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Intracellular pH Response to Anoxia in Acutely Dissociated Adult Rat Hippocampal CA1 Neurons

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Sheldon, Claire and John Church. Intracellular pH response to anoxia in acutely dissociated adult rat hippocampal CA1 neurons. *J Neurophysiol* 87: 2209–2224, 2002; 10.1152/jn.00637.2001. The effects of anoxia on intracellular pH (pH_i) were examined in acutely isolated adult rat hippocampal CA1 neurons loaded with the H^+-sensitive fluorophore, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein. During perfusion with HCO_3^-/CO_2- or HEPES-buffered media (pH 7.35) at 37°C, 5- or 10-min anoxic insults were typified by an intracellular acidification on the induction of anoxia, a subsequent rise in pH_i in the continued absence of O_2, and a further internal alkalinization on the return to normoxia. The steady-state pH_i changes were not consequent on changes in $[Ca^{2+}]_i$ and, examined in the presence of HCO_3^-, were not significantly affected by (DIDS). In the absence of HCO_3^-, the magnitude of the postanoxic alkalinization was attenuated when external Na^+ was reduced by substitution with *N*-methyl-D-glucamine ($NMDG^+$), but not Li^+, suggesting that increased Na^+/H^+ exchange activity contributes to this phase of the pH_i response. In contrast, 100–500 μM Zn^{2+}, a known blocker of H^+-conductive pathways, reduced the magnitudes of the internal alkalinizations that occurred both during and following anoxia. The effects of $NMDG^+$-substituted medium and Zn^{2+} to reduce the increase in pH_i that occurred after anoxia were additive. Consistent with the steady-state pH_i changes, rates of pH_i recovery from internal acid loads imposed immediately after anoxia were increased, and the application of Zn^{2+} and/or perfusion with $NMDG^+$-substituted medium slowed pH_i recovery. Reducing extracellular pH from 7.35 to 6.60, or reducing ambient temperature from 37°C to room temperature, also attenuated the increases in steady-state pH_i observed during and after anoxia and reduced rates of pH_i recovery from acid loads imposed in the immediate postanoxic period. Finally, inhibition of the cAMP/protein kinase A second-messenger system reduced the magnitude of the rise in pH_i after anoxia in a manner that was dependent on external Na^+; conversely, activation of the system with isoproterenol increased the postanoxic alkalinization, an effect that was attenuated by pretreatment with propranolol, *R*p-cAMPS, or when $NMDG^+$ (but not Li^+) was employed as an external Na^+ substitute. The results suggest that a Zn^{2+}-sensitive acid efflux mechanism, possibly a H^+-conductive pathway activated by membrane depolarization, contributes to the internal alkalinization observed during anoxia in adult rat CA1 neurons. The rise in pH_i after anoxia reflects acid extrusion via the H^+-conductive pathway and also Na^+/H^+ exchange, activation of the latter being mediated, at least in part, through a cAMP-dependent signaling pathway.

INTRODUCTION

The extra- and intracellular ionic changes that occur during and following anoxia and ischemia have been studied extensively (for reviews, see Erecinska and Silver 1994; Hansen 1985; Lipton 1999). While the contribution of Ca^{2+} ions has received particular attention, notably within the framework of the excitotoxic model of cell injury, Ca^{2+}-mediated excitotoxicity may not be a completely valid model for the direct actions of anoxia or ischemia on neurons, and there is renewed interest in the role of changes in intracellular pH (pH_i) in neurodegenerative phenomena. In part, this interest has been prompted by studies in nonneuronal cell types, such as cardiac myocytes, where anoxia/ischemia-induced changes in the activities of pH_i-regulating mechanisms, notably Na^+/H^+ exchange, have been found to play an important role in reperfusion injury (for reviews, see Herman et al. 1990; Karmazyn et al. 1999). The potential importance of similar events to ischemic neuropathology is suggested by findings that pharmacological blockers of Na^+/H^+ exchange exert a protective effect in neurons in which the antiport is sensitive to such compounds (Kuribayashi et al. 1999; Phillis et al. 1999; Vornov et al. 1996).

A number of mechanisms that act to regulate pH_i in mammalian central neurons have now been identified. In rat hippocampal CA1 neurons, Na^+/H^+ exchange (which, unusually, is insensitive to amiloride, amiloride analogues, and benzoylguanidinium compounds) and Na^+-dependent HCO_3^-/Cl^- exchange contribute to acid extrusion, whereas alkali extrusion is mediated by Na^+-independent HCO_3^-/Cl^- exchange (Baxter and Church 1996; Bevensee et al. 1996; Raley-Susman et al. 1991, 1993; Schwiening and Boron 1994; Smith et al. 1998). Although it has long been known that changes in neuronal pH_i occur during and following anoxia or ischemia in vivo and in slice preparations in vitro (for reviews, see Erecinska and Silver 1994; Lipton 1999; Siesjö et al. 1996), it is difficult under these experimental conditions to assess the contribution of intrinsic alterations in the activities of neuronal pH_i-regulating mechanisms to the pH_i changes observed. Anoxia and ischemia, for example, lead to complex changes in the microenvironment of neurons, many of which [e.g., changes in extracellular pH (pH_o), postsynaptic receptor activation] can affect the activities of pH_i-regulating mechanisms and steady-state pH_i. In this regard, isolated preparations offer an important advantage, and recent studies in cultured postnatal rat hippocampal (Diarra et al. 1999) and fetal mouse neocortical (Jørgensen et al. 1999) neurons point to the involvement of changes in Na^+/H^+ exchange activity and, in the case of rat

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hippocampal neurons, a Zn^{2+}-sensitive acid extrusion mechanism in the neuronal pH_i response to anoxia. However, both the sensitivity of mammalian central neurons to the damaging effects of anoxia (Friedman and Haddad 1993; Isagai et al. 1999; Kass and Lipton 1989; Roberts and Chih 1997) and the mechanisms that serve to regulate neuronal pH_i (Bevensee et al. 1996; Douglas et al. 2001; Raley-Susman et al. 1993; Roberts and Chih 1997) are developmentally regulated, and it remains unclear whether findings made in phenotypically immature cells in culture can be applied to more mature neurons, especially those such as rat hippocampal CA1 neurons that are particularly vulnerable to the damaging effects of anoxia.

In the present study, therefore we characterized the steady-state pH_i changes that occur during and following transient periods of anoxia in hippocampal CA1 neurons acutely isolated from adult rats and examined the mechanisms responsible for the increases in pH_i that were found to occur during and following anoxia. Some of these results have been presented in abstract form (Sheldon and Church 2000).

METHODS

Cell preparation

Acutely dissociated rat hippocampal CA1 neurons were prepared as previously described (Smith et al. 1998). In brief, male Wistar rats (200–260 g) were anesthetized with 3% halothane in air and decapitated. Transverse hippocampal slices (450 μm) were prepared and allowed to recover for $\geq$1 h in HCO_3^-/CO_2-buffered saline (see following text). To isolate CA1 neurons, slices were enzymatically digested in HCO_3^-/CO_2-buffered saline containing 1.5 mg/ml protease type XIV (Sigma Chemical, St. Louis, MO). The CA1 regions were then removed under a dissecting microscope and triturated with fire-polished Pasteur pipettes of diminishing tip diameters in 0.5 ml of HEPES-buffered saline (see following text). The triturated suspension was deposited onto a poly-D-lysine-coated glass coverslip mounted in a perfusion chamber so as to form the floor of the chamber, and neurons were allowed to adhere to the substrate for 15 min before being loaded with the acetoxymethyl ester (AM) forms of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; 2 μM for 15 min) or fura-2 (7 μM for 30 min). Neurons were then superfused at a rate of 2 ml/min for 15 min with the initial experimental solution at the appropriate experimental temperature prior to the start of an experiment.

Solutions and test compounds

The standard HCO_3^-/CO_2-free perfusion medium contained (mM) 136.5 NaCl, 3 KCl, 2 $CaCl_2$, 1.5 NaH_2PO_4, 1.5 $MgSO_4$, 17.5 D-glucose, and 10 HEPES and was titrated to the appropriate temperature-corrected pH with 10 M NaOH. In standard HCO_3^-/CO_2-containing media, HEPES was isosmotically replaced by NaCl and solutions contained either 19.5 mM (at 37°C) or 29 mM (room temperature; 20–22°C) $NaHCO_3$, by equimolar substitution for NaCl, together with the constituents listed in the preceding text. Normoxic HCO_3^-/CO_2-buffered solutions were equilibrated with 5% CO_2-95% air, giving a final pH value of 7.35; during perfusion with these media, the atmosphere in the recording chamber contained 5% CO_2-95% air. Solutions containing 20 mM NH_4Cl were prepared by equimolar substitution for NaCl. When external Na^+ was reduced to 2–4 mM, *N*-methyl-D-glucamine ($NMDG^+$) or Li^+ was employed as a substitute in HEPES-buffered media and solutions were titrated to pH 7.35 with 10 M HCl or 2 M LiOH, respectively. Given the use of sodium dithionite to induce anoxia (see following text) and the need to maintain $[Na^+]_o$ constant during an experiment, external Na^+-free media could not be employed. Nevertheless, 2–4 mM Na_o^+ is considerably less than the apparent K_m of the Na^+/H^+ exchanger in rat hippocampal neurons for external Na^+ (K_m = 23–26 mM) (Raley-Susman et al. 1991), and we have found that the rate of acid extrusion from acutely dissociated CA1 neurons in the complete absence of external Na^+ is not influenced by the addition of 2–4 mM Na^+ (C. Brett, C. Sheldon, and J. Church, unpublished observations). In experiments in which Na^+-free media could be employed (see Fig. 6), NaH_2PO_4 was omitted and $NMDG^+$ and/or KCl were employed as substitutes; solutions were titrated to pH 7.35 with 10 M HCl or 2 M KOH, respectively. For Ca^{2+}-free media, $CaCl_2$ was omitted, $[Mg^{2+}]$ was increased to 3.5 mM, and 200 μM EGTA was added. Neurons were superfused at a rate of 2 ml/min for the entire duration of an experiment, and, unless otherwise noted, all experiments were performed at 37°C and at pH_o 7.35. The pH of each experimental solution was rechecked at the end of every experiment.

Test compounds were obtained from Sigma Chemical with the exceptions of 2′,5′-dideoxyadenosine (Biomol Research Laboratories, Plymouth Meeting, PA), the *R*p-isomer of adenosine-3′,5′-cyclic monophosphorothioate (*R*p-cAMPS, Na^+ salt; Biolog Life Science Institute, La Jolla, CA), and omeprazole and SCH 28080 (generous gifts from, respectively, AstraZeneca, Mississauga, Ontario, Canada, and Schering Canada, Pointe-Claire, Quebec, Canada). BCECF-AM and fura-2-AM were obtained from Molecular Probes (Eugene, OR).

