

ANOXIA-EVOKED INTRACELLULAR pH AND  $\text{Ca}^{2+}$  CONCENTRATION CHANGES  
IN CULTURED POSTNATAL RAT HIPPOCAMPAL NEURONS

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**Abstract**—The ratiometric indicators 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein and Fura-2 were employed to examine, respectively, intracellular pH ( $\text{pH}_i$ ) and calcium ( $[\text{Ca}^{2+}]_i$ ) changes evoked by anoxia in cultured postnatal rat hippocampal neurons at 37°C. Under both  $\text{HCO}_3^-/\text{CO}_2$ - and HEPES-buffered conditions, 3-, 5- or 10-min anoxia induced a triphasic change in  $\text{pH}_i$ , consisting of an initial fall in  $\text{pH}_i$ , a subsequent rise in  $\text{pH}_i$  in the continued absence of  $\text{O}_2$  and, finally, a further rise in  $\text{pH}_i$  upon the return to normoxia, which recovered towards preanoxic steady-state  $\text{pH}_i$  values if the duration of the anoxic insult was  $\leq 5$  min. In parallel experiments performed on sister cultures, anoxia of 3, 5 or 10 min duration evoked rises in  $[\text{Ca}^{2+}]_i$  which, in all cases, commenced after the start of the fall in  $\text{pH}_i$ , reached a peak at or just following the return to normoxia and then declined towards preanoxic resting levels. Removal of external  $\text{Ca}^{2+}$  markedly attenuated increases in  $[\text{Ca}^{2+}]_i$ , but failed to affect the  $\text{pH}_i$  changes evoked by 5 min anoxia.

The latency from the start of anoxia to the start of the increase in  $\text{pH}_i$  observed during anoxia was increased by perfusion with media containing either 2 mM  $\text{Na}^+$ , 20 mM glucose or 1  $\mu\text{M}$  tetrodotoxin. Because each of these manoeuvres is known to delay the onset and/or attenuate the magnitude of anoxic depolarization, the results suggest that the rise in  $\text{pH}_i$  observed during anoxia may be consequent upon membrane depolarization. This possibility was also suggested by the findings that  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ , known blockers of voltage-dependent proton conductances, reduced the magnitude of the rise in  $\text{pH}_i$  observed during anoxia.

Under  $\text{HCO}_3^-/\text{CO}_2$ -free conditions, reduction of external  $\text{Na}^+$  by substitution with *N*-methyl-D-glucamine (but not  $\text{Li}^+$ ) attenuated the magnitude of the postanoxic alkalization, suggesting that increased  $\text{Na}^+/\text{H}^+$  exchange activity contributes to the postanoxic rise in  $\text{pH}_i$ . In support, rates of  $\text{pH}_i$  recovery from internal acid loads imposed following anoxia were increased compared to control values established prior to anoxia in the same neurons. In contrast, rates of  $\text{pH}_i$  recovery from acid loads imposed during anoxia were reduced, suggesting the possibility that  $\text{Na}^+/\text{H}^+$  exchange is inhibited during anoxia.

We conclude that the steady-state  $\text{pH}_i$  response of cultured rat hippocampal neurons to transient anoxia is independent of changes in  $[\text{Ca}^{2+}]_i$  and is characterized by three phases which are determined, at least in part, by alterations in  $\text{Na}^+/\text{H}^+$  exchange activity and, possibly, by a proton conductance which is activated during membrane depolarization. © 1999 IBRO. Published by Elsevier Science Ltd.

**Key words:** anoxia, intracellular pH, intracellular calcium,  $\text{Na}^+/\text{H}^+$  exchange, voltage-activated  $\text{H}^+$  conductance.

Recent studies suggest that changes in intracellular pH ( $\text{pH}_i$ ) may mediate at least some of the effects of anoxia and/or ischaemia on central neuronal function (reviewed in Refs 67 and 75). However, it remains unknown whether neuronal viability following transient anoxia is influenced primarily by changes in  $\text{pH}_i$  which occur during anoxia, or whether  $\text{pH}_i$  changes in the period immediately following anoxia are more important. In hepatocytes and myocytes, for example, tissue injury following transient anoxia appears to be mediated not by the fall in  $\text{pH}_i$  during the anoxic insult, but by activation of  $\text{Na}^+/\text{H}^+$  exchange in the immediate postanoxic period, and subsequent rises in  $\text{pH}_i$ , intracellular  $\text{Na}^+$  concentration ( $[\text{Na}^+]_i$ ) and, via reverse mode  $\text{Na}^+/\text{Ca}^{2+}$  exchange, intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ).<sup>5,28,34,39,43,53</sup> The importance of  $\text{Na}^+/\text{H}^+$  exchange in determining viability in these cell types is emphasized by

the fact that pharmacological inhibitors of the antiport are effective cardioprotective agents.<sup>53</sup> Similar mechanisms may operate in central neurons, given both the established involvement of reverse  $\text{Na}^+/\text{Ca}^{2+}$  exchange in anoxia-induced CNS white matter injury (reviewed in Ref. 72), and the correlation between poor metabolic recovery and the degree of intracellular alkalosis following ischaemia in the brain (e.g., Ref. 82). Indeed, the potential importance of alterations in  $\text{Na}^+/\text{H}^+$  exchange activity for neuronal viability following ischaemic/anoxic insults has been highlighted by the finding that inhibition of  $\text{Na}^+/\text{H}^+$  exchange in cultured rat neocortical neurons following the combined application of 2-deoxy-D-glucose and potassium cyanide results in neuroprotection, which was ascribed to a slower restoration of  $\text{pH}_i$  to normal values from the intracellular acidosis caused by metabolic inhibition and, possibly, reduced internal  $\text{Na}^+$  loading.<sup>77</sup>

Because  $\text{pH}_i$  may play an important role in the pathophysiology of cell death following anoxia and/or ischaemia, a knowledge of the  $\text{pH}_i$  changes evoked by anoxia in central neurons and the mechanisms involved in the regulation of neuronal  $\text{pH}_i$  both during and following anoxia may provide insights into the pathogenesis of neurodegenerative phenomena. Although anoxia is known to elicit changes in  $\text{pH}_i$  both *in vivo* and in slice preparations *in vitro* (e.g., Refs 21, 47, 54, 56, 57 and 67), it is difficult to separate the contribution of various cell types (including glia) to the changes observed under these experimental conditions, and a variety of factors

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**Abbreviations:** BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein;  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$  concentration; EGTA, ethylene-glycolbis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetra-acetate; EMEM, Eagle's minimum essential medium;  $g_{\text{H}^+}$ , voltage-activated proton conductance; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid;  $[\text{Na}^+]_i$ , intracellular  $\text{Na}^+$  concentration;  $[\text{Na}^+]_o$ , extracellular  $\text{Na}^+$  concentration; NMDG<sup>+</sup>, *N*-methyl-D-glucamine;  $\text{pH}_i$ , intracellular pH;  $\text{pH}_o$ , extracellular pH; TTX, tetrodotoxin.

(including variations in extracellular pH,  $pH_o$ ) may hinder the characterization of mechanisms which contribute to the changes in  $pH_i$ . In addition, although changes in neuronal  $pH_i$  associated with glutamate receptor-mediated excitotoxicity and metabolic inhibition have been described,<sup>27,77</sup> these insults are not a completely valid model for the direct action of anoxia on central neurons (see Ref. 18). In the present study, therefore, we have characterized the  $pH_i$  changes which occur in response to transient anoxia in cultured rat hippocampal neurons, an experimental preparation in which the  $pH_i$ -regulating mechanisms operating under normoxic conditions have been extensively characterized,<sup>2,60</sup> and in which changes in  $pH_i$  can be reliably quantified and their underlying mechanisms investigated.

#### EXPERIMENTAL PROCEDURES

All aspects of the study were conducted in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals, and were approved by the University of British Columbia Animal Care Committee.

#### Cell culture

Primary cultures of hippocampal neurons from four-day postnatal Wistar rats (Animal Care Centre, University of British Columbia) were prepared as described previously.<sup>1</sup> Briefly, rat pups were anaesthetized, decapitated and the hippocampi removed. The hippocampi were enzymatically and mechanically dissociated and the resulting cell suspension was underlain with fetal bovine serum and centrifuged at  $150 \times g$  at  $4^\circ\text{C}$  for 10 min. The cells were then re-suspended and plated at a density of  $4 \times 10^5$  neurons/cm<sup>2</sup> onto glass coverslips coated with poly-D-lysine and laminin. The initial growth medium was Eagle's minimum essential medium (EMEM; Life Technologies, Grand Island, NY, U.S.A.) supplemented with 5% horse serum and 5% fetal bovine serum. After 24 h, this medium was half-changed with N2-supplemented EMEM. The cultures were then fed every four to five days by half-changing the existing medium with N2-supplemented EMEM. Glial cell proliferation was inhibited 48 h after initial plating by adding cytosine arabinoside to a final concentration of 10  $\mu\text{M}$ . Neurons were used seven to 12 days after plating.

#### Solutions

The standard  $\text{HCO}_3^-/\text{CO}_2$ -free, HEPES-buffered perfusion medium contained (mM): NaCl 136.5, KCl 3,  $\text{NaH}_2\text{PO}_4$  1.5,  $\text{MgSO}_4$  1.5, D-glucose 10,  $\text{CaCl}_2$  2 and HEPES 10 (titrated with 10 M NaOH to pH 7.35 at  $37^\circ\text{C}$ ). The standard  $\text{HCO}_3^-/\text{CO}_2$ -buffered medium contained (mM): NaCl 126.5, KCl 3,  $\text{NaHCO}_3$  20,  $\text{NaH}_2\text{PO}_4$  1.5,  $\text{MgSO}_4$  1.5, D-glucose 10 and  $\text{CaCl}_2$  2; it was saturated with 5%  $\text{CO}_2/95\%$  air, giving a pH of 7.35 at  $37^\circ\text{C}$ . pH 6.8 and pH 7.75  $\text{HCO}_3^-/\text{CO}_2$ -buffered media contained 5.8 and 46 mM  $\text{NaHCO}_3$ , respectively; the changes in  $\text{NaHCO}_3$  concentration were balanced by equimolar changes in NaCl concentration. During perfusion with  $\text{HCO}_3^-$ -containing media, the atmosphere in the recording chamber contained 5%  $\text{CO}_2/95\%$  air. When 40 mM  $\text{NH}_4\text{Cl}$  was added, an equimolar amount of NaCl was omitted. In  $\text{HCO}_3^-/\text{CO}_2$ -free, HEPES-buffered media in which  $\text{Na}^+$  concentration was 2 mM (employed to assess the contribution of  $\text{Na}^+/\text{H}^+$  exchange to the  $pH_i$  response to anoxia), NaCl was reduced to 0.5 mM by substitution with either *N*-methyl-D-glucamine (NMDG<sup>+</sup>) or  $\text{Li}^+$ , and HCl or LiOH, respectively, were used to titrate the solutions to pH 7.35 at  $37^\circ\text{C}$ . In order to maintain extracellular  $\text{Na}^+$  concentration ( $[\text{Na}^+]_o$ ) constant during an experiment, media devoid of  $\text{Na}^+$  could not be employed because anoxia was induced with sodium dithionite (see below). However, 2 mM  $\text{Na}^+$  is considerably less than the apparent  $K_m$  of the  $\text{Na}^+/\text{H}^+$  exchanger in cultured rat hippocampal neurons for external  $\text{Na}^+$  ( $K_m = 23\text{--}26$  mM).<sup>60</sup> In addition, we confirmed that the recovery of  $pH_i$  from internal acid loads imposed by the  $\text{NH}_4^+$  prepulse technique was abolished during perfusion with HEPES-buffered medium containing 2 mM external  $\text{Na}^+$  ( $n = 3$ ; data not shown). For  $\text{Ca}^{2+}$ -free media,  $\text{Ca}^{2+}$  was omitted,  $[\text{Mg}^{2+}]$  was increased to 4 mM and 100  $\mu\text{M}$  EGTA was added. Solutions containing 20 mM glucose were prepared by iso-osmotic substitution for

NaCl. In solutions containing  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$ ,  $\text{H}_2\text{PO}_4^-$  and  $\text{SO}_4^{2-}$  were omitted.

