Adam-Vizi V. (2000) Exacerbated responses to oxidative stress by an Na^+ load in isolated nerve terminals: the role of ATP depletion and rise of $[\text{Ca}^{2+}]_i$. *J. Neurosci.* **20**, 2094–2103.
- Chow E. and Haddad G. G. (1998) Differential effects of anoxia and glutamate on cultured neocortical neurons. *Exp. Neurol.* **150**, 52–59.
- DeCoursey T. E. and Cherny V. V. (2000) Common themes and problems of bioenergetics and voltage-gated proton channels. *Biochim. Biophys. Acta* **1458**, 104–119.
- Demaurex N. and Grinstein S. (1994) Na^+/H^+ antiport: modulation by ATP and role in cell volume regulation. *J. Exp. Biol.* **196**, 389–404.
- Demaurex N., Romanek R. R., Orłowski J. and Grinstein S. (1997) ATP dependence of Na^+/H^+ exchange. Nucleotide specificity and assessment of the role of phospholipids. *J. Gen. Physiol.* **109**, 117–128.
- Diarra A., Sheldon C., Brett C. L., Baimbridge K. G. and Church J. (1999) Anoxia-evoked intracellular pH and Ca^{2+} concentration changes in cultured post-natal rat hippocampal neurons. *Neuroscience* **93**, 1003–1016.
- Diarra A., Sheldon C. and Church J. (2001) *In situ* calibration and $[\text{H}^+]$ sensitivity of the fluorescent Na^+ indicator, SBFI. *Am. J. Physiol.* **280**, C1623–C1633.
- Ercińska M. and Silver I. A. (1994) Ions and energy in mammalian brain. *Prog. Neurobiol.* **43**, 37–71.
- Friedman J. E. and Haddad G. G. (1994) Removal of extracellular sodium prevents anoxia-induced injury in freshly dissociated rat CA1 hippocampal neurons. *Brain Res.* **641**, 57–64.
- Hartley Z. and Dubinsky J. M. (1993) Changes in intracellular pH associated with glutamate excitotoxicity. *J. Neurosci.* **13**, 4690–4699.
- Horikawa N., Nishioka M., Itoh N., Kuribayashi Y., Matsui K. and Ohashi N. (2001) The Na^+/H^+ exchanger SM-20220 attenuates ischemic injury in *in vitro* and *in vivo* models. *Pharmacology* **63**, 76–81.
- Jean T., Frelin C., Vigne P., Barbry P. and Lazdunski M. (1985) Biochemical properties of the Na^+/H^+ exchange system in rat brain synaptosomes. *J. Biol. Chem.* **260**, 9678–9684.
- Jørgensen N. K., Petersen S. F., Damgaard I., Schousboe A. and Hoffman E. K. (1999) Increases in $[\text{Ca}^{2+}]_i$ and changes in intracellular pH during chemical anoxia in mouse neocortical neurons in primary culture. *J. Neurosci. Res.* **56**, 358–370.
- Kass I. S. and Lipton P. (1982) Mechanisms involved in irreversible anoxic damage to the *in vitro* rat hippocampal slice. *J. Physiol.* **332**, 459–472.
- Lamers J. M. J. (2001) Unmasking of a novel target for blocking harmful Na^+ coupled acid extrusion: electrogenic $\text{Na}^+/\text{HCO}_3^-$ symport. *Cardiovasc. Res.* **52**, 339–344.

- Lipton P. (1999) Ischemic cell death in brain neurons. *Physiol. Rev.* **79**, 1431–1568.
- Moor A. N., Gan X. T., Karmazyn M. and Fliegel L. (2001) Activation of Na⁺/H⁺ exchanger-directed protein kinases in the ischemic and ischemic-reperfused rat myocardium. *J. Biol. Chem.* **276**, 16113–16122.
- Mutch W. and Hansen A. (1984) Extracellular pH changes during spreading depression and cerebral ischemia: mechanisms of brain pH regulation. *J. Cereb. Blood Flow Metab.* **4**, 17–27.
- Obrenovitch T. P., Scheller D., Matsumoto T., Tegtmeier F., Höller M. and Symon L. (1990) A rapid redistribution of hydrogen ions is associated with depolarization and repolarization subsequent to cerebral ischemia reperfusion. *J. Neurophysiol.* **64**, 1125–1133.
- Park C.-O., Xiao X.-H. and Allen D. G. (1999) Changes in intracellular Na⁺ and pH in rat heart during ischemia: role of Na⁺/H⁺ exchanger. *Am. J. Physiol.* **276**, H1581–H1590.
- Phillis J. W., Estevez A. Y., Guyot L. L. and O'Regan M. H. (1999) 5-(N-ethyl-N-isopropyl)-amiloride, an Na⁺-H⁺ exchange inhibitor, protects gerbil hippocampal neurons from ischemic injury. *Brain Res.* **839**, 199–202.