Induction of anoxia

Anoxia was induced by the addition of 1 or 2 mM sodium dithionite, an O_2 scavenger, to the superfusing medium (see Diarra et al. 1999; Friedman and Haddad 1993). Solutions containing sodium dithionite were prepared immediately prior to use and were bubbled with either 5% CO_2-95% Ar (HCO_3^-/CO_2-buffered media) or 100% Ar (HEPES-buffered media); during perfusion with these media, the atmosphere in the recording chamber was switched, respectively, to 5% CO_2-95% Ar or 100% Ar. The P_{O_2} in media containing either 1 or 2 mM sodium dithionite was measured with a Radiometer ABL 500 blood gas analyzer calibrated for low P_{O_2} values; in samples obtained anaerobically from the recording chamber, P_{O_2} was $<$1 mmHg (n = 6 in each case). Similar P_{O_2} values were observed during experiments in which an oxygen electrode (ISO_2; World Precision Instruments, Sarasota, FL) was placed in the recording chamber.

To assess the possibility that sodium dithionite might induce changes in pH_i via mechanisms unrelated to its O_2 scavenging property, HCO_3^-/CO_2- and HEPES-buffered media were bubbled vigorously with ultra-high purity Ar (containing 5% CO_2 in the case of HCO_3^-/CO_2-buffered media) for periods of 1 to $\geq$18 h. In samples obtained anaerobically from the recording chamber, the P_{O_2} in media bubbled with Ar for 1 h was 25.3 $\pm$ 0.8 (SE) mmHg (n = 4), whereas, measured in eight different samples, the P_{O_2} in media bubbled with Ar for $\geq$18 h was $<$1 mmHg, a value the same as that measured in media containing 1 or 2 mM sodium dithionite. When a 5-min period of anoxia was imposed by exposing neurons to HCO_3^-/CO_2-buffered media that had been equilibrated with 5% CO_2-95% Ar for $\geq$18 h, the resultant steady-state pH_i changes were not significantly different to those observed when the P_{O_2} was reduced to $<$1 mmHg by the addition of sodium dithionite under identical buffering conditions (Table 1; Fig. 2*B*). Thus the steady-state pH_i changes evoked by exposure to media containing sodium dithionite reflect a reduction in P_{O_2} and are not secondary to any additional properties of the O_2 scavenger.

Recording techniques

Intracellular free calcium concentration ($[Ca^{2+}]_i$) and pH_i were measured using the dual-excitation ratio method, employing a fluorescence ratio-imaging system (Atto Instruments, Rockville, MD; Carl Zeiss Canada, Don Mills, Ontario, Canada). Details of the methods employed have been presented previously (Baxter and Church 1996; Church et al. 1998;

TABLE 1. *Anoxia-evoked changes in steady-state pH_i*

Duration of Anoxia, min	Buffering Condition	*n*	Magnitude (pH units)		
			pH_i decrease during anoxia	pH_i increase during anoxia	pH_i increase after anoxia
5 (Ar)*	HCO_3^-/CO_2	5	0.14 ± 0.01	0.08 ± 0.01	0.18 ± 0.07
5 (HPTS)†	HEPES	4	0.08 ± 0.02	0.06 ± 0.03	0.23 ± 0.07
5	HCO_3^-/CO_2	14	0.17 ± 0.02	0.07 ± 0.01	0.15 ± 0.04
10	HCO_3^-/CO_2	12	0.16 ± 0.02	0.11 ± 0.02‡	0.19 ± 0.02
5	HEPES	37	0.15 ± 0.01	0.05 ± 0.01	0.19 ± 0.01§
10	HEPES	15	0.16 ± 0.01	0.09 ± 0.02‡	0.27 ± 0.04§
10	HCO_3^-/CO_2 + 200 μM DIDS	8	0.13 ± 0.01	0.09 ± 0.02	0.24 ± 0.03

Experiments were performed at 37°C, pH_o 7.35. Unless otherwise noted, anoxia was induced with sodium dithionite and 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF) was employed as the pH indicator. The intracellular pH (pH_i) decrease during anoxia is the difference between the preanoxic steady-state pH_i value and the lowest pH_i value observed during anoxia. The pH_i increases during and after anoxia are, respectively, the difference between the pH_i value observed immediately prior to the return to normoxia and the minimum pH_i value observed during anoxia, and the difference between the highest pH_i value observed after anoxia and the preanoxic steady-state pH_i value. *, anoxia was induced by exposure to medium equilibrated with 5% CO_2-95% Ar for ≥18 hours. †, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was the pH indicator. ‡, indicates statistical significance between the corresponding parameter obtained in response to 5 min anoxia under the same buffering condition (ANOVA; $P < 0.05$). §, the pH_i increase under HEPES-buffered conditions was significantly larger than in the presence of HCO_3^-/CO_2 for the same duration of anoxia (ANOVA; $P < 0.05$).

Smith et al. 1998). In brief, fluorescence emissions measured at 520 or 510 nm from neurons loaded with BCECF or fura-2, respectively, were detected by an intensified charge-coupled device camera (Atto Instruments) and collected from regions of interest placed on individual neuronal somata. Raw emission intensity data at each excitation wavelength (488 and 452 nm for BCECF; 334 and 380 nm for fura-2) were corrected for background fluorescence prior to calculation of the ratio. Ratio pairs were acquired at 1- to 12-s intervals and analyzed off-line. Analysis was restricted to those neurons able to retain BCECF (as judged by raw emission intensity values recorded during excitation at 452 nm; see Fig. 2*A*) throughout the course of an experiment (see Bevensee et al. 1995). To reduce photobleaching of the fluorophores and cell damage, the output of the 100 W mercury arc lamp was attenuated electronically, neutral density filters were placed in the light path, and a high-speed shutter was employed to limit UV exposure to the periods required for data acquisition.

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 as described (Baxter and Church 1996; Smith et al. 1998). Parameters employed in the calculation of pH_i values were derived from nonlinear least-squares regression fits to background-subtracted ratio versus pH data, which, in turn, were obtained in full calibration experiments (see Baxter and Church 1996). For the 15 full-calibration experiments utilized in analyzing all BCECF-derived data, the mean values for $R_{n(max)}$ (the maximum obtainable value for the normalized ratio), $R_{n(min)}$ (the minimum obtainable value for the normalized ratio), and pK_a (the −log of the dissociation constant of BCECF) were (means ± SE) 1.89 ± 0.04, 0.43 ± 0.02, and 7.19 ± 0.02, respectively. These values were not dependent on the temperature at which the calibration was conducted (data not shown). To limit potential cross-contamination by nigericin, perfusion lines were replaced and the imaging chamber was decontaminated after each experiment by soaking first in ethanol and then in 20% Decon 75 (BDH, Toronto, Ontario, Canada) (see Bevensee et al. 1999; Richmond and Vaughan-Jones 1997). In addition, selected experiments were repeated using an experimental chamber that had never been exposed to the ionophore. Although the data from these experiments were not calibrated (and, therefore are not included in RESULTS), the BI_{488}/BI_{452} ratio values obtained were typical of those recorded during the course of equivalent experiments conducted in nigericin-decontaminated chambers. Thus we failed to reveal any evidence that nigericin contamination might have contributed to the results obtained in the study. Calibration of the fura-2 signal was not attempted and the effects of experimental maneuvers on $[Ca^{2+}]_i$ are presented as changes in background-corrected I_{334}/I_{380} ratio values. Nevertheless, under conditions identical to those employed in the present experiments, we have found that a BI_{334}/BI_{380} ratio value of ≈0.5 (as was observed in quiescent neurons in the present study; Fig. 3) represents an $[Ca^{2+}]_i$ ≈80 nM (see Church et al. 1998).

It has been reported that BCECF inhibits the plasmalemmal Ca^{2+}-ATPase in erythrocytes (IC_{50} 100 μM) (Gatto and Milanick 1993). Because the Ca^{2+}-ATPase in hippocampal neurons is a Ca_i^{2+}-H_o^+ exchanger (Trapp et al. 1996), the possibility existed that the rises in pH_i measured with BCECF under the high $[Ca^{2+}]_i$ conditions that pertain during and following anoxia (see RESULTS) might have been artifacts consequent on reduced background acid loading. Therefore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS; Molecular Probes), a fluorescent ratiometric H^+-sensitive indicator that is reported not to inhibit activity-dependent pH_i changes in snail neurons (Willoughby et al. 1998), was employed in a limited number of experiments to measure anoxia-evoked changes in pH_i. Loading of this membrane-impermeant dye was achieved by enzymatically treating and triturating hippocampal CA1 regions in the presence of 40 mM HPTS. The excitation wavelengths were 452 and 380 nm, and the one-point high-$[K^+]$/nigericin technique was employed to convert background-corrected HPTS emission intensity ratios (BI_{452}/BI_{380}) into pH_i values using the equation

$$pH = [pK_a + \log(1/\beta)] + \log[(R_n - R_{n(min)})/(R_{n(max)} - R_n)] \quad (1)$$

where R_n is the BI_{452}/BI_{380} ratio normalized to unity at pH 7.00 and $1/\beta = f_{n2a}/f_{n2b}$, where f_{n2a} and f_{n2b} are the normalized fluorescence intensities at the acidic and basic extremes while exciting the dye at 380 nm. The constant parameters of *Eq. 1* were derived from full calibration experiments (see preceding text). The increases in pH_i observed during and following 5 min anoxia in HPTS-loaded neurons were not significantly different to those observed in BCECF-loaded cells (Table 1; Fig. 2*C*), leading us to conclude that BCECF is an appropriate pH indicator for use in the present experiments. Our findings are in agreement with a recent report in cultured rat cerebellar granule cells in which activity-dependent changes in pH_i were recorded when BCECF was employed as the H^+-sensitive fluorophore (Wu et al. 1999).

Experimental procedures and data analysis

The effects of transient periods of anoxia were examined on both steady-state pH_i and on rates of pH_i recovery from internal acid loads imposed by the NH_4^+ prepulse technique. The parameters employed to compare the steady-state pH_i changes evoked by anoxia under the

various experimental conditions are illustrated in Fig. 1*A*. 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 neuron and the second being performed immediately following a 5-min anoxic insult (e.g., see Fig. 5*A*). Full details of the methods employed in analyzing the data obtained in acid load recovery experiments have been presented previously (Baxter and Church 1996; Smith et al. 1998). In brief, instantaneous rates of pH_i recovery under control and test conditions were plotted against absolute pH_i values (e.g., see Fig. 1*B*) and compared at corresponding absolute values of pH_i. The consistency of rates of pH_i recovery following two consecutive acid loads imposed in the absence of an anoxic insult was established in control experiments (Fig. 1*B*). In experiments where the composition of the external medium was altered during the recovery of pH_i from an intracellular acid load (see Figs. 5*C* and 6), individual portions of the recovery were fit to a linear equation, as described by Raley-Susman et al. (1991).

Data are reported as means ± SE with the accompanying *n* value referring to the number of neurons from which data were obtained. Statistical analyses were performed with Student's two-tailed unpaired *t*-test or two-way ANOVA, as appropriate. Significance was assumed at the 5% level.