#### Induction of anoxia

Anoxic media were prepared immediately prior to use by adding 1–2 mM sodium dithionite (an  $\text{O}_2$  scavenger)<sup>18</sup> to the media defined above, bubbling vigorously with 5%  $\text{CO}_2/95\%$   $\text{N}_2$  or argon ( $\text{HCO}_3^-/\text{CO}_2$ -buffered media) or 100%  $\text{N}_2$  or argon (HEPES-buffered media) and, if necessary, readjusting the pH of HEPES-buffered media to pH 7.35. During anoxia, the atmosphere in the recording chamber was switched to 5%  $\text{CO}_2/95\%$   $\text{N}_2$  or argon ( $\text{HCO}_3^-/\text{CO}_2$ -buffered media) or 100%  $\text{N}_2$  or argon (HEPES-buffered media). The  $p\text{O}_2$  in  $\text{Na}_2\text{S}_2\text{O}_4$ -containing media, measured with a Radiometer ABL 500 blood gas analyser calibrated for low  $p\text{O}_2$  values, was  $< 1$  mm Hg ( $n = 4$ ). In order to verify that the results obtained with sodium dithionite reflected only its  $\text{O}_2$  scavenging properties, control experiments were performed by inducing anoxia with media saturated for 24 h with 100% argon or 5%  $\text{CO}_2/95\%$  argon (for HEPES- and  $\text{HCO}_3^-/\text{CO}_2$ -buffered media, respectively). The measured  $p\text{O}_2$  values in these media were  $< 1$  mm Hg ( $n = 2$  in each case) and the  $pH_i$  changes evoked by a 5-min exposure to either of the solutions were indistinguishable from those induced by exposure to 1–2 mM  $\text{Na}_2\text{S}_2\text{O}_4$ -containing media (also see Ref. 18).

#### Microspectrofluorimetry

Fura-2 and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) were employed to estimate  $[\text{Ca}^{2+}]_i$  and  $pH_i$ , respectively. Full details of the methods employed have been described previously.<sup>2,7,8,70</sup> In brief, coverslips plated with neurons were placed in loading medium (which contained the same elements as the standard pH 7.35 HEPES-buffered medium with the iso-osmotic addition of 3 mM  $\text{NaHCO}_3$  in place of NaCl) containing either 5  $\mu\text{M}$  Fura-2 acetoxymethyl ester for 60 min at  $35^\circ\text{C}$  or 2  $\mu\text{M}$  BCECF acetoxymethyl ester for 30 min at room temperature. They were then mounted in a temperature-controlled perfusion chamber so as to form the base of the chamber and were superfused at a rate of 2 ml/min for 15 min with the initial experimental solution at  $37^\circ\text{C}$  prior to the start of an experiment. All experiments were performed at  $37^\circ\text{C}$ .

$[\text{Ca}^{2+}]_i$  and  $pH_i$  were measured using the dual-excitation ratio method, employing a digital fluorescence microscopy system (Atto Instruments Inc., Rockville, MD, U.S.A.; Carl Zeiss Canada Ltd., Don Mills, Ontario, Canada). The excitation wavelengths were 488 and 452 nm for BCECF, and 334 and 380 nm for Fura-2. Fluorescence emissions from individual neurons loaded with either BCECF or Fura-2 were obtained simultaneously from multiple neuronal somata and raw emission intensity values at each excitation wavelength were corrected for background fluorescence prior to calculation of ratios, which were acquired every 3–20 s. Calibration of the Fura-2 signal was not attempted (see Ref. 7) and the effects of experimental manoeuvres on  $[\text{Ca}^{2+}]_i$  are presented as changes in background-corrected emission intensity ratio ( $I_{334}/I_{380}$ ) values. The one-point high- $\text{K}^+$ /nigericin technique was employed to convert  $I_{488}/I_{452}$  ratios into  $pH_i$  values. Analysis was restricted to those neurons able to retain BCECF (as judged by raw emission intensity values) throughout the course of an experiment.<sup>3</sup> In studies where information was required on the effects of anoxia on both  $[\text{Ca}^{2+}]_i$  and  $pH_i$ , measurements of  $[\text{Ca}^{2+}]_i$  and  $pH_i$  were performed separately in parallel experiments conducted on sister cultures.

#### Data analysis

In order to compare results obtained under different experimental conditions, a number of parameters were defined and measured.

Figure 1A illustrates the typical changes in  $pH_i$  observed in response to a 5-min anoxic insult. The  $pH_i$  response was characterized by: (i) a decrease in  $pH_i$  following the induction of anoxia; (ii) a rise in  $pH_i$  in the continued absence of  $\text{O}_2$ ; and (iii) a further intracellular alkalization upon the return to normoxia which, in some cases (see Results), gradually recovered towards preanoxic steady-state  $pH_i$  values. The measured parameters were as follows. (1) The magnitude of the acidic shift induced by anoxia ( $\Delta pH_{i(\text{acid})}$ ), i.e. the difference between the preanoxic steady-state  $pH_i$  and the minimum  $pH_i$  observed during anoxia. (2) The magnitude of the rise in  $pH_i$  during anoxia ( $\Delta pH_{i(\text{anoxia})}$ ), i.e. the difference between the minimum  $pH_i$  reached during anoxia and the  $pH_i$  immediately prior to the return to normoxia. This measurement

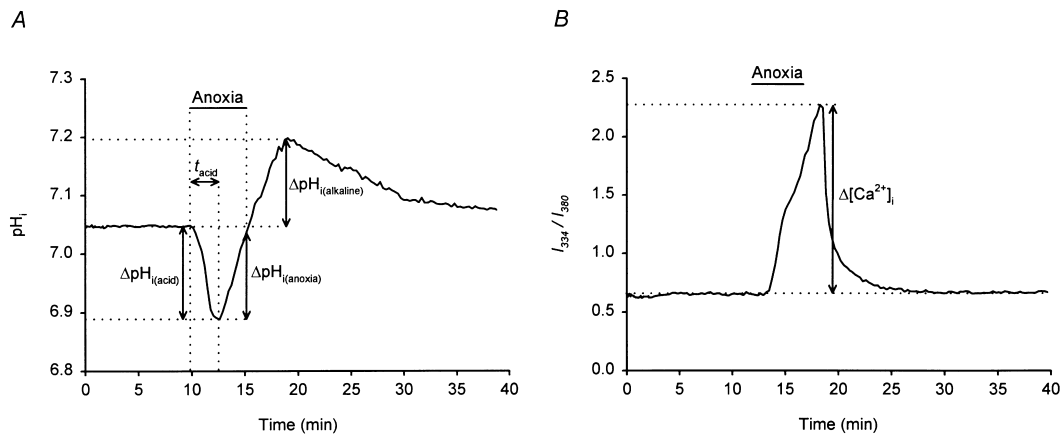


Fig. 1. Parameters measured in the study. (A) A representative trace of the  $pH_i$  changes evoked by a 5-min period of anoxia in rat hippocampal neurons. The response to anoxia was characterized by a fall in  $pH_i$  following the induction of anoxia, followed by a rise in  $pH_i$  in the continued absence of  $O_2$ . Upon the return to normoxia, a further internal alkalization occurred which, in the example shown, recovered towards the preanoxic steady-state  $pH_i$  level. Indicated on the trace are the parameters measured in the study: (i) the magnitude of the acidic shift induced by anoxia ( $\Delta pH_{i(\text{acid})}$ ); (ii) the magnitude of the rise in  $pH_i$  during anoxia ( $\Delta pH_{i(\text{anoxia})}$ ); (iii) the magnitude of the alkaline shift observed following the return to normoxia ( $\Delta pH_{i(\text{alkaline})}$ ); and (iv) the interval between the induction of anoxia and the time-point at which  $pH_i$  started to increase in the continued absence of  $O_2$  ( $t_{\text{acid}}$ ). The trace is a mean of data obtained simultaneously from 20 neurons on a single coverslip. (B) A representative trace of changes in background-subtracted  $I_{334}/I_{380}$  ratio values in response to 5-min anoxia in rat hippocampal neurons. The response to anoxia was characterized by an increase in the  $I_{334}/I_{380}$  ratio value (representing an increase in  $[Ca^{2+}]_i$ ), followed by the recovery of the  $I_{334}/I_{380}$  ratio value to preanoxic resting levels. The parameter measured was the magnitude of the increase in the  $I_{334}/I_{380}$  ratio value evoked by anoxia ( $\Delta[Ca^{2+}]_i$ ). The trace is a mean of data obtained simultaneously from 18 neurons on a single coverslip.

Table 1. Effects of anoxia on intracellular pH

Duration of anoxia (min)	Buffer	$\Delta pH_{i(\text{acid})}$ (pH units)	$t_{\text{acid}}$ (s)	$pH_i$ recovery (%)	$\Delta pH_{i(\text{alkaline})}$ (pH units)	<i>n</i>
3	$HCO_3^-/CO_2$	$0.15 \pm 0.01$	$92 \pm 12$	$8 \pm 2$	$0.12 \pm 0.01$	3
	HEPES	$0.18 \pm 0.03$	$97 \pm 12$	$14 \pm 5$	$0.12 \pm 0.02$	5
5	$HCO_3^-/CO_2$	$0.14 \pm 0.01$	$120 \pm 12$	$108 \pm 9$	$0.18 \pm 0.03$	14
	HEPES	$0.17 \pm 0.02$	$106 \pm 9$	$102 \pm 24$	$0.25 \pm 0.03^*$	17
10	$HCO_3^-/CO_2$	$0.14 \pm 0.02$	$110 \pm 13$	$262 \pm 34$	$0.29 \pm 0.02$	7
	HEPES	$0.16 \pm 0.02$	$109 \pm 9$	$290 \pm 43$	$0.36 \pm 0.03^*$	8

$\Delta pH_{i(\text{acid})}$  is the magnitude of the acidic shift induced by anoxia;  $t_{\text{acid}}$  is the time from the induction of anoxia to the time at which  $pH_i$  started to increase in the continued absence of  $O_2$ ;  $pH_i$  recovery is the percentage recovery of  $pH_i$  towards preanoxic resting levels in the continued absence of  $O_2$  calculated according to the equation: percentage  $pH_i$  recovery =  $100 \times (\Delta pH_{i(\text{anoxia})} / \Delta pH_{i(\text{acid})})$ , where  $\Delta pH_{i(\text{anoxia})}$  is the magnitude of the rise of  $pH_i$  observed during anoxia;  $\Delta pH_{i(\text{alkaline})}$  is the magnitude of the alkaline shift observed following the return to normoxia. \* $P < 0.05$  for difference from corresponding value obtained under  $HCO_3^-/CO_2$ -buffered conditions. In all cases,  $pH_o$  was 7.35.

was employed to calculate the percentage recovery of  $pH_i$  towards preanoxic resting levels in the continued absence of  $O_2$  according to the equation: percentage  $pH_i$  recovery =  $100 \times (\Delta pH_{i(\text{anoxia})} / \Delta pH_{i(\text{acid})})$ . In the example shown in Fig. 1A, the percentage  $pH_i$  recovery calculated according to this equation was 94%. (3) The magnitude of the alkaline shift observed following the return to normoxia ( $\Delta pH_{i(\text{alkaline})}$ ), i.e. the difference between the preanoxic steady-state  $pH_i$  and the maximum  $pH_i$  observed following the anoxic insult. (4)  $t_{\text{acid}}$ , defined as the time between the induction of anoxia and the time-point at which  $pH_i$  started to increase in the continued absence of  $O_2$ .