- Pirttilä T.-R. M. and Kauppinen R. A. (1992) Recovery of intracellular pH in cortical brain slices following anoxia studied by nuclear magnetic resonance spectroscopy: role of lactate removal, extracellular sodium and sodium/hydrogen exchange. *Neuroscience* **47**, 155–164.
- Pirttilä T.-R. M. and Kauppinen R. A. (1994) Regulation of intracellular pH in guinea pig cerebral cortex *ex vivo* studied by ³¹P and ¹H nuclear magnetic resonance spectroscopy: role of extracellular bicarbonate and chloride. *J. Neurochem.* **62**, 656–664.
- Raley-Susman K. M., Cragoe E. J., JrSapolsky R. M. and Kopito R. R. (1991) Regulation of intracellular pH in cultured hippocampal neurons by an amiloride-insensitive Na⁺/H⁺ exchanger. *J. Biol. Chem.* **266**, 2739–2745.
- Richmond P. H. and Vaughan-Jones R. D. (1997) Assessment of evidence for K⁺-H⁺ exchange in isolated type-1 cells of neonatal rat carotid body. *Pflügers Arch.* **434**, 429–437.
- Satoh H., Sugiyama S., Nomura N., Terada H. and Hayashi H. (2001) Importance of glycolytically derived ATP for Na⁺ loading via Na⁺/H⁺ exchange during metabolic inhibition in guinea pig ventricular myocytes. *Clin. Sci.* **101**, 243–251.
- Schmitt B. M., Berger U. V., Douglas R. M., Bevensee M. O., Hediger M. A., Haddad G. G. and Boron W. F. (2000) Na/HCO₃ cotransporters in rat brain: Expression in glia, neurons, and choroid plexus. *J. Neurosci.* **20**, 6839–6848.
- Schwiening C. J. and Boron W. F. (1994) Regulation of intracellular pH in pyramidal neurones from the rat hippocampus by Na⁺-dependent Cl⁻ HCO₃⁻ exchange. *J. Physiol.* **475**, 59–67.
- Sheldon C. and Church J. (2002) Intracellular pH response to anoxia in acutely dissociated adult rat hippocampal CA1 neurons. *J. Neurophysiol.* **87**, 2209–2224.
- Sheldon C., Diarra A. and Church J. (2001) Anoxia-evoked changes in intracellular sodium concentration in rat hippocampal neurons. *Soc. Neurosci. Abstract* **27**, 868.8.
- Sheline C. T., Behrens M. M. and Choi D. W. (2000) Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. *J. Neurosci.* **20**, 3139–3146.
- Siesjö B. K., Katsura K. and Kristián T. (1996) Acidosis-related damage. In: *Advances in Neurology: Cellular and Molecular Mechanisms of Ischemic Brain Damage*, Vol. 71 (Siesjö, B. K. and Wieloch, T., eds), pp. 209–236. Lippincott-Raven, Philadelphia.
- Smith G. A. M., Brett C. L. and Church J. (1998) Effects of noradrenaline on intracellular pH in acutely dissociated adult rat hippocampal CA1 neurones. *J. Physiol.* **512**, 487–505.
- Sun G. Y. and Hsu C. Y. (1996) Poly-phosphoinositide-mediated messengers in focal cerebral ischemia and reperfusion. *J. Lipid Med. Cell Signalling* **14**, 137–145.
- Szabó E. Z., Numata M., Shull G. E. and Orłowski J. (2000) Kinetic and pharmacological properties of human brain Na⁺/H⁺ exchanger isoform 5 stably expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **275**, 6302–6307.
- Taylor D. L., Obrenovitch T. P. and Symon L. (1996) Changes in extracellular acid-base homeostasis in cerebral ischemia. *Neurochem. Res.* **21**, 1013–1021.
- Vornov J. J., Thomas A. G. and Jo D. (1996) Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. *J. Neurochem.* **67**, 2379–2389.
- Wakabayashi S., Shigekawa M. and Pouyssegur J. (1997) Molecular physiology of vertebrate Na⁺/H⁺ exchangers. *Physiol. Rev.* **77**, 51–74.
- Wu M.-L. and Vaughan-Jones R. D. (1994) Effect of metabolic inhibitors and second messengers upon Na⁺-H⁺ exchange in the sheep cardiac Purkinje fibre. *J. Physiol.* **478**, 301–313.
- Wu M.-L. and Vaughan-Jones R. D. (1997) Interaction between Na⁺ and H⁺ ions on Na-H exchange in sheep cardiac Purkinje fibers. *J. Mol. Cell. Cardiol.* **29**, 1131–1140.
- Yao H., Gu X.-Q., Douglas R. M. and Haddad G. G. (2001) Role of Na⁺/H⁺ exchanger during O₂ deprivation in mouse CA1 neurons. *Am. J. Physiol.* **281**, C1205–C1210.
- Yao H., Gu X.-Q. and Haddad G. G. (2003) The role of HCO₃⁻ -dependent mechanisms in pH_i regulation during O₂ deprivation. *Neuroscience* **117**, 29–35.