RESULTS

Resting pH_i values under normoxic conditions

Under HCO_3^-/CO_2-buffered conditions at pH_o 7.35, resting pH_i was distributed in a Gaussian manner around a mean of 7.30 ± 0.17 (range pH, 6.90–7.60; $n = 39$). In nominally HCO_3^-/CO_2-free, HEPES-buffered medium at pH 7.35, steady-state pH_i was 7.30 ± 0.21 (range pH, 6.40–7.80; $n = 277$) and the distribution of resting pH_i values was fit with the sum of two Gaussian distributions with means at pH_i 6.93 ± 0.17 and pH_i 7.37 ± 0.14. The mean resting pH_i values and their distributions under both HCO_3^-/CO_2- and HEPES-buffered conditions were similar to those reported previously by this laboratory (Smith et al. 1998) and others (Bevensee et al. 1996) for acutely isolated mature rat hippocampal CA1 neurons at 37°C.

Steady-state pH_i response to anoxia

The steady-state pH_i changes evoked by 5- and 10-min periods of anoxia were first examined under HCO_3^-/CO_2-buffered conditions at pH_o 7.35. The results are presented in Table 1, and typical responses are illustrated in Fig. 2*A*. In each case, anoxia elicited a triphasic pattern of steady-state pH_i changes that consisted of an initial acidic shift following the induction of anoxia, a subsequent rise in pH_i in the continued absence of O_2, and, finally, a further internal alkalinization on the return to normoxia that, in the case of 5 min anoxic insults, recovered slowly toward resting pH_i values. A clear change in the rate of increase of pH_i was observed during the transition from anoxia to normoxia (see Fig. 2*A*) in 10/14 and 8/12 neurons subjected, respectively, to 5 and 10 min anoxia under HCO_3^-/CO_2-buffered conditions (corresponding changes were observed in 29/37 and 14/15 neurons under nominally HCO_3^-/CO_2-free conditions; see following text and Fig. 2*D*).

To assess the potential contribution of HCO_3^- and HCO_3^--dependent pH_i regulating mechanisms to anoxia-evoked changes in steady-state pH_i, experiments were repeated under nominally HCO_3^-/CO_2-free, HEPES-buffered conditions. Irrespective of the duration of the anoxic insult, the decreases in pH_i typically observed during anoxia were not significantly different in the absence or presence of HCO_3^- (Table 1) (also see Pirttilä and Kauppinen 1994). Similarly, although the magnitudes of the rises in pH_i observed in the continued absence of O_2 increased under both buffering conditions as the duration of the anoxic insult increased, for a given duration of anoxia, no

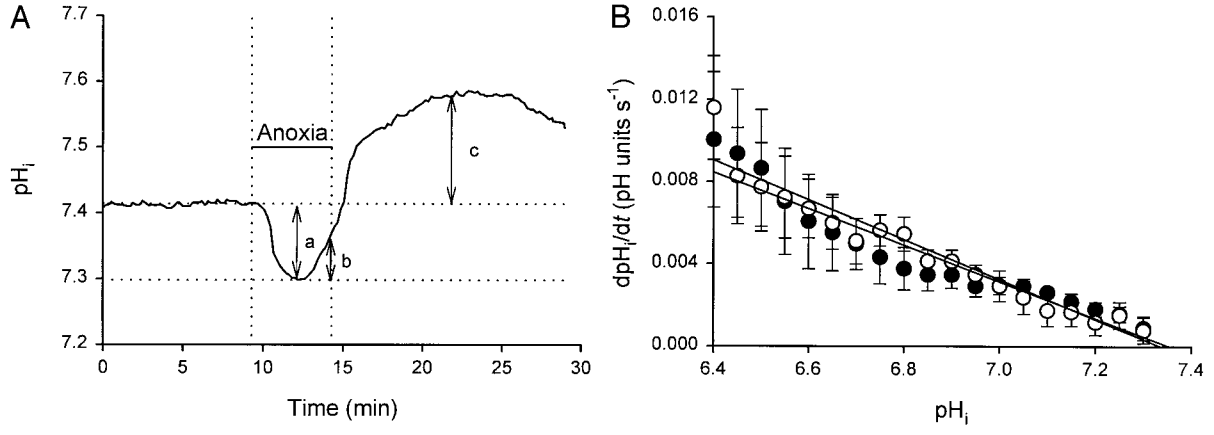

FIG. 1. Steady-state intracellular pH (pH_i) changes measured in the study and rates of pH_i recovery from internal acid loads imposed under control conditions. *A*: a representative record of the steady-state pH_i changes evoked by 5 min anoxia under HEPES-buffered conditions (pH_o 7.35, 37°C). The pH_i response to anoxia was characterized by a fall in pH_i on the induction of anoxia, a subsequent rise in pH_i in the continued absence of O_2, and, finally, a further internal alkalinization in the immediate postanoxic period. The parameters measured were: a) the magnitude of the internal acidic shift induced by anoxia, which is the difference between the preanoxic steady-state pH_i value and the lowest pH_i value observed during anoxia; b) the magnitude of the rise in pH_i observed in the continued absence of O_2, which is the difference between the pH_i value observed immediately prior to the return to normoxia and the minimum pH_i value observed during anoxia; and c) the magnitude of the internal alkaline shift observed following the return to normoxia, which is the difference between the highest pH_i value observed after anoxia and the preanoxic steady-state pH_i value. *B*: the pH_i dependencies of rates of pH_i recovery following an initial (○) and a 2nd (●) internal acid load imposed under HEPES-buffered control conditions. Rates of pH_i recovery were evaluated at 0.05 pH unit intervals of pH_i and error bars ($n = 18$) represent SE. Continuous lines represent the weighted nonlinear regression fits to the data points indicated for the 1st and 2nd acid loads (see Motulsky and Ransnas 1987).

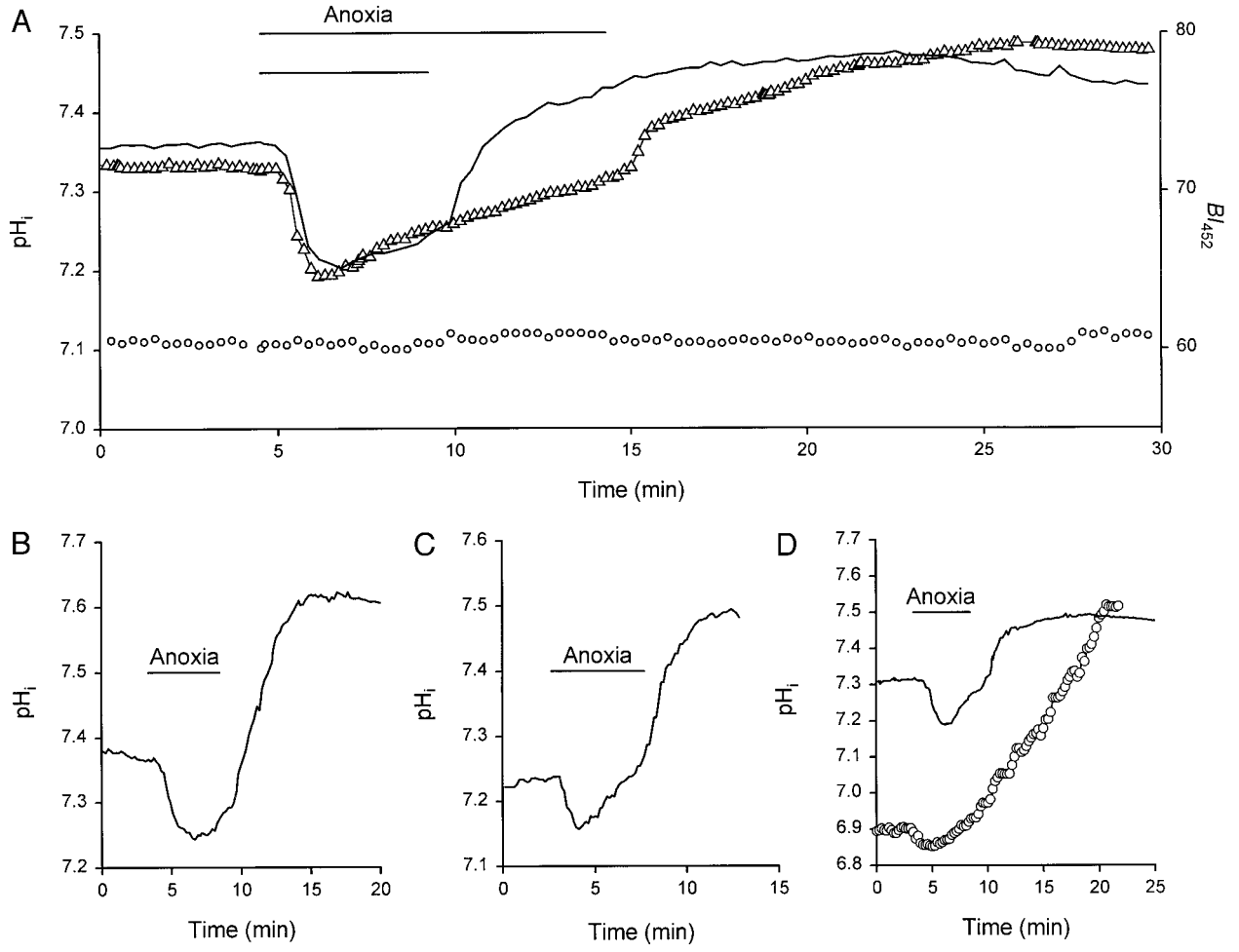

FIG. 2. Steady-state pH_i changes evoked by transient periods of anoxia. *A*: shown are the steady-state pH_i changes evoked by 5 (—) and 10 (△) min anoxia, indicated by the respective bars above the traces, in 2 different neurons with similar steady-state pH_i values prior to the induction of anoxia. In each case, anoxia was imposed under HCO_3^-/CO_2-buffered conditions by exposure to medium containing sodium dithionite. Beneath the pH_i traces are shown the BI_{452} values (○) that were employed in the measurement of the pH_i response to 5 min anoxia. The stability of the BI_{452} values indicates that the relatively persistent nature of the increase in pH_i observed after anoxia is not an artifact produced by a decline in BI_{452} values consequent on a deterioration of membrane integrity (see METHODS). *B*: a 5-min period of anoxia was imposed by exposure to HCO_3^-/CO_2-buffered medium that had been bubbled vigorously with 5% CO_2-95% ultrahigh purity Ar for 20 h. *C*: the pH_i changes evoked by 5 min anoxia (sodium dithionite) in a neuron in which 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was employed as the pH_i indicator. *D*: "typical" (—) and "atypical" (○) pH_i responses to 5 min anoxia in 2 different BCECF-loaded neurons exposed to sodium dithionite-containing, HEPES-buffered medium. Note the low resting pH_i value in the neuron that responded to anoxia with a small reduction in pH_i that, in turn, gave way to a large internal alkalinization that started during anoxia and continued into the postanoxic period. In *A–D*, records were obtained at 37°C and pH_o was 7.35 throughout.

significant difference was found between the rises in pH_i under HCO_3^-/CO_2- or HEPES-buffered conditions. However, irrespective of the duration of anoxia, the increases in pH_i observed following the return to normoxia were significantly larger under HEPES- than under HCO_3^-/CO_2-buffered conditions, suggesting that, as in other cell types (Bevensee and Boron 2000; Pirttilä and Kauppinen 1994), changes in the activities of HCO_3^--dependent pH_i-regulating mechanisms might influence this phase of the pH_i response to anoxia in adult rat CA1 neurons. However, although the rise in pH_i observed after a 10-min period of anoxia imposed under HCO_3^-/CO_2-buffered conditions increased in the presence of 200 μM DIDS, this failed to reach statistical significance (Table 1).