Typical changes in  $I_{334}/I_{380}$  ratio values evoked by 5-min anoxia are illustrated in Fig. 1B. The measured parameter was the magnitude of the increase in the  $I_{334}/I_{380}$  ratio value evoked by anoxia ( $\Delta[Ca^{2+}]_i$ ), i.e. the difference between the preanoxic steady-state  $I_{334}/I_{380}$  ratio value and the peak  $I_{334}/I_{380}$  ratio value evoked by anoxia.

$Na^+/H^+$  exchange activity was assessed by measuring the rate of recovery of  $pH_i$  from internal acid loads imposed by the  $NH_4^+$  prepulse technique under  $HCO_3^-/CO_2$ -free conditions. Full details of the methods employed have been presented previously.<sup>2,70</sup> In brief, in each experiment in which rates of  $pH_i$  recovery were examined, two consecutive intracellular acid loads were imposed, the first being employed to calculate control rates of  $pH_i$  recovery for a given population of neurons and the second being performed either during or immediately following an anoxic insult (see Fig. 6A and D). We have

established previously that rates of  $pH_i$  recovery are consistent between two consecutive internal acid loads imposed in the absence of a test treatment.<sup>70</sup> In view of the fact that changes in  $pH_i$  elicit changes in  $Na^+/H^+$  exchange activity,<sup>44</sup> control rates of  $pH_i$  recovery were compared to rates of  $pH_i$  recovery under a test condition at the same absolute values of  $pH_i$ .

A given experimental manoeuvre was performed on a minimum of three (usually five or more) populations of cultured cells. Data are presented as means  $\pm$  S.E.M., with the accompanying *n* value referring to the number of cell populations (i.e. the number of coverslips) from which data were obtained. The total number of neurons from which data were obtained was 1386. Statistical comparisons were carried out using Student's two-tailed *t*-test, paired or unpaired as appropriate, with a 95% confidence limit.

## RESULTS

### Characterization of the effects of transient anoxia on intracellular pH

Initially, the effects on  $pH_i$  of anoxic insults of 3, 5 and 10 min duration were examined under  $HCO_3^-/CO_2$ -buffered conditions at a constant  $pH_o$  (7.35). The results are

summarized in Table 1. The  $\text{pH}_i$  changes in response to 5-min anoxia were typified by a decrease in  $\text{pH}_i$  following the induction of anoxia, a subsequent rise in  $\text{pH}_i$  in the continued absence of  $\text{O}_2$  and, finally, a further intracellular alkalinization upon the return to normoxia which, in nine of 14 cell populations examined, gradually recovered towards preanoxic steady-state  $\text{pH}_i$  values (Figs 1A, 2B). This triphasic pattern of  $\text{pH}_i$  changes was also observed in response to 3- and 10-min anoxic insults (Fig. 2A and C, respectively). The magnitude of the acidic shift observed during anoxia ( $\Delta\text{pH}_{i(\text{acid})}$ ) was not affected significantly by the duration of the anoxic insult (Table 1). However, both the extent of the rise in  $\text{pH}_i$  observed in the continued absence of  $\text{O}_2$  (measured as percentage  $\text{pH}_i$  recovery) and the magnitude of the alkaline shift observed following the return to normoxia ( $\Delta\text{pH}_{i(\text{alkaline})}$ ) appeared to increase progressively as the duration of the anoxic insult increased. At least a partial ( $> 50\%$ ) recovery of  $\text{pH}_i$  from the postanoxic intracellular alkalinization was observed in all of three and in none of seven cell populations subjected to 3- and 10-min anoxia, respectively (see Fig. 2). The sustained increases in  $\text{pH}_i$ , sometimes observed following anoxia were not due to a decline in  $I_{452}$  values consequent upon a deterioration in membrane integrity (see Ref. 70).

#### Role of changes in intracellular free $\text{Ca}^{2+}$ concentration

It is well established that a complex relationship exists in neurons between changes in intracellular  $\text{H}^+$  concentration and  $[\text{Ca}^{2+}]_i$  (e.g., Refs 45 and 51). Furthermore, as noted in the Introduction, in peripheral cell types, activation of  $\text{Na}^+/\text{H}^+$  exchange in the postanoxic period (see below) may contribute to anoxia-evoked increases in  $[\text{Ca}^{2+}]_i$ . In order to examine the possible relationship between changes in  $[\text{Ca}^{2+}]_i$  and  $\text{pH}_i$  evoked by transient anoxia, we measured changes in  $I_{334}/I_{380}$  ratio values evoked by 3-, 5- and 10-min periods of anoxia under  $\text{HCO}_3^-/\text{CO}_2$ -buffered conditions in sister cultures to those employed in the  $\text{pH}_i$  measurements.

Typical changes in  $I_{334}/I_{380}$  ratio values observed in response to 3-, 5- and 10-min anoxic insults are illustrated in Fig. 2A–C. The  $[\text{Ca}^{2+}]_i$  response to 5-min anoxia was characterized by a  $1.63 \pm 0.14$  ( $n = 14$ ) increase in the  $I_{334}/I_{380}$  ratio value, which reached a maximum at or immediately following the end of the anoxic insult and then recovered towards preanoxic resting levels (Fig. 2B). The magnitude of the increase in  $I_{334}/I_{380}$  ratio values ( $\Delta[\text{Ca}^{2+}]_i$ ) was a function of the duration of the anoxic insult, being  $0.67 \pm 0.21$  ( $n = 3$ ) and  $3.42 \pm 0.43$  ( $n = 7$ ) in response to 3- and 10-min anoxic insults, respectively ( $P < 0.05$  for the differences between 3- and 5-min anoxic groups and 5- and 10-min anoxic groups). Recovery of  $I_{334}/I_{380}$  ratio values to preanoxic resting levels was observed in all cell populations following 3- or 5-min anoxia (see Fig. 2A and B), and in the majority of neurons (82/100 neurons in the seven neuronal populations examined) subjected to 10-min anoxia (Fig. 2C). The observation that  $[\text{Ca}^{2+}]_i$  is not permanently elevated in the immediate postanoxic period reflects findings made in cultured neurons following exposure to excitotoxic concentrations of glutamate<sup>1,12</sup> or following transient ischaemia *in vivo*.<sup>15</sup> In addition, an anoxic insult did not leave neurons refractory to stimulation. Thus, in three neuronal populations in which  $[\text{Ca}^{2+}]_i$  had recovered to preanoxic steady-state levels following a 10-min anoxic insult, transient application of  $20 \mu\text{M}$  *N*-methyl-D-aspartate (see Ref. 8) evoked transient increases in

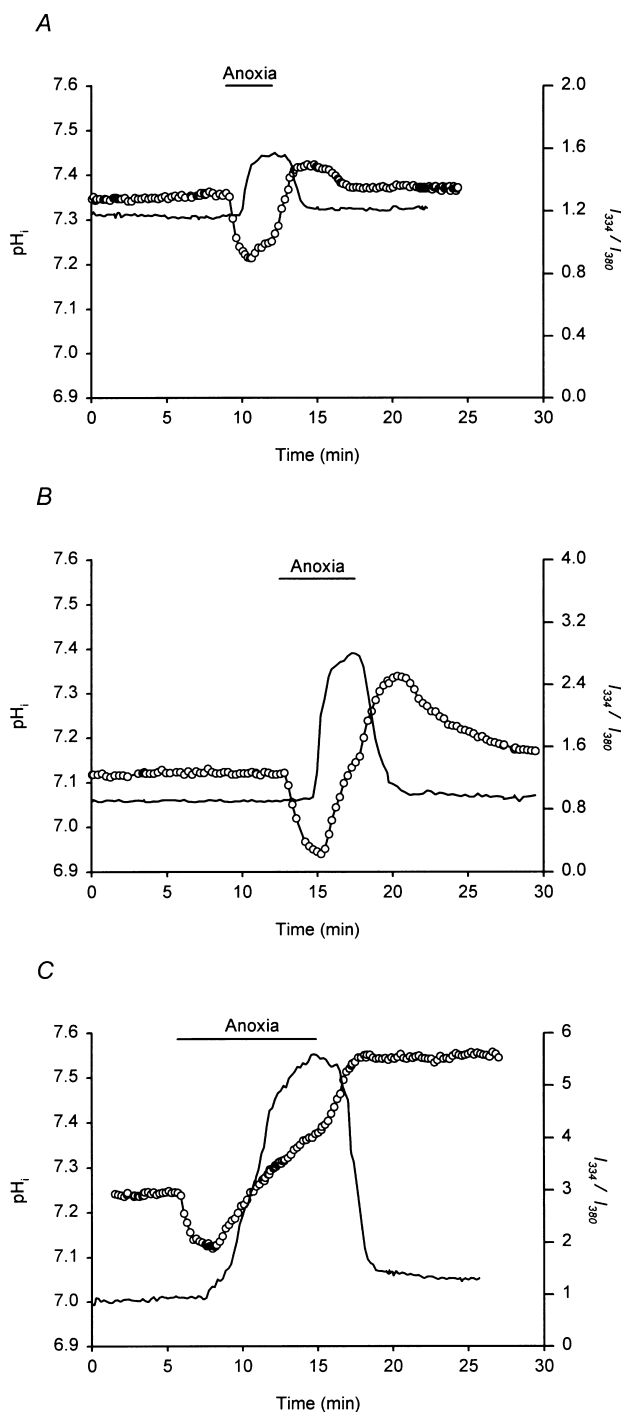


Fig. 2. Changes in  $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  evoked by transient anoxic insults. Illustrated are changes in  $\text{pH}_i$  (open circles) and background-subtracted  $I_{334}/I_{380}$  ratio values (representing changes in  $[\text{Ca}^{2+}]_i$ ; continuous lines) in response to 3- (A), 5- (B) and 10-min (C) anoxic insults. In A–C,  $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  measurements were performed on sister cultures in parallel experiments under identical  $\text{HCO}_3^-/\text{CO}_2$ -buffered conditions. In each case, the  $\text{pH}_i$  response to transient anoxia was characterized by an initial fall in  $\text{pH}_i$  (which commenced prior to the increase in the  $I_{334}/I_{380}$  ratio value), a subsequent rise in  $\text{pH}_i$  in the continued absence of  $\text{O}_2$  (which commenced at a similar time as the increase in the  $I_{334}/I_{380}$  ratio value), and a further internal alkalinization in the postanoxic period.  $I_{334}/I_{380}$  ratio values recovered towards preanoxic resting levels under each experimental condition, whereas  $\text{pH}_i$  remained elevated following the 10-min anoxic insult. Records are means of data obtained simultaneously from 16 and 18 ( $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  traces, respectively) neurons in A, 20 and 20 neurons in B, and 25 and 15 neurons in C. Note the change in scale for  $I_{334}/I_{380}$  ratio values from A to C.

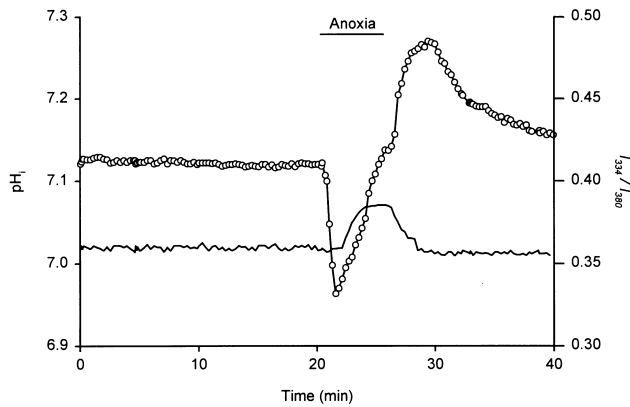


Fig. 3. Changes in  $pH_i$  and  $[Ca^{2+}]_i$  evoked by anoxia in the absence of external  $Ca^{2+}$ . Compared to responses obtained in the presence of 2 mM external  $Ca^{2+}$  (see Fig. 2B), the rise in background-subtracted  $I_{334}/I_{380}$  ratio values (continuous line) evoked by 5-min anoxia was markedly reduced in the absence of external  $Ca^{2+}$  (note the change in scale for the  $I_{334}/I_{380}$  ratio values between this figure and Fig. 2B). In contrast, the removal of external  $Ca^{2+}$  failed to significantly modify any of the components of the  $pH_i$  response (open circles) to anoxia. Records are means of  $pH_i$  and  $I_{334}/I_{380}$  ratio value data obtained simultaneously from 10 and 19 neurons, respectively, in two experiments performed on sister cultures in parallel experiments under identical  $HCO_3^-/CO_2$ -buffered conditions.

$[Ca^{2+}]_i$  indistinguishable from those observed in neurons which had not been previously subjected to anoxia (data not shown; see Ref. 12).

We also compared the time-courses of the changes in  $I_{334}/I_{380}$  ratio values and the  $pH_i$  changes evoked by corresponding anoxic insults, and found that reductions in  $pH_i$  started to occur after a significantly shorter delay following the induction of anoxia than increases in  $I_{334}/I_{380}$  ratio values in each of the 3-, 5- and 10-min anoxia groups (see Fig. 2A–C). In the 5-min group, for example, the time required for  $I_{334}/I_{380}$  ratio values to start to increase in response to anoxia was  $102 \pm 15$  s ( $n = 14$ ), compared to  $35 \pm 5$  s ( $n = 14$ ) for  $pH_i$  to begin to fall ( $P < 0.05$ ). Indeed, at 100 s following the start of 5-min anoxia (i.e. at approximately the time-point at which  $I_{334}/I_{380}$  ratio values started to rise),  $pH_i$  had reached 89% of the maximum anoxia-evoked acidic shift. Although anoxia-evoked reductions in  $pH_i$  occurred more quickly than anoxia-evoked increases in  $I_{334}/I_{380}$  ratio values, the subsequent rise in  $pH_i$  which took place in the continued absence of  $O_2$  started to occur at a similar time to the rise in the  $I_{334}/I_{380}$

ratio value (see Fig. 2). Thus, the latency to the start of the  $pH_i$  increase following the induction of 5-min anoxia was  $120 \pm 12$  s ( $n = 14$ ), whereas, in 14 sister cultures,  $I_{334}/I_{380}$  ratio values started to increase after  $102 \pm 15$  s ( $P > 0.05$ ).

The above observations indicate that activation of  $Na^+/H^+$  exchange in the immediate postanoxic period (see below) is unlikely to mediate the increases in  $[Ca^{2+}]_i$  evoked by anoxia because, in the majority of cases,  $[Ca^{2+}]_i$  in the postanoxic period declined to normal resting levels, even under conditions where the postanoxic intracellular alkalinization was maintained (see Fig. 2C). Furthermore, the results suggest that rises in  $[Ca^{2+}]_i$  do not initiate the internal acidic shift observed during anoxia, a possibility supported by experiments in which anoxia was imposed under external  $Ca^{2+}$ -free conditions. In the absence of external  $Ca^{2+}$ , the  $I_{334}/I_{380}$  ratio value increase evoked by 5-min anoxia was reduced to  $0.03 \pm 0.01$  ( $n = 4$ ;  $P < 0.05$  for difference in the rise in  $I_{334}/I_{380}$  ratio values observed in response to 5-min anoxia in the presence of external  $Ca^{2+}$ ; Fig. 3; also see Ref. 18). In contrast, the magnitudes of the acidic shift observed during anoxia and the postanoxic alkaline shift were not significantly different to those observed in the presence of external  $Ca^{2+}$  (Table 2, Fig. 3).

#### Effects of changing extracellular pH

Because  $pH_o$  declines under anoxic conditions *in vivo*,<sup>67</sup> we examined the effects of reducing  $pH_o$  on the steady-state  $pH_i$  response to 5-min anoxia. The results are summarized in Table 2 and a typical response is illustrated in Fig. 4A. Reducing  $pH_o$  to 6.80 from the normal value of 7.35 reduced steady-state  $pH_i$  prior to the induction of anoxia from  $7.22 \pm 0.10$  ( $n = 8$ ) to  $6.81 \pm 0.11$ , confirming the steep dependence of  $pH_i$  on  $pH_o$  in rat hippocampal neurons.<sup>7</sup> The magnitude of the fall in  $pH_i$  evoked by anoxia during perfusion with pH 6.80 medium was not significantly different to that observed under  $pH_o$  7.35 conditions. In contrast, the magnitudes of the rise in  $pH_i$  observed during anoxia and the postanoxic internal alkaline shift were significantly reduced. Recovery from the postanoxic internal alkalization occurred in all of eight cell populations subjected to 5-min anoxia under  $pH_o$  6.80 conditions (see Fig. 4A). The effect of a reduction in  $pH_o$  was also examined on the rise in  $[Ca^{2+}]_i$  evoked in sister cultures by 5-min anoxia (Fig. 4B). In agreement with previous reports (e.g., Ref. 38), the anoxia-evoked

Table 2. Effects of changes in perfusate composition on the intracellular pH response to 5-min anoxia

Perfusate	Buffer	$\Delta pH_{i(acid)}$ (pH units)	$t_{acid}$ (s)	$pH_i$ recovery (%)	$\Delta pH_{i(alkaline)}$ (pH units)	<i>n</i>
0 $[Ca^{2+}]_o$	$HCO_3^-/CO_2$	$0.13 \pm 0.01$	$103 \pm 24$	$100 \pm 10$	$0.17 \pm 0.04$	4
$pH_o = 6.80$	$HCO_3^-/CO_2$	$0.15 \pm 0.01$	$144 \pm 9^*$	$61 \pm 12^*$	$0.08 \pm 0.01^*$	8
$pH_o = 7.75$	$HCO_3^-/CO_2$	$0.07 \pm 0.03^*$	$137 \pm 29$	$146 \pm 10^*$	$0.26 \pm 0.03^*$	4
2 mM $[Na^+]_o$ (NMDG <sup>+</sup> )	HEPES	$0.12 \pm 0.01^*$	$241 \pm 13^*$	$30 \pm 7^*$	$0.05 \pm 0.04^*$	6
2 mM $[Na^+]_o$ (Li <sup>+</sup> )	HEPES	$0.15 \pm 0.03$	$189 \pm 29^*$	$53 \pm 12^*$	$0.28 \pm 0.03$	8
+ TTX (1 $\mu$ M)	HEPES	$0.16 \pm 0.01$	$182 \pm 12^*$	$35 \pm 8^*$	$0.22 \pm 0.02$	6
20 mM glucose	HEPES	$0.30 \pm 0.03^*$	$144 \pm 8^*$	$40 \pm 6^*$	$0.21 \pm 0.03$	6
+ $Zn^{2+}$ (250–1000 $\mu$ M)	HEPES	$0.18 \pm 0.02$	$125 \pm 11$	$48 \pm 9^*$	$0.18 \pm 0.05$	8
+ $Cd^{2+}$ (1000 $\mu$ M)	HEPES	$0.21 \pm 0.03$	$120 \pm 11$	$64 \pm 9^*$	$0.14 \pm 0.06$	4

Unless noted otherwise,  $pH_o = 7.35$  and glucose concentration = 10 mM.  $\Delta pH_{i(acid)}$  is the magnitude of the acidic shift induced by anoxia;  $t_{acid}$  is the time from the induction of anoxia to the time at which  $pH_i$  started to increase in the continued absence of  $O_2$ ;  $pH_i$  recovery is the percentage recovery of  $pH_i$  towards preanoxic resting levels in the continued absence of  $O_2$  calculated according to the equation: percentage  $pH_i$  recovery =  $100 \times (\Delta pH_{i(anoxia)}/\Delta pH_{i(acid)})$ , where  $\Delta pH_{i(anoxia)}$  is the magnitude of the rise of  $pH_i$  observed during anoxia;  $\Delta pH_{i(alkaline)}$  is the magnitude of the alkaline shift observed following the return to normoxia.  $[Ca^{2+}]_o$ , extracellular  $Ca^{2+}$  concentration. \* $P < 0.05$  for difference from control value obtained under the same buffering conditions (see Table 1).