In 13 additional neurons examined under HEPES-buffered conditions, 5 min anoxia elicited a different pattern of pH_i changes to that described in the preceding text. In these neurons, the fall in pH_i during anoxia was significantly smaller (0.04 ± 0.01 pH units) than the response observed in the majority of cells examined under identical buffering conditions, and the small acidification gave way to a marked internal alkalinization (0.53 ± 0.05 pH units) that started during anoxia and continued into the postanoxic period (Fig. 2*D*). This atypical pattern of pH_i changes was also observed in HPTS-loaded neurons (data not shown) and, interestingly, is reported to be the usual response of mouse CA1 hippocampal neurons to O_2 deprivation under HEPES-buffered conditions (Yao et al. 2001). Although no attempt was made to characterize the mechanism(s) underlying the atypical pH_i response to anoxia, it is noteworthy that neurons that exhibited the response had low resting pH_i values (mean pH_i prior to the induction of anoxia was 6.92 ± 0.07). This finding is consistent with the

possibility (Bevensee et al. 1996) that a "low pH_i" population of mature rat hippocampal CA1 neurons exists that exhibits a distinct pattern of pH_i regulation.

Because the typical steady-state pH_i changes evoked by anoxia in adult rat hippocampal CA1 neurons under HCO_3^-/CO_2-buffered conditions were not significantly affected by DIDS, subsequent experiments were conducted in the nominal absence of HCO_3^-/CO_2 and, in all cases, employed a 5-min anoxic insult.

Steady-state $[Ca^{2+}]_i$ response to anoxia

In adult CA1 neurons, anoxia leads to a disruption of internal ion homeostasis that is associated with energy failure and an abrupt depolarization of the plasma membrane (reviewed by Hansen 1985; Lipton 1999; also see Rader and Lanthorn 1989; Silver and Erecinska 1990; Tanaka et al. 1997). In the present study, anoxia evoked a 2.0 ± 0.4 ($n = 8$) unit increase in the fura-2 BI_{334}/BI_{380} ratio value, which commenced at approximately 2 min after the induction of anoxia (as did the rise in pH_i; Fig. 3*A*) and which remained elevated after the return to normoxia for as long as stable recordings could be maintained (≤25 min following the end of an anoxic insult) (also see Friedman and Haddad 1993; Kubo et al. 2001).

The potential contribution of changes in $[Ca^{2+}]_i$ to the changes in steady-state pH_i evoked by anoxia was assessed by imposing anoxia under external Ca^{2+}-free conditions. As shown in Fig. 3*B*, exposure to Ca^{2+}-free medium caused a 0.30 ± 0.02 ($n = 6$) ratio unit decrease in resting BI_{334}/BI_{380} values, and anoxia failed to induce the rapid and marked rises in BI_{334}/BI_{380} values that were observed in the presence of Ca^{2+} (the increase in the BI_{334}/BI_{380} value observed under external Ca^{2+}-free conditions was 0.02 ± 0.01 ratio units). Interestingly, in 4/6 neurons examined under Ca^{2+}-free conditions, a small 0.08 ± 0.02 ratio unit increase in BI_{334}/BI_{380} values was observed in the immediate postanoxic period (Fig. 3*B*); although the basis for this transient increase was not investigated, it may reflect Ca^{2+} release from intracellular stores consequent on anoxia-evoked changes in pH_i (see Ou Yang et al. 1994). In parallel experiments in BCECF-loaded neurons, exposure to Ca^{2+}-free medium evoked an increase in steady-state pH_i of 0.11 ± 0.04 pH units ($n = 6$), as previously reported (Smith et al. 1998). Once a new steady-state pH_i value had been reached, a 5-min period of anoxia induced a triphasic pH_i response, the individual components of which were not significantly different to those observed in the presence of 2 mM Ca_o^{2+} (Figs. 3*B* and 4).

Mechanisms underlying the increases in pH_i observed during and after anoxia

Although a fall in pH_i appears to be a universal response of mammalian central neurons to anoxia or ischemia, the increases in pH_i that occurred during and after anoxia in the present study have been observed only relatively infrequently in neurons in vivo or in slice preparations in vitro (see Fujiwara et al. 1992; Mabe et al. 1983; Melzian et al. 1996; Pirttilä and Kauppinen 1992). In subsequent experiments, therefore we examined the mechanisms responsible for these increases in pH_i.

Under HCO_3^-/CO_2-free conditions, Na^+/H^+ exchange is the dominant acid extrusion mechanism in rat hippocampal neurons, but, unusually, this transporter is insensitive to amiloride, amiloride derivatives, and benzoylguanidinium compounds (Baxter and Church 1996; Bevensee et al. 1996; Raley-Susman et al. 1991; Schwiening and Boron 1994). To inhibit Na^+/H^+ exchange therefore, neurons were perfused with reduced-Na_o^+, $NMDG^+$-substituted medium. Under these conditions, the increases in pH_i observed during and following anoxia were, respectively, statistically unaffected and reduced, compared with the corresponding changes measured in the presence of normal Na_0^+ (Fig. 4). Unlike $NMDG^+$, Li^+ can act as a substrate for Na^+/H^+ exchange in hippocampal neurons (Baxter and Church 1996; Raley-Susman et al. 1991). Under reduced-

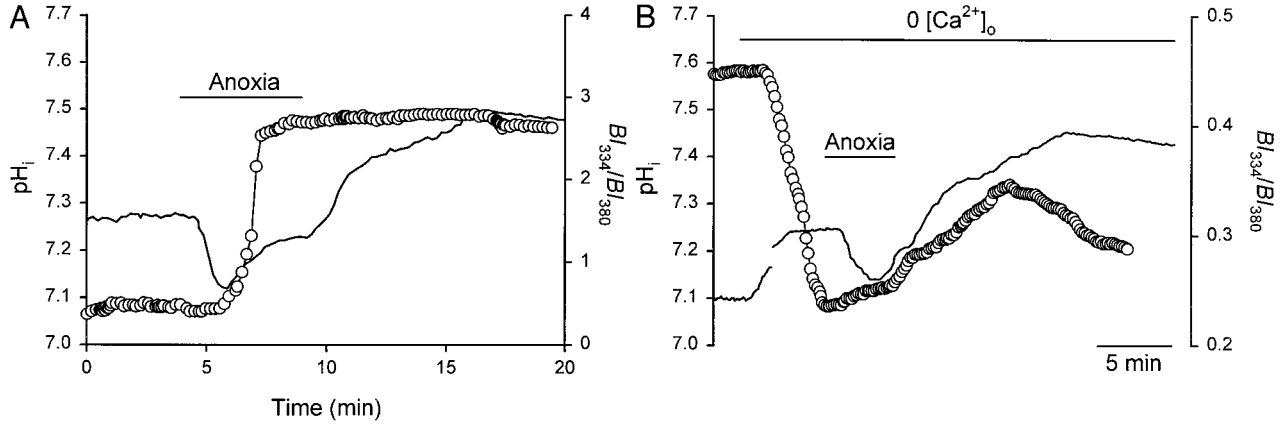

FIG. 3. Effects of anoxia on steady-state pH_i and BI_{334}/BI_{380} ratio values in the presence and absence of external Ca^{2+}. *A*: in the presence of 2 mM external Ca^{2+}, 5 min anoxia imposed under HEPES-buffered conditions induced a typical pattern of pH_i changes (—). Compare with Fig. 2*A* (—), the same experiment conducted under HCO_3^-/CO_2-buffered conditions. ○, the changes in BI_{334}/BI_{380} ratio values (representing changes in $[Ca^{2+}]_i$) evoked by 5 min anoxia in a sister neuron in a parallel experiment under identical conditions. *B*: on exposure to Ca^{2+}-free medium, pH_i (—) increased to a new steady-state value. The break in the pH_i trace indicates a 2-min gap in the recording. When a new steady-state pH_i value had been reached, 5 min anoxia induced a triphasic pattern of pH_i changes, none of the components of which were significantly different from those observed in the presence of 2 mM Ca_o^{2+}. In contrast, the rise in BI_{334}/BI_{380} ratio values (○) was significantly attenuated (note the change of scale for the BI_{334}/BI_{380} axis between *A* and *B*). There was also a small, reversible rise in BI_{334}/BI_{380} ratio values in the immediate postanoxic period (see text). All records were obtained at 37°C under HEPES-buffered conditions at pH_o 7.35.

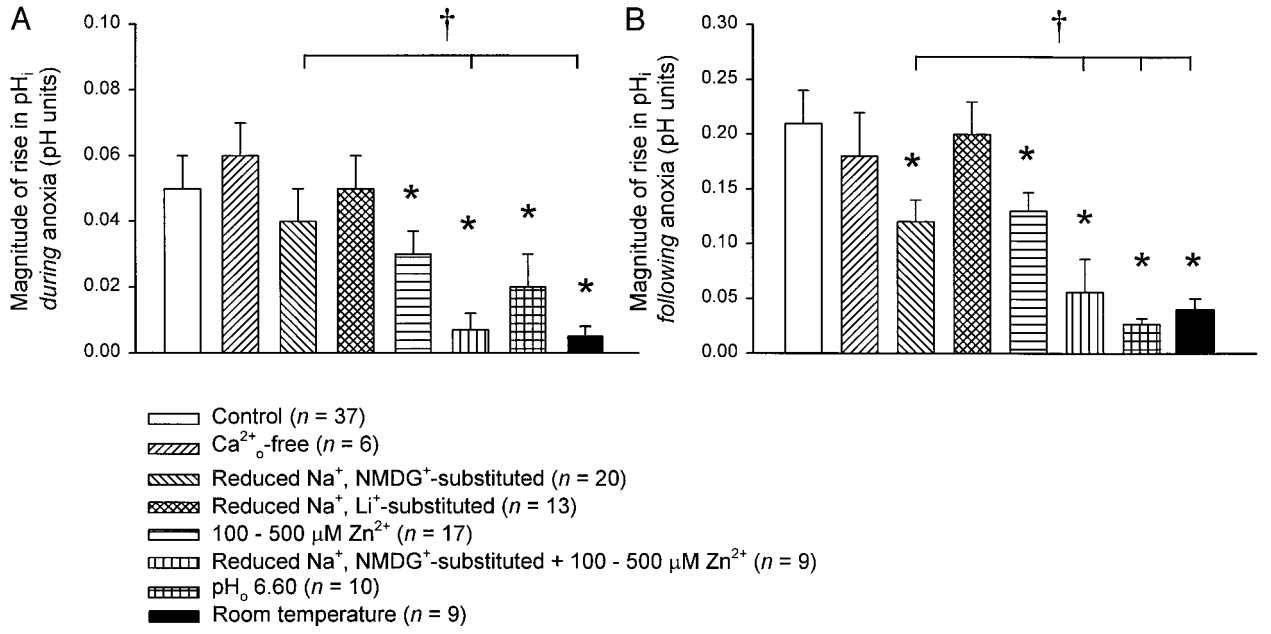

FIG. 4. Effects of changes in perfusate composition on the increases in pH_i observed during (*A*) and following (*B*) 5 min anoxia. All experiments were performed under nominally HCO_3^-/CO_2-free, HEPES-buffered conditions; error bars are SE. *, a statistically significant difference ($P < 0.05$) compared with control (pH_o 7.35, 37°C; shown in the *1st column* in both *A* and *B*). †, a statistically significant difference ($P < 0.05$) compared with the value obtained under reduced-Na_o^+, $NMDG^+$-substituted conditions (shown in the *3rd column* in both *A* and *B*).

Na_o^+, Li^+-substituted conditions, the rise in pH_i during anoxia was again not significantly affected, but the increase in pH_i after anoxia was restored to control levels (Fig. 4). The results are consistent with the possibility that Na^+/H^+ exchange contributes to the production of the postanoxic internal alkalinization. However, because blockade of Na^+/H^+ exchange failed to affect the magnitude of the rise in pH_i during anoxia and did not abolish the pH_i increase after anoxia (also see Pirttilä and Kauppinen 1994), additional mechanism(s) must contribute to these phases of the anoxic pH_i response.

As noted in the preceding text, the internal alkalinizations that occurred during and after anoxia appeared to be associated temporally with marked and persistent increases in $[Ca^{2+}]_i$, raising the possibility that they may reflect H^+ efflux through a H^+-conductive pathway activated by anoxic membrane depolarization. In all cell types studied to date, voltage-gated H^+ conductances (g_{H^+}s) are blocked by micromolar concentrations of Zn^{2+} (for reviews, see DeCoursey and Cherny 1994a, 2000; Eder and DeCoursey 2001). Therefore we examined the effects of 100–500 μM Zn^{2+} on the rises in pH_i observed during and following anoxia. While the application of Zn^{2+} did not change resting pH_i prior to anoxia, there was a significant reduction in the magnitudes of the rises in pH_i during and after anoxia (Fig. 4). When Zn^{2+} was applied under reduced-$[Na^+]_o$, $NMDG^+$-substituted conditions, the magnitudes of the rises in pH_i observed during and after anoxia were reduced to values significantly less than those observed under reduced-Na_o^+ ($NMDG^+$-substituted) conditions alone (Fig. 4). The results suggest that acid efflux via a Zn^{2+}-sensitive mechanism, possibly a H^+-conductive pathway activated as a consequence of membrane depolarization, contributes to the production of the internal alkalinizations observed during and after anoxia in acutely isolated adult rat CA1 neurons.

pH_i recovery from internal acid loads imposed immediately after the return to normoxia

To further investigate the possibilities that Na^+/H^+ exchange and a presumed g_{H^+} contribute to the internal alkalinization observed after anoxia, we compared rates of pH_i recovery from internal acid loads imposed prior to and immediately following anoxia. Examined in 17 neurons, instantaneous rates of pH_i recovery were increased significantly after anoxia at all absolute values of pH_i (Fig. 5, *A* and *B*). 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) were similar prior to and after anoxia (0.24 ± 0.02 and 0.21 ± 0.02 pH unit increases, respectively; $n = 17$ in each case; $P > 0.05$), suggesting that alterations in intracellular buffering power are unlikely to underlie the changes in the rates of pH_i recovery observed after anoxia. Next, internal acid loads were imposed under reduced-Na_o^+, $NMDG^+$-substituted conditions ($n = 5$); rates of pH_i recovery after anoxia were significantly slower than the corresponding rates observed in the presence of Na_o^+ (Fig. 5*B*). Consistent with the possibility that Na^+/H^+ exchange activation was occurring in the immediate postanoxic period, plots of the differences between rates of pH_i recovery under Na_o^+-containing and reduced-Na_o^+ conditions both prior to and after anoxia (Fig. 5*B*, *inset*) revealed an increased contribution from a Na_o^+-dependent mechanism to pH_i recovery from acid loads immediately after anoxia. However, even under reduced-Na_o^+ conditions, rates of pH_i recovery increased after anoxia at every absolute value of pH_i, compared with rates established prior to anoxia under the same conditions (Fig. 5*B*). The latter finding is consistent with the steady-state pH_i results detailed in the preceding text and suggests that Na^+/H^+ exchange cannot

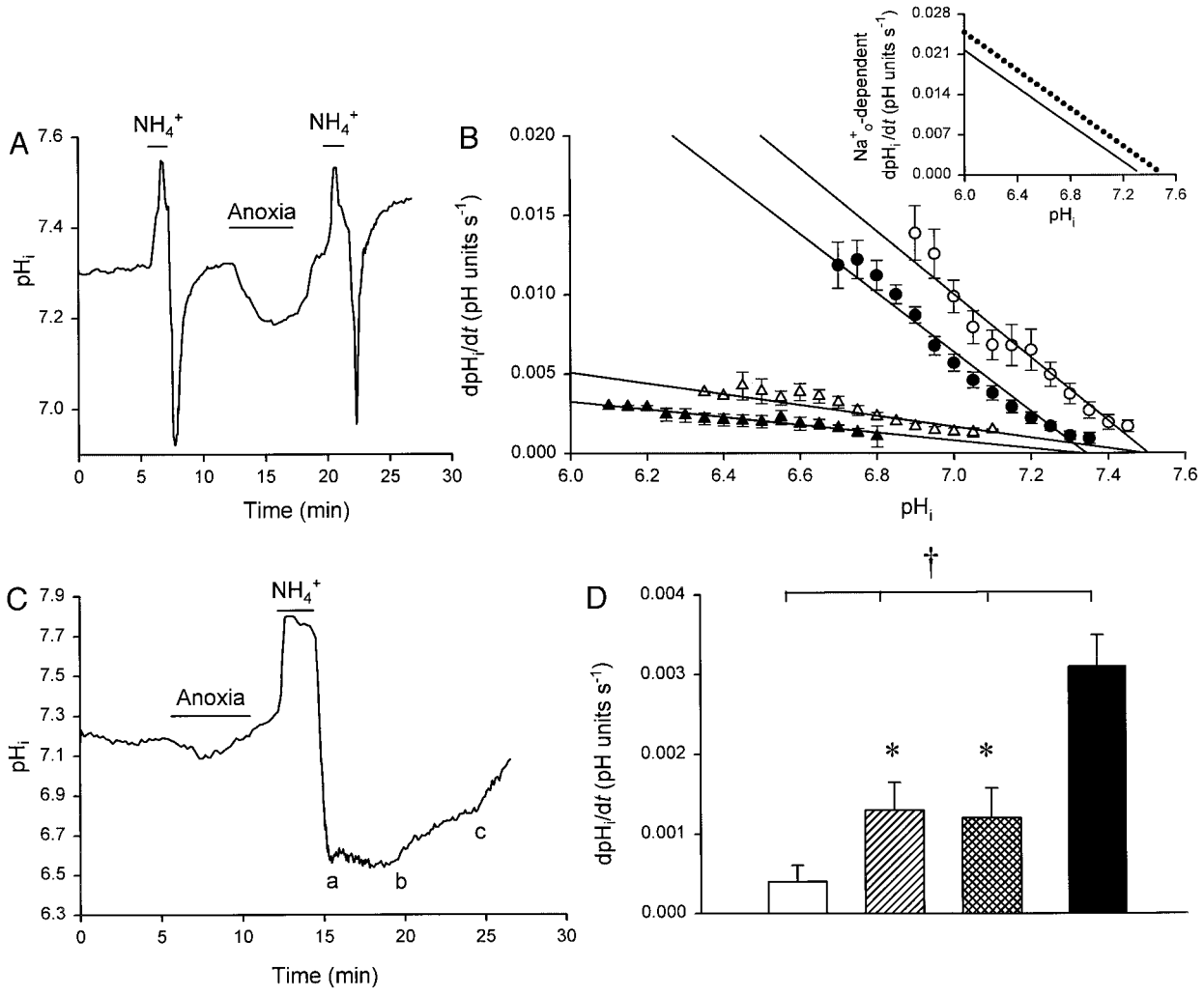

FIG. 5. Recovery of pH_i from internal acid loads imposed immediately after anoxia. *A*: following the 1st NH_4^+-induced intracellular acid load, pH_i was allowed to recover. A 2nd acid load was then applied after 5 min anoxia. The rate of recovery of pH_i was increased in the immediate postanoxic period, compared with the rate of pH_i recovery observed prior to anoxia. *B*: rates of pH_i recovery prior to (● and ▲) and immediately after (○ and △) 5 min anoxia under control (Na_o^+-containing; ●, ○) and reduced-Na_o^+, $NMDG^+$-substituted (▲ and △) conditions. Under both conditions, rates of pH_i recovery were increased following anoxia ($P < 0.05$ at each absolute value of pH_i); data points were obtained from 17 and 5 experiments, respectively, of the type shown in *A*. —, the weighted nonlinear regression fits to the data points indicated for each experimental condition ($r^2 > 0.93$ in all cases). Where missing, standard error bars lie within the symbol areas. *Inset*, the Na_o^+-dependent component of pH_i recovery prior to (—) and after (···) anoxia revealed by plotting the differences between the regression fits obtained under Na_o^+-containing and reduced-Na_o^+ ($NMDG^+$-substituted) conditions. *C*: following a 5-min period of anoxia, an internal acid load was imposed under control (Zn^{2+}-free, Na_o^+-containing) conditions. At the peak of the acidification, the perfusate was changed to a reduced-Na_o^+, $NMDG^+$-substituted medium containing 100 μM Zn^{2+} (a to b). From b to c, Zn^{2+} was removed, and pH_i recovery was allowed to proceed under reduced-Na_o^+, $NMDG^+$-substituted conditions. At c, the neuron was reperfused with control medium. *D*: rates of pH_i recovery from internal acid loads imposed immediately after anoxia during perfusion with reduced-Na_o^+, $NMDG^+$-substituted medium containing 100 μM Zn^{2+} ($n = 11$; □); reduced-Na_o^+, $NMDG^+$-substituted medium ($n = 7$; ▨); Na_o^+-containing medium in the presence of 100 μM Zn^{2+} ($n = 6$; ▩); and control (Zn^{2+}-free, normal Na_o^+-containing) medium ($n = 16$; ■). Error bars are SE. †, a statistically significant difference ($P < 0.05$) compared with control (Zn^{2+}-free, normal Na_o^+). *, a statistically significant difference ($P < 0.05$) compared with the value obtained under reduced-Na_o^+, $NMDG^+$-substituted conditions in the presence of 100 μM Zn^{2+}. In *A–D*, data were obtained at 37°C during perfusion with HEPES-buffered media at pH_o 7.35.

be the sole mechanism responsible for the increased rate of pH_i recovery observed in the immediate postanoxic period under control (normal Na_o^+-containing) conditions.