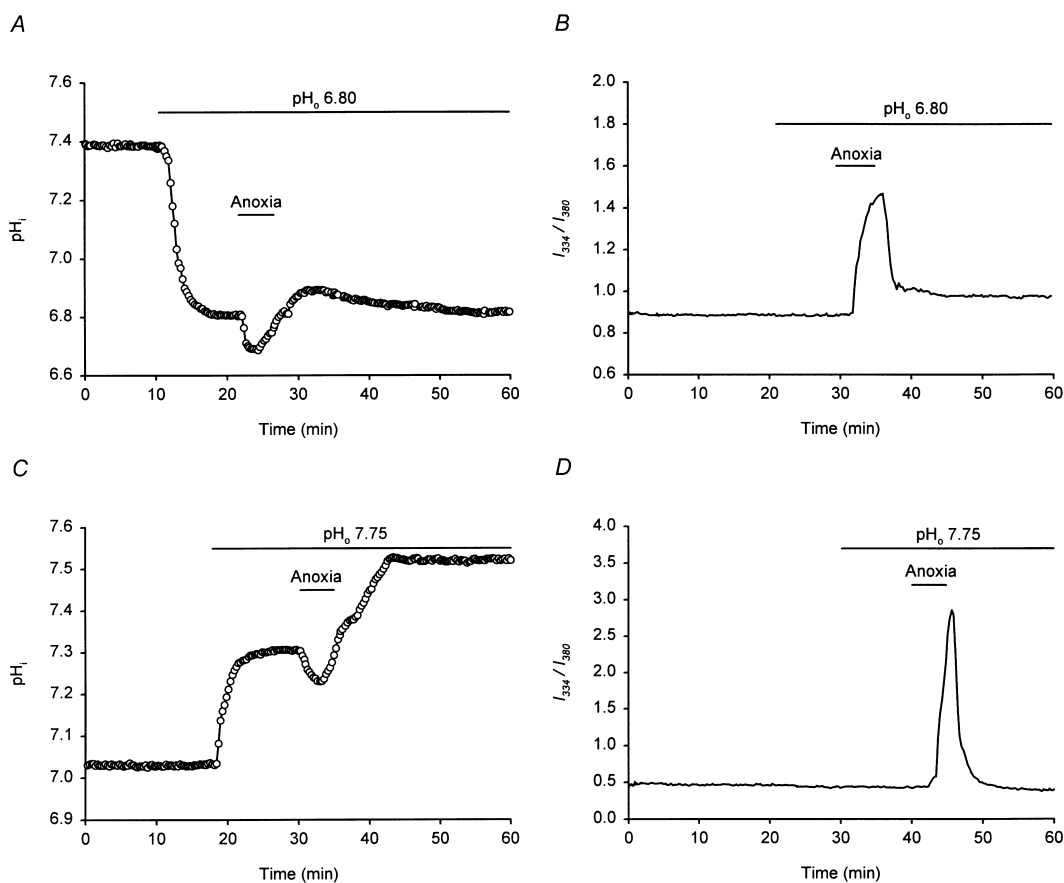


Fig. 4. Effect of changing  $pH_o$  on  $pH_i$  and  $[Ca^{2+}]_i$  responses to anoxia. Experiments in A and B, and in C and D, were performed in sister cultures under identical  $HCO_3^-/CO_2$ -buffered conditions. (A) Reducing  $pH_o$  from 7.35 to 6.80 produced a fall in  $pH_i$ . When  $pH_i$  had stabilized at the new steady-state level, a 5-min period of anoxia evoked a fall in  $pH_i$ , the magnitude of which was similar to that observed during 5-min anoxia under  $pH_o$  7.35 conditions, followed by a rise in  $pH_i$  in the continued absence of  $O_2$  and a postanoxic internal alkaline transient, the magnitudes of which were smaller than those observed following 5-min anoxia under  $pH_o$  7.35 conditions (compare with Fig. 2B, the same experiment performed at  $pH_o$  7.35 under identical buffering conditions). The trace is a mean of data obtained simultaneously from 10 neurons. (B)  $pH_o$  was reduced from 7.35 to 6.80 and a 5-min period of anoxia evoked a rise in  $I_{334}/I_{380}$  ratio values which was smaller than that observed under  $pH_o$  7.35 conditions (compare with Fig. 2B). Note the incomplete recovery of the  $I_{334}/I_{380}$  ratio values to preanoxic resting levels under  $pH_o$  6.80 conditions, possibly reflecting inhibition of  $Ca^{2+}$  extrusion mechanisms under low  $pH_o/pH_i$  conditions (see Discussion; also Ref. 37). The trace is a mean of data obtained simultaneously from seven neurons. (C) Increasing  $pH_o$  from 7.35 to 7.75 evoked an increase in steady-state  $pH_i$ . A 5-min anoxic insult produced a fall in  $pH_i$ , the magnitude of which was less than that observed under  $pH_o$  7.35 conditions, and a postanoxic internal alkalization, the magnitude of which was greater than that observed under  $pH_o$  7.35 conditions.  $pH_i$  failed to recover from the postanoxic alkalization (compare with Fig. 2B). The trace is a mean of data obtained simultaneously from 15 neurons. (D)  $pH_o$  was increased from 7.35 to 7.75, and a 5-min period of anoxia evoked a rise in  $I_{334}/I_{380}$  ratio values which was larger than that observed under  $pH_o$  7.35 conditions (compare with Fig. 2B). The trace is a mean of data obtained simultaneously from eight neurons.

rise in  $I_{334}/I_{380}$  ratio values was reduced to  $0.65 \pm 0.12$  ( $n = 5$ ) during perfusion with pH 6.80 medium ( $P < 0.05$  for the difference from the magnitude of the rise in  $I_{334}/I_{380}$  ratio values evoked by 5-min anoxia under  $pH_o$  7.35 conditions; see above).

We also examined the  $pH_i$  and  $[Ca^{2+}]_i$  responses to anoxia under high  $pH_o$  conditions. Increasing  $pH_o$  from 7.35 to 7.75 increased steady-state  $pH_i$  prior to the induction of anoxia from  $7.11 \pm 0.06$  to  $7.39 \pm 0.05$  ( $n = 4$ ). When a 5-min anoxic insult was imposed under  $pH_o$  7.75 conditions, the magnitude of the anoxia-evoked fall in  $pH_i$  was reduced compared to the change observed at  $pH_o$  7.35, whereas the magnitudes of the rises in  $pH_i$  both during and following anoxia were increased (Table 2, Fig. 4C). Recovery from the postanoxic internal alkalization occurred in none of four cell populations subjected to 5-min anoxia under  $pH_o$  7.75 conditions (see Fig. 4C). Examined in four sister cultures, the rise in  $[Ca^{2+}]_i$  evoked by 5-min anoxia under  $pH_o$  7.75 conditions was greater than that observed under control ( $pH_o$  7.35) conditions, the rise in  $I_{334}/I_{380}$  ratio values being  $3.15 \pm 0.28$

( $n = 4$ ; Fig. 4D) during perfusion with pH 7.75 medium ( $P < 0.05$  for the difference from the magnitude of the rise in  $I_{334}/I_{380}$  ratio values evoked by 5-min anoxia under  $pH_o$  7.35 conditions; see above).

#### Role of $HCO_3^-$

In order to assess the possible contribution of  $HCO_3^-$  to the changes in steady-state  $pH_i$  evoked by anoxia, we imposed 3-, 5- and 10-min anoxic insults under nominally  $HCO_3^-/CO_2$ -free, HEPES-buffered conditions. The results are summarized in Table 1. The magnitudes of the acidic shifts observed under  $HCO_3^-$ -free conditions were similar during 3-, 5- and 10-min anoxic insults, and were not statistically different to the corresponding values obtained under  $HCO_3^-/CO_2$ -buffered conditions. In addition, there was no statistical difference between the magnitudes of the postanoxic alkaline shifts observed after 3-min anoxia under  $HCO_3^-/CO_2$ -buffered or  $HCO_3^-/CO_2$ -free conditions. Following 5- or 10-min anoxia, however, the postanoxic alkaline shifts observed under

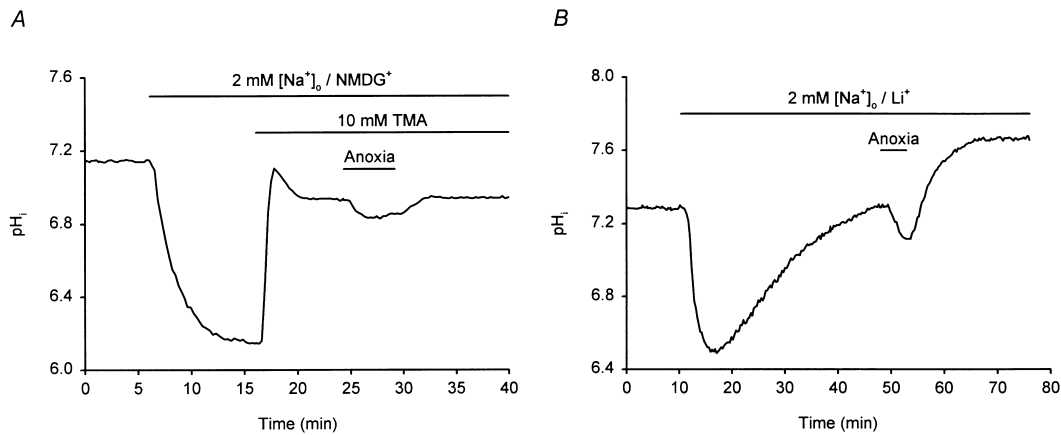


Fig. 5. Steady-state  $pH_i$  changes evoked by anoxia under reduced  $[Na^+]_o$  conditions. The experiments shown in A and B were performed during perfusion with  $HCO_3^-/CO_2$ -free, HEPES-buffered media. (A) The external  $Na^+$  dependence of anoxia-evoked changes in  $pH_i$  was examined by substituting NMDG<sup>+</sup> for all but 2 mM external  $Na^+$ . This produced a fall in  $pH_i$  which was elevated towards normal resting values by addition of the weak base trimethylamine (TMA; 10 mM). Under these conditions, a 5-min period of anoxia evoked a fall in  $pH_i$ , the magnitude of which was significantly smaller than that observed under normal  $[Na^+]_o$  conditions. The magnitude of the postanoxic intracellular alkalization was also markedly attenuated. The trace represents a mean of data obtained simultaneously from 23 neurons. (B) The replacement of all but 2 mM external  $Na^+$  with  $Li^+$  evoked a transient intracellular acidification followed by a recovery of  $pH_i$ , despite continued exposure to reduced  $Na^+$  medium (see Refs 2 and 70). Under these conditions, a 5-min period of anoxia evoked a fall in  $pH_i$  and a postanoxic internal alkalization, the magnitudes of which were not statistically different from those observed in the presence of normal  $[Na^+]_o$ . The trace represents a mean of data obtained simultaneously from 17 neurons.

HEPES-buffered conditions were significantly larger than the corresponding changes observed under  $HCO_3^-/CO_2$ -buffered conditions (Table 1). In a manner similar to that observed under  $HCO_3^-/CO_2$ -buffered conditions, none of eight cell populations examined under  $HCO_3^-$ -free conditions exhibited recovery of  $pH_i$  from the postanoxic alkaline shift following 10-min anoxia, whereas 10 of 17 and four of five cell populations subjected to 5- and 3-min anoxia, respectively, showed at least a partial ( $> 50\%$ ) recovery of  $pH_i$  towards preanoxic resting levels.

#### Role of $Na^+/H^+$ exchange

The  $Na^+/H^+$  exchanger in rat hippocampal neurons is insensitive to amiloride, amiloride analogues and benzoyl guanidinium compounds (e.g., HOE 694);  $Na^+/H^+$  exchange was therefore blocked by substituting all but 2 mM external  $Na^+$  with NMDG<sup>+</sup> under  $HCO_3^-/CO_2$ -free, HEPES-buffered conditions.<sup>2,3,60,66,70</sup> Under these conditions, where the fall in  $pH_i$  observed upon the introduction of  $Na^+$ -reduced medium had been compensated for by the addition of the weak base trimethylamine,<sup>2,70</sup> the magnitudes of both the anoxia-evoked acid shift and the postanoxic rise in  $pH_i$  were significantly reduced compared to changes observed in the presence of normal  $[Na^+]_o$  (Table 2, Fig. 5A). The reduced magnitude of the acidic shift might reflect a reduction in ATP hydrolysis under reduced  $[Na^+]_o$  conditions.<sup>16,17</sup> The markedly reduced magnitude of the postanoxic alkaline shift, on the other hand, suggests that activation of  $Na^+/H^+$  exchange contributes to the rise in steady-state  $pH_i$  observed following the return to normoxia. This possibility was supported by experiments in which  $Li^+$  was employed as the external  $Na^+$  substitute ( $Li^+$ , in contrast to NMDG<sup>+</sup>, can act as a substrate for  $Na^+/H^+$  exchange).<sup>44</sup> Thus, in the presence of  $Li^+$ , the magnitude of the rise in  $pH_i$  after a 5-min anoxic insult was not significantly different from that observed in the presence of normal external  $Na^+$  (Table 2; Fig. 5B).