Next, internal acid loads were applied immediately after anoxia and pH_i recovery was allowed to proceed in the presence of 100–500 μM Zn^{2+} and/or under reduced-Na_o^+, $NMDG^+$-substituted conditions. As illustrated in Fig. 5*C*, pH_i recovery was markedly inhibited under reduced-Na_o^+ conditions in the presence of Zn^{2+}; there was a significant increase in the rate of pH_i recovery when Zn^{2+} was removed from the low-Na^+ medium, and the rate of recovery increased further on the reintroduction of normal external Na^+. Similar results were obtained in experiments in which pH_i recovery from an acid load was allowed to proceed initially under control conditions (i.e., in the presence of normal $[Na^+]_o$ and absence of Zn^{2+}); in these experiments, reducing external Na^+ and/or adding Zn^{2+} also slowed significantly the rate at which pH_i recovered. The pooled results from these series of experiments are pre-

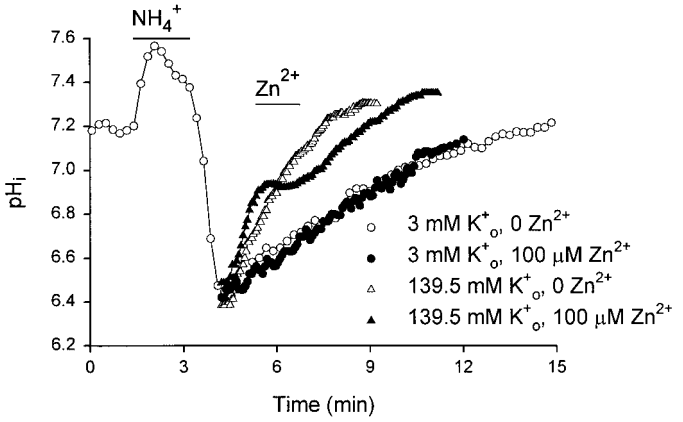

FIG. 6. Effect of high $[K^+]_o$ on pH_i recovery from intracellular acid loads under nominally HCO_3^--free, HEPES-buffered conditions (pH_o 7.35) in the absence of external Na^+. Under control conditions (3 mM KCl; ○), an internal acid load was applied by the NH_4^+ prepulse technique and pH_i slowly recovered (see Bevensee et al. 1996). The rate of pH_i recovery was faster under high-K_o^+ conditions (139.5 mM KCl; △) compared with control, and the brief application of 100 μM Zn^{2+} slowed the rate of pH_i recovery under high K_o^+-conditions (▲) but had no effect at normal $[K^+]_o$ (●). Records were obtained from 4 different neurons that exhibited similar minimum pH_i values in response to the NH_4^+ prepulse (with the exception of the control response, NH_4^+ prepulses have been omitted for clarity).

sented in Fig. 5*D*. Taken together, the results are entirely consistent with the possibilities, raised in light of the steady-state pH_i data (see preceding text), that Na^+/H^+ exchange and a Na_o^+-independent, Zn^{2+}-sensitive mechanism contribute to acid extrusion after anoxia in acutely isolated CA1 neurons.

To support the possibility that the Zn^{2+}-sensitive component of the recovery of pH_i from acid loads imposed after anoxia might be activated by membrane depolarization (see preceding text), internal acid loads were applied during normoxia under Na_o^+-free conditions (i.e., Na^+/H^+ exchange blocked). As illustrated in Fig. 6, pH_i recovery in the absence of external Na^+ (see Bevensee et al. 1996) was more than twofold faster under depolarizing (139.5 mM K_o^+) than under control (3 mM K_o^+) conditions ($n = 9$ in each case). Furthermore, whereas 100 μM Zn^{2+} failed to affect pH_i recovery under control conditions, the rate of pH_i recovery under high-$[K^+]_o$ conditions was reduced from $4.1 \pm 0.9 \times 10^{-3}$ pH units/s prior to the application of Zn^{2+} to $0.5 \pm 0.3 \times 10^{-3}$ pH units/s in its presence ($n = 9$ in each case; $P < 0.05$; Fig. 6). In contrast, the effect of high-$[K^+]_o$ to increase rates of pH_i recovery from acid loads was not affected by the P-type H^+,K^+-ATPase inhibitors omeprazole (25–50 μM, $n = 7$) or SCH 28080 (500 μM, $n = 2$) or by the V-type H^+-ATPase inhibitor bafilomycin A_1 (2 μM, $n = 5$).

Effects of changes in pH_o and temperature on the pH_i response to anoxia

Anoxia and ischemia in vivo and in slice preparations in vitro lead to reductions in pH_o (e.g., Obrenovitch et al. 1990; Roberts and Chih 1997; Silver and Erecinska 1990, 1992). In addition, the activities of Na^+/H^+ exchangers and g_{H^+}s are reduced by falls in pH_o (DeCoursey and Cherny 2000; Green et al. 1988; Ritucci et al. 1998; Vaughan-Jones and Wu 1990; Wu and Vaughan-Jones 1997). Therefore we examined the effects of lowering pH_o on the increases in pH_i observed during and after anoxia. Reducing pH_o from 7.35 to 6.60 caused a 0.49 ± 0.03 pH unit fall in pH_i ($n = 19$) (also see Church et al. 1998). Once pH_i had stabilized at a new resting level, anoxia evoked an internal acidification followed by increases in pH_i during and after anoxia that were significantly smaller than those observed at pH_o 7.35 (Fig. 4, *A* and *B*). Next, intracellular acid loads were imposed prior to and following anoxia at pH_o 6.60 (Fig. 7*A*). Prior to anoxia, rates of pH_i recovery were decreased at pH_o 6.60, compared with rates of pH_i recovery observed at the same absolute values of pH_i under control (pH_o 7.35) conditions (Fig. 7*B*). When acid loads were imposed immediately after anoxia, rates of pH_i recovery increased, compared with rates of recovery established prior to anoxia also at pH_o 6.60 ($n = 9$; Fig. 7*A*). Plots of the pH_i dependence of the rates of pH_i recovery obtained at pH_o 6.60 (Fig. 7*B*) indicated that, at all absolute values of pH_i, rates of pH_i recovery after anoxia were reduced at pH_o 6.60 compared with rates established after anoxia at pH_o 7.35. Taken together, the results are consistent with contributions from Na^+/H^+ exchange and a g_{H^+} to the pH_i response to anoxia. The data also suggest that, even at pH_o

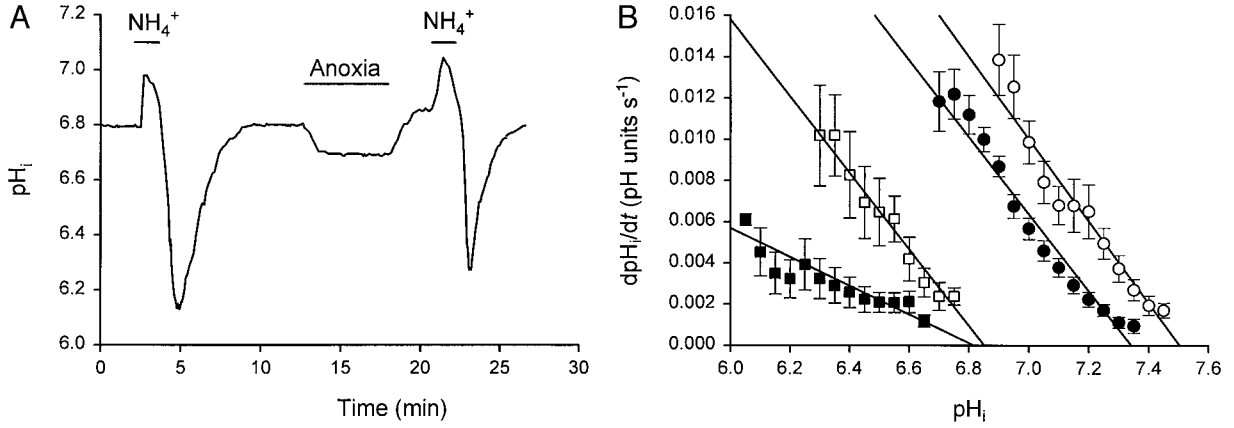

FIG. 7. Effects of reduced pH_o on rates of pH_i recovery from acid loads imposed in the immediate postanoxic period. *A*: an initial acid load was imposed at pH_o 6.60, and pH_i was allowed to recover. The neuron was then exposed to anoxia for 5 min and a 2nd acid load was applied after the return to normoxia. *B*: rates of pH_i recovery from acid loads imposed prior to (●, ■) and immediately after (○, □) anoxia at pH_o 6.60 (■, □) and pH_o 7.35 (●, ○). —, the weighted nonlinear regression fits to the data points indicated for each experimental condition. Data collected at pH_o 6.60 were obtained from 9 experiments of the type shown in *A*; where missing, standard error bars lie within the symbol areas. Absolute rates of pH_i recovery were significantly faster following anoxia at both pH_o 7.35 and pH_o 6.60 ($P < 0.05$ at each absolute value of pH_i).

6.60, the mechanism(s) can continue to participate in acid extrusion after anoxia. Qualitatively opposite results were obtained under pH_o 7.60 conditions (data not shown).

A reduction in temperature also inhibits Na^+/H^+ exchange and g_{H^+}s (see Baxter and Church 1996; Eder and DeCoursey 2001). Consistent with the possibilities that Na^+/H^+ exchange and a g_{H^+} contribute to acid extrusion during and/or following anoxia, the increases in pH_i observed during and after anoxia at pH_o 7.35 were significantly reduced at room temperature, compared with corresponding changes observed at 37°C (Figs. 4 and 8*A*). Similar effects were observed when anoxia was imposed under HCO_3^-/CO_2-buffered conditions (pH_o 7.35) at 20–22°C ($n = 13$; data not shown). In addition, rates of pH_i recovery from acid loads imposed after anoxia were reduced at room temperature, compared with rates obtained at 37°C, although rates of pH_i recovery after anoxia continued to be faster than rates established prior to anoxia when both acid loads were imposed at room temperature (Fig. 8, *B* and *C*).

Role of cAMP/cAMP-dependent protein kinase in the pH_i response to anoxia

Of particular interest is the finding, detailed in the preceding text, that activation of Na^+/H^+ exchange occurred in the immediate postanoxic period even though external pH was held at a constant value (i.e., pH 7.35). Thus a return to normal pH_o values from an external acidification, as would occur after anoxia in vivo, is not an absolute requirement for the postanoxic activation of Na^+/H^+ exchange in isolated hippocampal neurons. One mechanism that could contribute to the activation of Na^+/H^+ exchange after anoxia is an anoxia-induced change in the activity of intracellular second-messenger system(s) which, in turn, act to regulate Na^+/H^+ exchange. In hippocampal neurons, the intracellular concentration of adenosine-3′,5′-cyclic monophosphate ($[cAMP]_i$) rises rapidly in the immediate postanoxic period (e.g., Domanska-Janik 1996; Small et al. 1996; Whittingham et al. 1984), and we have shown previously that increases in $[cAMP]_i$, acting via cAMP-dependent protein kinase (PKA), activate Na^+/H^+ exchange in acutely isolated rat CA1 neurons under normoxic conditions (Smith et al. 1998). Therefore we investigated the effect of modulating the activity of the cAMP/PKA system on the rise in steady-state pH_i observed after anoxia.

As previously reported (Smith et al. 1998), the selective PKA inhibitor *R*p-cAMPS (50 μM) failed to affect steady-state pH_i under HEPES-buffered, normoxic conditions ($n = 12$). However, as illustrated in Fig. 9, *A* and *B*, the magnitude of the internal alkalinization observed after anoxia was significantly reduced in the presence of *R*p-cAMPS. In contrast, 50 μM *R*p-cAMPS failed to significantly affect the residual increase in pH_i observed after anoxia under reduced-Na_o^+ ($NMDG^+$-substituted) conditions (see Fig. 9*B*). Similar results were obtained following pretreatment with the adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine (100 μM), which reduced the magnitude of the increase in pH_i after anoxia to 0.11 ± 0.03 pH units ($n = 9$; $P < 0.05$ for the difference to the increase in pH_i observed under control conditions). Finally, the effect of β adrenergic agonists to increase $[cAMP]_i$ is potentiated after ischemia (Domanska-Janik 1996; Lin et al. 1983). As shown in Fig. 9, *A* and *B*, stimulation of the cAMP/PKA pathway with the β-adrenoceptor agonist isoproterenol (10 μM) significantly increased the magnitude of the postanoxic alkalinization, an effect that was attenuated by *R*p-cAMPS or the full β-adrenoceptor antagonist, propranolol. Furthermore, the isoproterenol-evoked increase in the magnitude of the postanoxic alkalinization was significantly attenuated under reduced-Na_o^+ ($NMDG^+$-substituted) conditions and was restored to control values when Li^+ was employed as the Na^+-substitute (Fig. 9*B*). Taken together, the results are consistent with the possibility that anoxia-induced changes in the activity of the cAMP/PKA second-messenger system may contribute to the activation of Na^+/H^+ exchange in rat hippocampal CA1 neurons immediately after anoxia.

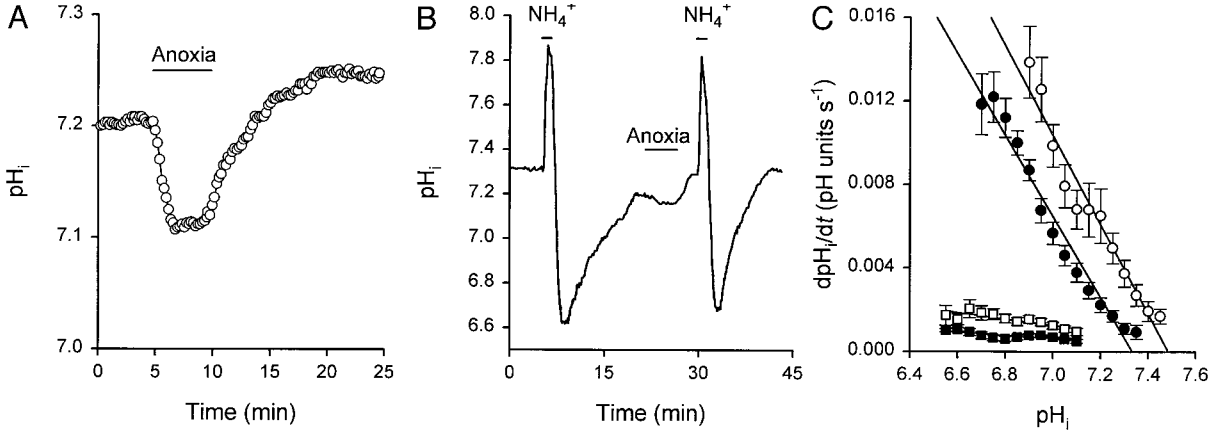

FIG. 8. pH_i responses to anoxia at room temperature. *A*: under HEPES-buffered conditions at 22°C, 5 min anoxia evoked a fall in pH_i, a small rise in pH_i in the continued absence of O_2, and a further small internal alkalinization on the return to normoxia. *B*: following an initial NH_4^+-induced intracellular acid load, pH_i was allowed to recover. A 2nd acid load was applied after 5 min anoxia. The rate of recovery of pH_i was increased in the immediate postanoxic period, compared with the rate of pH_i recovery observed prior to anoxia. *C*: rates of pH_i recovery from internal acid loads imposed prior to (■) and immediately after (□) anoxia at room temperature, at pH_i values shown on the abscissa. Also plotted are the rates of pH_i recovery from acid loads imposed prior to (●) and after (○) anoxia at 37°C (see Fig. 5*B*). —, the weighted nonlinear regression fits to the data points indicated for each experimental condition. Data collected at room temperature were obtained from 11 experiments of the type shown in *B*; where missing, SE bars lie within the symbol areas. Absolute rates of pH_i recovery in the postanoxic period were significantly slower at 20–22°C than at 37°C ($P < 0.05$ at each absolute value of pH_i). All data were obtained at pH_o 7.35.

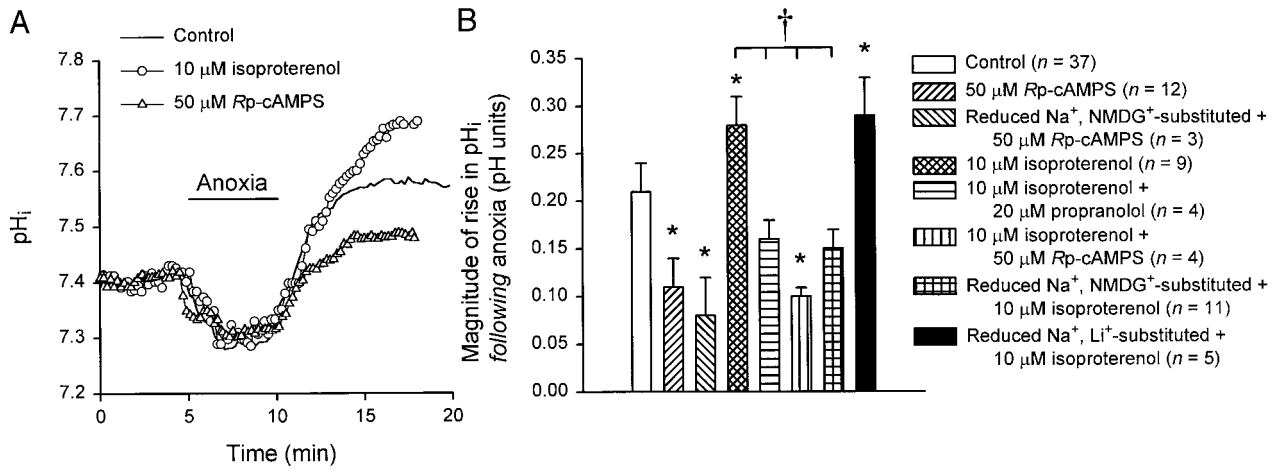

FIG. 9. Effects of modulating the activity of the cAMP/protein kinase A (PKA) pathway on the pH_i response to anoxia. *A*: the magnitude of the internal alkalinization observed following 5 min anoxia under control conditions (—) was increased in the presence of the β-adrenoceptor agonist isoproterenol (10 μM; ○) and reduced in the presence of the PKA inhibitor, *R*p-cAMPS (50 μM; △). The records shown were obtained at 37°C from 3 different neurons with similar resting pH_i values immediately prior to the induction of anoxia. Pharmacological treatments were applied for ≥10 min prior to the induction of anoxia and were maintained throughout the records shown. *B*: effects of the test conditions shown on the figure on the increases in pH_i observed after 5 min anoxia. All experiments were performed under nominally HCO_3^-/CO_2-free, HEPES-buffered conditions at 37°C, pH_o 7.35; error bars are SE. *, statistically significant difference ($P < 0.05$) compared with control (shown in the 1st column). †, a statistically significant difference ($P < 0.05$) compared with the value obtained in the presence of 10 μM isoproterenol (shown in the 4th column).

DISCUSSION

Steady-state pH_i changes evoked by anoxia

The typical steady-state pH_i response to anoxia in acutely isolated adult rat hippocampal CA1 neurons consisted of an initial fall in pH_i on the induction of anoxia, a subsequent rise in pH_i in the continued absence of O_2, and a further internal alkalinization on the return to normoxia. These pH_i changes were observed under constant external conditions and, as such, represent the intrinsic pH_i response of the neurons to anoxia. Although anoxia is known to elicit changes in neuronal pH_i both in vivo and in slice preparations in vitro (for reviews see Erecinska and Silver 1994; Hansen 1985; Lipton 1999; Siesjö et al. 1996), it is difficult to separate the contribution of various cell types, including glia, to the changes observed from volume-averaged measurements and additional confounds, such as concurrent changes in pH_o, $[K^+]_o$ and neurotransmitter release (each of which can affect pH_i), complicate the characterization of underlying mechanisms (see Erecinska and Silver 1994; Pirttilä and Kauppinen 1994). Indeed, although increases in neuronal pH_i during anoxia and pH_i "overshoots" immediately after anoxia have occasionally been observed in slices and in vivo (see Fujiwara et al. 1992; Mabe et al. 1983; Melzian et al. 1996; Pirttilä and Kauppinen 1992), the more usual response in these preparations comprises a fall in pH_i during anoxia and a gradual restoration of pH_i toward normal resting levels in the period following the return to normoxia (e.g., see Roberts and Chih 1997; Silver and Erecinska 1992). The internal alkalinization during anoxia and the pH_i overshoot on the return to normoxia observed in the present study were attenuated at pH_o 6.60 compared with pH_o 7.35, suggesting that the decreases in interstitial pH that occur during and following anoxia in slice preparations and in vivo may be an important determinant of these apparent discrepancies.

Although studies in hippocampal slices have suggested that there are developmental changes in the neuronal pH_i response to anoxia (see Roberts and Chih 1997), the typical pattern of pH_i changes observed in the present study was similar in most respects to that found in cultured postnatal rat hippocampal neurons (Diarra et al. 1999) and cultured fetal mouse neocortical neurons (Jørgensen et al. 1999). The major differences between our previous study in cultured postnatal neurons (Diarra et al. 1999) and the present work in acutely isolated adult CA1 neurons are the relatively persistent increases in $[Ca^{2+}]_i$ and pH_i that were observed even after 5 min anoxia and that, in cultured postnatal neurons, occur only after ≥10 min of O_2 deprivation. These differences may reflect, at least in part, the more marked and more persistent membrane depolarization that occurs in adult, compared with fetal or postnatal, hippocampal neurons on withdrawal of metabolic substrates (Bickler et al. 1993; Isagai et al. 1999; Nabetani et al. 1997; Tanaka et al. 1997, 1999). Although electrophysiological recordings will be required to substantiate or refute this possibility, it may also account for the fact that Zn^{2+} exerted a greater inhibitory effect on the increases in pH_i observed during and after anoxia in the present study in adult neurons than in our previous study (Diarra et al. 1999) in postnatal neurons.

In contrast to excitotoxin-evoked reductions in pH_i that, under normoxic conditions, are largely consequent on increases in $[Ca^{2+}]_i$ and the subsequent activation of a plasmalemmal Ca^{2+}-ATPase (e.g., Hartley and Dubinsky 1993; Irwin et al. 1994; Trapp et al. 1996; Wu et al. 1999), changes in $[Ca^{2+}]_i$ do not appear to be a major determinant of anoxia-evoked changes in pH_i in hippocampal CA1 neurons. Thus as previously reported in cultured postnatal rat hippocampal (Diarra et al. 1999) and fetal mouse neocortical (Jørgensen et al. 1999) neurons, despite the marked reduction in anoxia-evoked increases in $[Ca^{2+}]_i$ observed in the absence of Ca_o^{2+}, the pH_i response to anoxia was not significantly affected. Experiments in which HPTS was employed as the pH_i indicator did not

support the possibility that inhibition of the plasmalemmal Ca^{2+}-ATPase by BCECF might account for the increases in pH_i observed during and after anoxia in the presence of external Ca^{2+}. Rather, our findings are consistent with the possibility that Ca^{2+}-ATPase activity may be inhibited by metabolic insults (e.g., see Castilho et al. 1998; Chinopoulos et al. 2000; Kass and Lipton 1989; Pereira et al. 1996; Wu et al. 1999; Zaidi and Michaelis 1999) and therefore contribute little to background acid loading despite the marked increase in $[Ca^{2+}]_i$ evoked by anoxia in adult CA1 neurons.