To further assess the possibility that anoxia produces changes in the activity of the  $Na^+/H^+$  exchanger, intracellular

acid loads were imposed both during anoxia and immediately following the return to normoxia. The experiments were performed in HEPES-buffered media, under which conditions recovery of  $pH_i$  from an imposed acid load in hippocampal neurons reflects  $Na^+/H^+$  exchange activity.<sup>2,3,60,66,70</sup> As illustrated in Fig. 6A,  $pH_i$  recovery from an imposed acid load was slowed during anoxia. Rates of  $pH_i$  recovery from acid loads imposed under anoxic conditions were compared to control rates of  $pH_i$  recovery at the same absolute values of  $pH_i$ , and the resulting plots of the  $pH_i$  dependence of the rates of  $pH_i$  recovery before and during anoxia are presented in Fig. 6B. Measured at the common test  $pH_i$  of 7.05, the mean rate of  $pH_i$  recovery from internal acid loads imposed during anoxia was reduced by 43% ( $n = 5$ ;  $P < 0.02$ ; Fig. 6C). In contrast to results obtained during anoxia, rates of  $pH_i$  recovery increased when internal acid loads were imposed in the period immediately following the return to normoxia (Fig. 6D). Rates of  $pH_i$  recovery from acid loads imposed following the return to normoxia were compared to control rates of  $pH_i$  recovery at the same absolute values of  $pH_i$  (Fig. 6E) and, as illustrated in Fig. 6F,  $pH_i$  recovery rates measured at the common test  $pH_i$  of 7.05 were increased by 249% ( $n = 6$ ;  $P < 0.01$ ) in the postanoxic period.

Taken together, the results suggest that  $Na^+/H^+$  exchange activity is reduced during anoxia and strongly activated following the return to normoxia. In particular, the findings are consistent with the possibility, raised earlier, that the increase in steady-state  $pH_i$  observed following anoxia reflects, at least in part, an increase in  $Na^+/H^+$  exchange activity.

#### Effects of tetrodotoxin and high glucose concentration

The results presented above suggest that changes in  $pH_i$  evoked by transient anoxia in rat hippocampal neurons reflect, at least in part, alterations in  $Na^+/H^+$  exchange activity. However, the  $Na^+/H^+$  exchanger is an electroneutral transport mechanism<sup>10</sup> and anoxia leads to membrane depolarization. In order to investigate whether membrane depolarization

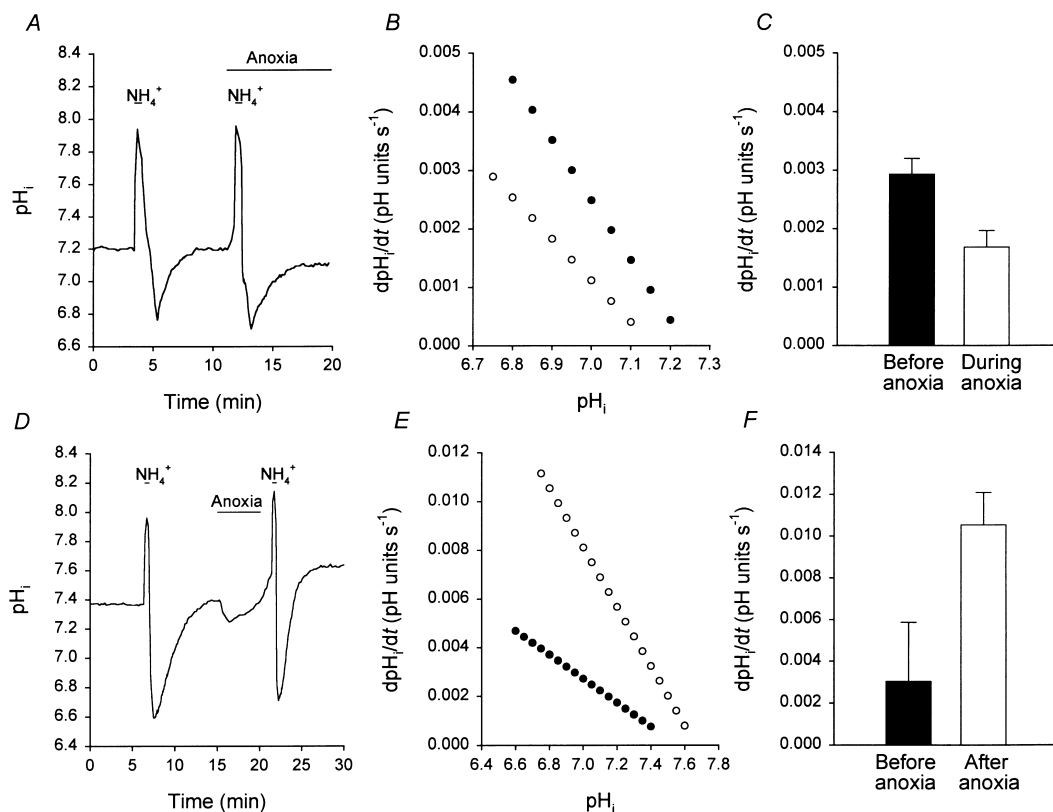


Fig. 6. The effects of anoxia on the recovery of  $pH_i$  from imposed intracellular acid loads. Traces in A and D were obtained under identical  $HCO_3^-/CO_2$ -free, HEPES-buffered conditions. (A) Following the first  $NH_4^+$ -induced internal acid load,  $pH_i$  was allowed to recover to normal resting values. The neurons were then exposed to an anoxic medium and a second internal acid load was performed. The recovery of  $pH_i$  from the acid load imposed under anoxic conditions was slowed. The trace represents a mean of data obtained simultaneously from eight neurons. (B) The  $pH_i$  sensitivity of the rate of  $pH_i$  recovery plotted from data obtained in the experiment shown in A. (●) Control data from recovery prior to anoxia; (○) data obtained during anoxia. Rates were evaluated at 0.05-unit intervals of  $pH_i$ . (C) Mean rate of  $pH_i$  recovery ( $\pm$  S.E.M.;  $n = 5$ ) measured at a test  $pH_i$  of 7.05. (■) Control; (□) during anoxia. Rates of  $pH_i$  recovery were reduced significantly under anoxic conditions ( $P < 0.02$ ). (D) Following an initial internal acid load,  $pH_i$  was allowed to recover to normal resting values. A 5-min period of anoxia was then imposed, which resulted in a normal decline in  $pH_i$  followed by a return of  $pH_i$  towards normal resting values in the continued absence of  $O_2$ . Following the return to normoxia, a second acid load was performed. The recovery of  $pH_i$  from the acid load imposed following the transient anoxic period was hastened. The trace represents a mean of data obtained simultaneously from 20 neurons. (E) The  $pH_i$  sensitivity of the rate of  $pH_i$  recovery plotted from data obtained in the experiment shown in D. (●) Control data from recovery prior to anoxia; (○) data obtained following anoxia. Rates were evaluated at 0.05-unit intervals of  $pH_i$ . (F) Mean rate of  $pH_i$  recovery ( $\pm$  S.E.M.;  $n = 6$ ) measured at a test  $pH_i$  of 7.05. (■) Control; (□) following anoxia. Rates of  $pH_i$  recovery were increased significantly following the transient anoxic insult ( $P < 0.01$ ).

might influence the  $pH_i$  response to anoxia, we examined the effects of tetrodotoxin (TTX) and perfusion with 20 mM glucose-containing medium, both of which are known to delay the onset and/or reduce the magnitude of anoxic depolarization.<sup>13,22,24,26,38,40,72,79,81</sup> The results are summarized in Table 2.

Neither the magnitude of the decrease in  $pH_i$  observed during anoxia nor the magnitude of the postanoxic internal alkalization was significantly affected by 1  $\mu$ M TTX (Fig. 7A). Interestingly, however, TTX significantly prolonged the latency to the rise in  $pH_i$  observed during anoxia ( $t_{acid}$ ) and concomitantly reduced the extent of the rise in  $pH_i$  observed in the absence of  $O_2$ . Because TTX is known to delay the onset of anoxic depolarization, the results are consistent with the possibility that the rise in  $pH_i$  observed during anoxia may be determined, at least in part, by a mechanism activated in response to membrane depolarization. The latter possibility was also supported by findings that the effects of TTX in delaying the onset and reducing the magnitude of the rise in  $pH_i$  observed during anoxia were mimicked by reducing  $[Na^+]_o$  to 2 mM (Table 2). In addition, both TTX and low- $[Na^+]_o$  ( $Li^+$ -substituted) medium increased the time between the end of anoxia and the time-point at which  $pH_i$  reached a

maximum value in the postanoxic period; under control conditions (5-min anoxia, HEPES-buffered medium), this interval was  $3.3 \pm 0.2$  min ( $n = 17$ ), whereas in the presence of TTX the interval was  $8.9 \pm 1.0$  min ( $n = 6$ ;  $P < 0.05$ ; Fig. 7A) and during perfusion with  $Li^+$ -substituted medium the interval was  $9.3 \pm 1.3$  min ( $n = 8$ ;  $P < 0.05$ ; Fig. 5B).

Next, we examined the effects on  $pH_i$  of 5-min anoxia imposed in the presence of 20 mM glucose (neurons were superfused for 10 min with the high-glucose medium prior to the induction of anoxia). As illustrated in Fig. 7B, exposure to medium containing 20 mM glucose evoked a slow increase in  $pH_i$  of  $0.15 \pm 0.02$  pH units ( $n = 6$ ); although the basis for this effect was not examined, the rise in  $pH_i$  may reflect activation of  $Na^+/H^+$  exchange, as described in some peripheral cell types (e.g., Ref. 32). Once  $pH_i$  had stabilized at the new resting level, a 5-min anoxic insult resulted in an acidic shift which was significantly larger than the corresponding change observed under control (10 mM glucose) conditions (Table 2; also see Refs 40, 61 and 67). However, in a manner reminiscent of the effects of TTX and reduced  $[Na^+]_o$ , the latency to the start of the rise in  $pH_i$  during anoxia and the extent to which  $pH_i$  increased in the absence of  $O_2$  were increased and reduced, respectively, under high-glucose conditions

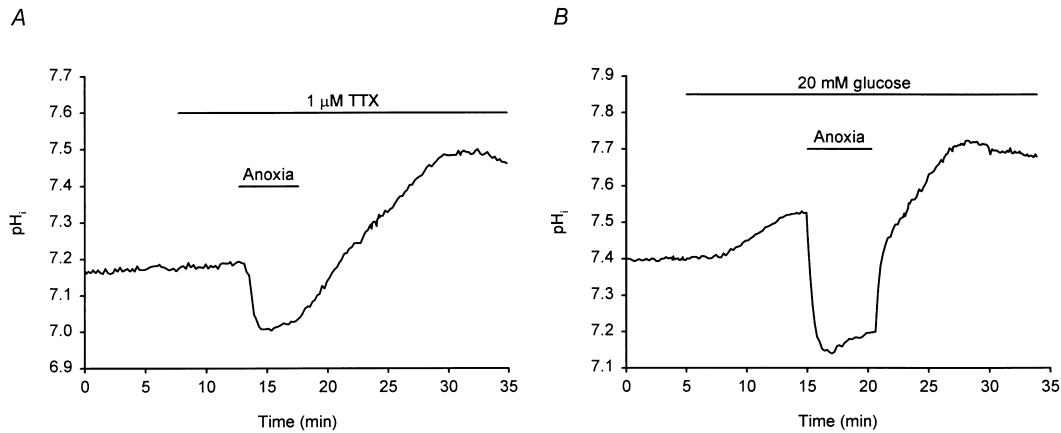


Fig. 7. Effects of TTX and high glucose concentration on the pH<sub>i</sub> response to anoxia. Records in A and B were obtained under HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub>-free, HEPES-buffered conditions. (A) A 5-min period of anoxia imposed in the presence of 1 μM TTX produced a fall in pH<sub>i</sub> which was followed by a small rise in pH<sub>i</sub> in the continued absence of O<sub>2</sub>, and a postanoxic internal alkalization. The trace is a mean of data obtained simultaneously from 12 neurons. (B) Increasing the concentration of glucose from the normal value of 10 mM to 20 mM evoked a slow rise in steady-state pH<sub>i</sub>. When pH<sub>i</sub> had stabilized at the new resting level, a 5-min period of anoxia imposed in the presence of 20 mM glucose evoked a large fall in pH<sub>i</sub> but, reminiscent of the effects of TTX (see A), only a limited recovery of pH<sub>i</sub> occurred in the continued absence of O<sub>2</sub>. The trace is a mean of data obtained simultaneously from 19 neurons.

(Table 2). The magnitude of the postanoxic internal alkalization was not significantly affected by 20 mM glucose, compared to control responses obtained during perfusion with medium containing 10 mM glucose (Table 2). However, again in a manner reminiscent of the effects of TTX and reduced [Na<sup>+</sup>]<sub>o</sub>, high glucose concentration increased the latency to the time-point at which pH<sub>i</sub> reached a maximum value in the postanoxic period to  $6.8 \pm 0.6$  min ( $n=6$ ;  $P < 0.05$  for the difference from control values obtained during perfusion with 10 mM glucose; Fig. 7B).

#### Possible contribution of a voltage-activated proton conductance

The results obtained with TTX and under reduced [Na<sup>+</sup>]<sub>o</sub> and high-glucose conditions suggest that manoeuvres which are known to delay or attenuate anoxic depolarization reduce the extent to which pH<sub>i</sub> increases in the absence of O<sub>2</sub> and reduce the rate of rise of pH<sub>i</sub> in the immediate postanoxic period. In turn, the findings suggest the possibility that an alkalizing mechanism activated by depolarization may contribute to the pH<sub>i</sub> response to anoxia in hippocampal neurons. One such alkalizing mechanism might be a voltage-activated proton conductance ( $g_{H^+}$ ), an identifying characteristic of which is blockade by Zn<sup>2+</sup> and Cd<sup>2+</sup>.<sup>41,46</sup> We therefore examined the effects of 250–1000 μM Zn<sup>2+</sup> on the pH<sub>i</sub> response to 5-min anoxia; data obtained in the presence of different concentrations of Zn<sup>2+</sup> were not significantly different and the results, which are presented in Table 2, were therefore pooled. When 5-min anoxia was imposed in the presence of Zn<sup>2+</sup>, the magnitudes of the acidic shift observed during anoxia and the alkaline shift observed after anoxia were not significantly different from values obtained in the absence of Zn<sup>2+</sup> (Fig. 8). However, in a manner reminiscent of the effects of TTX, reduced [Na<sup>+</sup>]<sub>o</sub> and high glucose concentration (see above), Zn<sup>2+</sup> reduced the extent of the rise in pH<sub>i</sub> observed in the absence of O<sub>2</sub> (Table 2) and delayed the onset of the peak alkalization observed following anoxia (which occurred at  $5.1 \pm 0.4$  min in the presence of Zn<sup>2+</sup>;  $n=8$ ; Fig. 8). Similar results were obtained when anoxia was imposed in the presence of 1 mM Cd<sup>2+</sup> (Table 2).

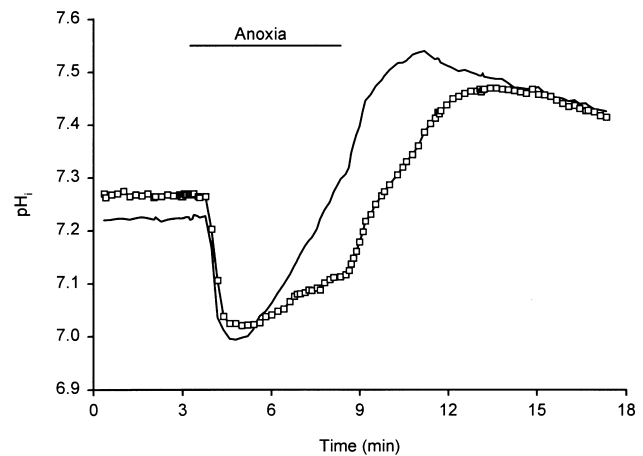


Fig. 8. Effect of Zn<sup>2+</sup> on the pH<sub>i</sub> response to anoxia. Under control, HEPES-buffered conditions (continuous line), a 5-min period of anoxia evoked a fall in pH<sub>i</sub> followed by a marked increase in pH<sub>i</sub> to above preanoxic resting levels in the continued absence of O<sub>2</sub> and, upon the return to normoxia, an alkaline transient which slowly recovered towards preanoxic steady-state pH<sub>i</sub> levels. A parallel experiment was performed on a sister culture in the presence of 250 μM Zn<sup>2+</sup> (open squares). In this case, the magnitudes of the decrease in pH<sub>i</sub> observed during anoxia and the increase in pH<sub>i</sub> observed following anoxia were not significantly different from the respective control values. In contrast, the extent of the rise in pH<sub>i</sub> observed during anoxia was diminished. Traces represent the means of data obtained simultaneously from 16 and 14 neurons in the absence and presence of Zn<sup>2+</sup>, respectively.

#### DISCUSSION

Cultured postnatal rat hippocampal neurons respond to anoxia with an initial fall in pH<sub>i</sub>, a subsequent increase in pH<sub>i</sub> in the continued absence of O<sub>2</sub> and, finally, a postanoxic internal alkalization which recovers towards preanoxic pH<sub>i</sub> levels provided the duration of the anoxic insult is  $\leq 5$  min. The removal of external HCO<sub>3</sub><sup>-</sup> failed to affect the changes in pH<sub>i</sub> observed during anoxia although, following 5- and 10-min periods of anoxia, the magnitudes of the postanoxic alkaline shifts observed under HEPES-buffered conditions were greater than the corresponding changes observed in the presence of HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub>. The latter findings may reflect a greater intracellular buffering power in the presence than in the absence of HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub>,<sup>2,3</sup> although we cannot rule out a

possible contribution from changes in the activities of  $\text{HCO}_3^-$ -dependent  $\text{pH}_i$ -regulating mechanisms in the postanoxic period.<sup>55,57</sup> Given the association between an internal alkalosis and poor cell survival following transient ischaemia (see Introduction), the greater postanoxic alkalization observed in the present experiments under HEPES-buffered conditions may be related to previous findings that cellular damage induced by anoxia is worsened when buffering power is reduced.<sup>55,71</sup>

#### *The fall in intracellular pH during anoxia*

A fall in  $\text{pH}_i$  appears to be a universal response of central neurons to anoxia and/or ischaemia.<sup>16,40,67</sup> In the present study, anoxia evoked a decrease in  $\text{pH}_i$  before any measurable rise in  $[\text{Ca}^{2+}]_i$ , a finding which is consistent with the *in vivo* intracellular  $\text{H}^+$  concentration and  $[\text{Ca}^{2+}]_i$  measurements of Silver and Erecinska,<sup>68</sup> and the magnitude of the fall in  $\text{pH}_i$  was not affected by the removal of external  $\text{Ca}^{2+}$ . These observations indicate that a rise in  $[\text{Ca}^{2+}]_i$ , and subsequent displacement of protons from shared intracellular binding sites, activation of  $\text{Ca}^{2+}/\text{H}^+$  exchange and/or  $\text{Ca}^{2+}$  sequestration by internal organelles,<sup>45,51</sup> does not initiate the fall in  $\text{pH}_i$  evoked by anoxia. On the other hand, the fact that  $\text{pH}_i$  declined before  $[\text{Ca}^{2+}]_i$  increased raises the possibility that anoxia-evoked falls in  $\text{pH}_i$  may play a role in promoting the rise in  $[\text{Ca}^{2+}]_i$ , for example, by displacing  $\text{Ca}^{2+}$  ions from shared binding sites,<sup>45</sup> impairing  $\text{Ca}^{2+}$  uptake and/or evoking  $\text{Ca}^{2+}$  release from intracellular stores,<sup>51</sup> inhibiting  $\text{Ca}^{2+}$  extrusion,<sup>37</sup> blocking  $\text{K}^+$  conductances<sup>7</sup> and/or hastening the onset of energy failure.<sup>16</sup>

It has been suggested that  $\text{Na}^+/\text{H}^+$  exchange contributes only minimally to  $\text{H}^+$  extrusion from brain tissue during hypoxia.<sup>54</sup> In the present study, rates of  $\text{pH}_i$  recovery from acid loads imposed during anoxia were reduced compared to rates of  $\text{pH}_i$  recovery established in the same neurons prior to the induction of anoxia. The result suggests that inhibition of  $\text{Na}^+/\text{H}^+$  exchange may occur during anoxia, although this possibility must be tempered by the fact that an increased rate of production of intracellular acid may contribute to the reduction in the rate of  $\text{pH}_i$  recovery observed during anoxia (see Ref. 33). Of particular interest was the fact that the apparent reduction in  $\text{Na}^+/\text{H}^+$  exchange activity during anoxia occurred even when  $\text{pH}_o$  was held at a constant value (i.e. pH 7.35); thus, in hippocampal neurons, a fall in  $\text{pH}_o$  (as occurs *in vivo*) may not be an absolute requirement for reduced antiporter activity. An alternative possibility is that the decline in intracellular ATP concentration evoked by anoxia<sup>4,16</sup> may mediate the reduction in  $\text{Na}^+/\text{H}^+$  exchange activity. Although the ATP-dependence of the  $\text{Na}^+/\text{H}^+$  exchanger in hippocampal neurons has not been examined, it is established that ATP depletion directly inhibits  $\text{Na}^+/\text{H}^+$  exchange activity in peripheral cell types and in cell lines stably transfected with  $\text{Na}^+/\text{H}^+$  exchanger isoforms.<sup>11</sup> Given the sensitivity of  $\text{Na}^+/\text{H}^+$  exchangers to reductions in  $\text{pH}_o$ ,<sup>31,44</sup> the external acidosis which occurs during anoxia *in vivo* or in slice preparations *in vitro* would act to augment any direct inhibitory effect of anoxia on  $\text{Na}^+/\text{H}^+$  exchange activity.

#### *The rise in intracellular pH during anoxia*

The fall in  $\text{pH}_i$  observed during anoxia was followed by a rise in  $\text{pH}_i$  in the continued absence of  $\text{O}_2$ . Residual  $\text{Na}^+/\text{H}^+$

exchange activity is unlikely to be responsible for this rise in  $\text{pH}_i$ , because there were no significant differences between either the latencies to or the magnitudes of the rises in  $\text{pH}_i$  observed during anoxia when external  $\text{Na}^+$  was replaced by  $\text{NMDG}^+$  (under which circumstances  $\text{Na}^+/\text{H}^+$  exchange is blocked) or  $\text{Li}^+$  (under which circumstances the  $\text{Na}^+/\text{H}^+$  exchanger remains active). This finding parallels observations made in peripheral cell types, where anoxia-evoked falls in  $\text{pH}_i$  are not augmented by pharmacological blockade of  $\text{Na}^+/\text{H}^+$  exchange (e.g., Ref. 62). Although it has been suggested that rat CNS neurons possess a monocarboxylate transporter which contributes to  $\text{pH}_i$  regulation,<sup>49</sup> in our hands the transport inhibitor  $\alpha$ -cyano-4-hydroxycinnamic acid (5–10 mM)<sup>58</sup> fails to affect steady-state  $\text{pH}_i$  in the great majority of rat hippocampal neurons and, examined in five populations of cultured neurons under HEPES-buffered conditions, 5 mM  $\alpha$ -cyano-4-hydroxycinnamic acid paradoxically increased the percentage recovery of  $\text{pH}_i$  observed during 5 min anoxia (Diarra A., unpublished observations). Thus, the rise in  $\text{pH}_i$  observed in the absence of  $\text{O}_2$  is unlikely to be mediated by lactate/ $\text{H}^+$  co-transport, a finding consistent with those of others who have reported that lactate transport does not play a major role in  $\text{pH}_i$  homeostasis during anoxia.<sup>21,55,56</sup>

Two findings suggest, albeit indirectly, that the rise in  $\text{pH}_i$  observed during anoxia may be consequent upon membrane depolarization. First, the latencies from the start of anoxia to the onset of the rises in  $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  were similar, and it is well established that the rapid rise in  $[\text{Ca}^{2+}]_i$  evoked by anoxia coincides with membrane depolarization.<sup>67,73,79</sup> Second, manoeuvres which are known to delay the onset and/or attenuate the magnitude of anoxic depolarization, including perfusion with low pH (Fig. 4),<sup>74</sup> reduced  $\text{Na}^+$  concentration (Fig. 5),<sup>73</sup> or high-glucose (Fig. 7)<sup>13,38,61</sup> media and the application of TTX (Fig. 7),<sup>22,79,81</sup> also delayed the onset of the rise in  $\text{pH}_i$  observed under anoxic conditions. In addition, we have observed that a rise in steady-state  $\text{pH}_i$  occurs in cultured postnatal rat hippocampal neurons during membrane depolarization evoked by exposure to media containing either veratridine (20  $\mu\text{M}$ ) or high concentrations (25–50 mM) of  $\text{K}^+$  (Diarra A., unpublished observations; also see Ref. 63). Depolarization-induced internal alkalinizations have been described in a variety of cell types, where they are mediated variously by  $\text{Na}^+/\text{HCO}_3^-$  co-transport (e.g., Ref. 9), voltage-activated proton conductances (e.g., Ref. 46) or a reduction in the driving force for passive  $\text{H}^+$  entry across the plasma membrane (e.g., Ref. 63). This laboratory<sup>2</sup> and others<sup>66</sup> have been unable to demonstrate  $\text{Na}^+/\text{HCO}_3^-$  co-transport in rat hippocampal neurons and, furthermore, the rise in  $\text{pH}_i$  which occurred under anoxic conditions was not affected by the removal of external  $\text{HCO}_3^-$ . On the other hand, the fact that both  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  reduced the magnitude of the rise in  $\text{pH}_i$  suggests that a  $g_{\text{H}^+}$  activated during anoxic depolarization might contribute to the  $\text{pH}_i$  change. Although we have confirmed the effect of  $\text{Zn}^{2+}$  in attenuating the rise in  $\text{pH}_i$  observed during anoxia in acutely dissociated adult rat hippocampal CA1 neurons (Sheldon C., unpublished observations), given the many effects of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  on ligand- and/or voltage-gated ionic conductances,<sup>6</sup> electrophysiological studies will be required to substantiate or refute the possibility that a  $g_{\text{H}^+}$  may contribute to the  $\text{pH}_i$  response to transient anoxia in hippocampal neurons. The residual rise in  $\text{pH}_i$  observed during anoxia in the presence of  $\text{Zn}^{2+}$  (see Fig. 8) or  $\text{Cd}^{2+}$  may reflect a reduction in net  $\text{H}^+$  influx consequent

upon a reduction in the inward driving force on the proton during membrane depolarization,<sup>63</sup> but this possibility also requires further investigation.

Voltage-dependent  $H^+$  currents and/or a reduction in passive  $H^+$  entry could provide mechanisms for limiting falls in  $pH_i$  under conditions of energy failure (such as anoxia), where  $Na^+/H^+$  exchange is inhibited, in particular because both processes proceed without increasing  $[Na^+]_i$  and, in consequence, without further reducing the cellular energy state.<sup>10,23,67</sup> However, although recoveries of  $pH_i$  from anoxia-induced intracellular acidifications have been observed previously in the CA1 region of hippocampal slices subjected to combined anoxic and hypoglycaemic insults,<sup>21,47</sup> in the majority of such studies (as well as *in vivo*)  $pH_i$  decreases and does not recover in the continued absence of  $O_2$  (e.g., Refs 61 and 69). Given the fact that voltage-activated proton conductances are inhibited by reductions in  $pH_o$ ,<sup>41</sup> the difference between the latter reports and the present findings (at  $pH_o$  7.35) may be related to the reduction in  $pH_o$  which occurs during anoxia *in vivo* and in slice preparations *in vitro*. Indeed, in the present study, when  $pH_o$  was reduced from 7.35 to 6.80, the magnitude of the rise in  $pH_i$  observed during anoxia was reduced.

#### The rise in intracellular pH following anoxia

The final phase of the  $pH_i$  response was a postanoxic internal alkalization. In contrast to the rise in  $pH_i$  which occurred during anoxia (see above), the rise in  $pH_i$  observed after anoxia appeared to be mediated, at least in part, by activation of  $Na^+/H^+$  exchange in the immediate postanoxic period. This possibility does not exclude potential contributions from other mechanisms, such as synthesis of ATP (see Ref. 16) and alkalizing mechanism(s) activated by continued membrane depolarization (e.g., voltage-activated proton conductances) in the period immediately following anoxia (see above and Refs 10 and 73). Given the known inhibitory effect of reductions in  $pH_o$  on  $Na^+/H^+$  exchange activity,<sup>31,44</sup> a reduced interstitial pH in the immediate postanoxic period would act to limit any increase in  $Na^+/H^+$  exchange activity at this time. Indeed, in the present study, the magnitude of the postanoxic internal alkalization was significantly reduced when  $pH_o$  was lowered from 7.35 to 6.80 (also see Ref. 77), suggesting a possible reason for the fact that internal pH "overshoots" have been observed only infrequently following transient anoxic insults *in vivo* or in slice preparations *in vitro*.<sup>42,54,82</sup>

Activation of  $Na^+/H^+$  exchange following anoxia/ischaemia has been extensively documented in peripheral cell types<sup>28,39,43,53</sup> and, recently,  $Na^+/H^+$  exchange has been shown to mediate the recovery of  $pH_i$  following metabolic inhibition in cultured rat neocortical neurons.<sup>77</sup> In peripheral cell types, postanoxic activation of  $Na^+/H^+$  exchange is thought to occur as an integrated response to both the fall in  $pH_i$  which occurs during anoxia and the restoration of normal  $pH_o$  values in the postanoxic period. In this regard, of particular interest are the present findings that postanoxic activation of  $Na^+/H^+$  exchange in hippocampal neurons occurs even when  $pH_i$  upon the return to normoxia is not reduced from preanoxic resting levels and even when  $pH_o$  is maintained at a constant value throughout the pre- and postanoxic periods. These findings indicate, respectively, that neither an anoxia-evoked fall in  $pH_i$  nor a return to

normal  $pH_o$  values is an absolute requirement for  $Na^+/H^+$  exchange activation in the postanoxic period. Although the factor(s) which promote(s) increased  $Na^+/H^+$  exchange activity in hippocampal neurons following anoxia remain(s) unknown, one possibility is an anoxia-induced change in the activities of intracellular second messenger system(s) which, in turn, act to regulate  $Na^+/H^+$  exchange. For example, intracellular cyclic AMP concentration increases in the immediate postanoxic period (e.g., Ref. 78) and we have recently found that increases in intracellular cyclic AMP concentration activate  $Na^+/H^+$  exchange in rat hippocampal neurons.<sup>70</sup>

Although  $Na^+/H^+$  exchange was stimulated in the immediate postanoxic period,  $[Ca^{2+}]_i$  returned to normal resting values. This finding indicates that postanoxic activation of  $Na^+/H^+$  exchange does not necessarily lead to an increase in  $[Ca^{2+}]_i$  in hippocampal neurons, even though it would be expected to promote a rise in  $[Na^+]_i$  and, in this way, favour reverse  $Na^+/Ca^{2+}$  exchange (see Refs 4, 20, 29 and 80). Indeed, an increasing body of evidence suggests that activation of  $Na^+/H^+$  exchange and a return to physiological  $pH_i$ , rather than cytosolic  $Ca^{2+}$  overload *per se*, precipitates injury in peripheral cell types following anoxia/ischaemia (e.g., Refs 5 and 65). In this regard, activation of  $Na^+/H^+$  exchange following metabolic inhibition in cultured rat neocortical neurons has been found to promote cell death,<sup>77</sup> and the present results suggest the possibility that a similar association may exist in hippocampal neurons subjected to anoxia. In particular, reducing  $pH_o$  or  $[Na^+]_o$  (NMDG<sup>+</sup> substitution), manoeuvres which inhibit  $Na^+/H^+$  exchange and thereby reduce the magnitude of the postanoxic internal alkaline shift, are known to be neuroprotective (e.g., Refs 19 and 74).

Activation of  $Na^+/H^+$  exchange in the immediate postanoxic period could modulate neurodegenerative processes not only via changes in  $pH_i$  and  $pH_o$ ,<sup>75</sup> but also by contributing to the internal  $Na^+$  load at a time at which  $Na^+$  influx via voltage-activated  $Na^+$  channels is declining due to membrane repolarization.<sup>20,25,48,65,79</sup> The rapid return to normal or above-normal  $pH_i$  values in the immediate postanoxic period could, for example, trigger the onset of the mitochondrial permeability transition.<sup>59</sup> On the other hand, an increase in  $[Na^+]_i$  and a reduction in the transmembrane  $Na^+$  gradient may contribute to a worsening of energy state,<sup>14,23</sup> a reduction in  $Ca^{2+}$  extrusion and  $Ca^{2+}$  buffering by internal organelles,<sup>35,36</sup> a decline in glutamate reuptake or even an increase in  $Ca^{2+}$ -independent glutamate release,<sup>50,52,64,80</sup> potentiation of *N*-methyl-D-aspartate receptor-mediated currents,<sup>83</sup> and early osmotic injury.<sup>30</sup> Further experiments are required to differentiate between these, and other, possibilities.

#### CONCLUSIONS

The intrinsic  $pH_i$  response of cultured postnatal rat hippocampal neurons to transient anoxia consists of perianoxic acidic and postanoxic alkaline shifts, both of which are independent of changes in  $[Ca^{2+}]_i$  and may reflect, at least in part, changes in the activity of the acid-extruding  $Na^+/H^+$  exchanger. In addition, a  $g_{H^+}$  activated by membrane depolarization may contribute to a rise in  $pH_i$  which occurs in the continued absence of  $O_2$  and to the internal alkalization which occurs immediately following the return to normoxia. Although the magnitudes of the  $pH_i$  shifts observed in the present study are

sufficient to affect a variety of processes critical to neuronal function, including the activities of ionic conductances (e.g., Refs 7 and 76), the contribution, if any, of the  $\text{pH}_i$  changes to  $\text{O}_2$  deprivation injury in cultured rat hippocampal neurons remains to be determined.

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