Potential contribution of Na^+/H^+ exchange to the increases in pH_i during and after anoxia

Examination of the role of Na^+/H^+ exchange in acid extrusion from rat hippocampal neurons is complicated by the lack of a pharmacological inhibitor. Nevertheless, the observation that the increase in pH_i during anoxia was not inhibited by marked reductions in $[Na^+]_o$ indicates that Na^+/H^+ exchange does not make a major contribution to this phase of the pH_i response. A similar finding has been made in rat central neurons in slice preparations (Pirttilä and Kauppinen 1992) and in primary culture (Diarra et al. 1999), although it appears contrary to a recent report in mouse hippocampal neurons (Yao et al. 2001). While the mechanistic basis for the observed lack of Na^+/H^+ exchange activity during anoxia remains unknown, measurements of pH_i (present study; also Diarra et al. 1999) and $[Na^+]_i$ (Diarra et al. 2001) in rat hippocampal neurons indicate that the quotient Na_o^+/Na_i^+ continues to be larger than H_0^+/H_i^+ during anoxia, such that the driving force acting on Na^+/H^+ exchange at this time will continue to favor forward-mode transport activity (i.e., net H^+ efflux) (see Kaila and Vaughan-Jones 1987; Vaughan-Jones and Wu 1990). This is in agreement with studies in cardiac myocytes (e.g., Park et al. 1999) and indicates that factor(s) other than changes in the transmembrane Na^+ and/or H^+ gradients must contribute to the lack of observable Na^+/H^+ exchange activity during anoxia under the present experimental conditions. One such factor might be intracellular ATP depletion, which not only occurs rapidly after the induction of anoxia in adult CA1 neurons (e.g., Kass and Lipton 1989; Whittingham et al. 1984) but also reduces Na^+/H^+ exchange activity (e.g., Green et al. 1988; Wakabayashi et al. 1997).

In contrast to the rise in pH_i during anoxia, the present results support the possibility that Na^+/H^+ exchange in hippocampal neurons is activated immediately after anoxia and contributes to the internal alkalinization observed at this time. Thus pH_i overshoots following anoxia were reduced either when $NMDG^+$ (but not Li^+) was employed as a Na_o^+ substitute or when pH_o or temperature were lowered; in contrast to $NMDG^+$, Li^+ can act as a substrate for Na^+/H^+ exchange, and it is established that Na^+/H^+ exchange activity can be reduced by falls in pH_o or temperature (Baxter and Church 1996; Green et al. 1988; Ritucci et al. 1998; Vaughan-Jones and Wu 1990; Wu and Vaughan-Jones 1997). Consistent with the steady-state pH_i results, rates of pH_i recovery from acid loads increased in the period immediately following the return to normoxia, and these increases were attenuated either when $NMDG^+$ was employed as an external Na^+ substitute or when pH_o or temperature were reduced. The increase in the Na_o^+-dependent component of pH_i recovery from acid loads observed after anoxia (Fig. 5*B*, *inset*) is also consistent with activation of Na^+/H^+ exchange in the immediate postanoxic period. Although is remains unknown whether cellular ATP and/or $[Na^+]_i$ recover sufficiently quickly in acutely isolated adult CA1 neurons to allow the rapid activation of forward-mode Na^+/H^+ exchange after anoxia, the present results are consistent with previous reports not only in cultured postnatal rat hippocampal neurons (where $[Na^+]_i$ and $[ATP]_i$ do recover quickly following the return to normoxia) (Diarra et al. 1999, 2001; Lipton 1999) but also in other isolated neuronal preparations in which an involvement of Na^+/H^+ exchange in the restoration of pH_i following anoxia has been demonstrated with selective pharmacological inhibitors (Jørgensen et al. 1999; Vornov et al. 1996; Yao et al. 2001). The results of the present study also support previous suggestions that Na^+/H^+ exchange activity may contribute to the acidotic $[H^+]_o$ shift that occurs in vivo and in slice preparations during early reperfusion (see Obrenovitch et al. 1990; Ohno et al. 1989).

In cardiac myocytes, it has been proposed that Na^+/H^+ exchange activity is inhibited during anoxia/ischemia by the extracellular acidosis that occurs at this time and that the rapid normalization of pH_o immediately on reperfusion relieves this inhibition and thereby contributes to the activation of Na^+/H^+ exchange (Lazdunski et al. 1985). In the present study, however, stimulation of Na^+/H^+ exchange activity occurred after anoxia even when pH_o was maintained at a constant value throughout the anoxic and postanoxic periods and even when pH_i immediately prior to the return to normoxia may not have been markedly decreased from the resting level observed prior to anoxia. Thus neither a decrease in pH_i during anoxia nor a return to normal pH_o values in the immediate postanoxic period are absolute requirements for the rapid postanoxic activation of Na^+/H^+ exchange in adult rat CA1 neurons. In cardiac myocytes, protein kinase C activation also contributes to the rapid activation of Na^+/H^+ exchange activity during reperfusion (Ikeda et al. 1988; Yasutake and Avkiran 1995), and the present study points to an analogous contribution from anoxia-evoked changes in the activity of the cAMP/PKA second-messenger system in mediating the activation of Na^+/H^+ exchange in hippocampal neurons in the immediate postanoxic period. Thus not only do rapid increases in $[cAMP]_i$ occur in hippocampal neurons immediately on reperfusion but also these increases can be maintained for $\leq$60 min (e.g., Blomqvist et al. 1985; Domanska-Janik 1996; Kobayashi et al. 1977; Small et al. 1996; Whittingham et al. 1984). In addition, we have shown previously that, under normoxic conditions, β-adrenoceptor activation, acting via cAMP and PKA, evokes a sustained increase in Na^+/H^+ exchange activity in acutely isolated adult CA1 neurons by producing an alkaline shift in the pH_i dependence of the transport mechanism (Smith et al. 1998). Consistent with these previous findings, in the present study there was an alkaline shift in the pH_i dependence of Na_o^+-dependent acid extrusion following anoxia. Furthermore, inhibition of adenylate cyclase or PKA reduced the magnitude of the Na_o^+-dependent component of the pH_i overshoot after anoxia (also see Yao et al. 2001), whereas β-adrenoceptor activation augmented the postanoxic rise in pH_i (an effect that was blocked by propranolol, *R*p-cAMPS and under conditions where $NMDG^+$, but not Li^+, was employed as a Na_o^+ substitute). The effects of modulating the activity of the cAMP/PKA system on the increase in pH_i observed immediately after

anoxia are not only consistent with a contribution from Na^+/H^+ exchange to the postanoxic increase in pH_i but also provide an example of the potential importance of the regulation of neuronal Na^+/H^+ exchange by second messenger systems.

Potential contribution of a g_{H^+} to the increases in pH_i during and after anoxia

In contrast to the effects of inhibiting Na^+/H^+ exchange, micromolar concentrations of Zn^{2+} attenuated the increases in pH_i observed both during *and* following anoxia. Although concurrent imaging and electrophysiological recordings will be required to substantiate or refute the possibility that the effects of Zn^{2+} may be due to the inhibition of H^+ efflux through a H^+-conductive pathway activated as a consequence of membrane depolarization, there is precedence for external Na^+- and HCO_3^--independent H^+ extrusion from hippocampal neurons under anoxic conditions (Diarra et al. 1999; Ohno et al. 1989; Pirttilä and Kauppinen 1994), and the possible contribution of a g_{H^+} to the rises in pH_i observed during and immediately after anoxia in the present experiments is suggested by a number of lines of evidence.

First, inhibition by Zn^{2+} is an identifying characteristic of g_{H^+}s (for reviews, see DeCoursey and Cherny 1994a, 2000; Eder and DeCoursey 2001), and although Zn^{2+} failed to affect steady-state pH_i under normoxic conditions, it attenuated the increases in pH_i that occurred during and after anoxia under both Na_o^+-containing and reduced-Na_o^+ conditions. It is important to note that, although Zn^{2+} ions modulate the activities of a variety of membrane-bound ion channels (for reviews, see Harrison and Gibbons 1994; Smart et al. 1994), under the constant perfusion conditions employed in the present experiments, the pH_i changes evoked by anoxia are unaffected by *N*-methyl-D-aspartate (NMDA), AMPA, or $GABA_A$ receptor antagonists, or inhibitors of high-voltage-activated Ca^{2+} channels (A. Diarra, C. Sheldon, and J. Church, unpublished observations). The effect of Zn^{2+} to markedly reduce the magnitudes of the alkalinizations observed during and after anoxia under $NMDG^+$-substituted conditions (Fig. 4) may reflect the established coupling between Na^+/H^+ exchange activity and g_{H^+}s (DeCoursey and Cherny 1994b; Demaurex et al. 1995). Thus inhibition of Na^+/H^+ exchange activity under $NMDG^+$-substituted conditions will potentially act to increase the relative contribution of the Zn^{2+}-sensitive g_{H^+} to acid extrusion under the depolarizing conditions that occur during and immediately after anoxia. *Second*, consistent with the steady-state pH_i results, Zn^{2+} decreased rates of pH_i recovery from acid loads imposed after anoxia, both under control conditions and under conditions where Na^+/H^+ exchange was inhibited by the substitution of $NMDG^+$ for external Na^+. *Third*, the fact that the Zn^{2+}-sensitive increases in pH_i observed during and after anoxia were inhibited by a reduction in pH_o is consistent with the established sensitivity of voltage-gated H^+-conducting pathways to the transplasmalemmal pH gradient (DeCoursey and Cherny 1994a, 2000). *Fourth*, the Zn^{2+}-inhibitable internal alkalinizations that occurred during and after anoxia were associated temporally with marked and persistent increases in $[Ca^{2+}]_i$ that, in turn, are known to occur in adult CA1 neurons in response to anoxic depolarization (Rader and Lanthorn 1989; Silver and Erecinska 1990; Tanaka et al. 1997). In this regard, we found not only that the recovery of pH_i from internal acid loads imposed during normoxia in the absence of external Na^+ was faster under depolarizing (139.5 mM K_o^+) than under control (3 mM K_o^+) conditions, but also that Zn^{2+} only slowed the rate of recovery of pH_i in the former case. In addition, we have previously observed that maneuvers that delay the onset of anoxic depolarization in cultured postnatal rat hippocampal neurons (e.g., the application of TTX) also delay the onset of the Zn^{2+}-inhibitable rise in pH_i during anoxia (Diarra et al. 1999). Arguing against the possibility that the Zn^{2+}-sensitive acid extrusion mechanism might be a H^+-conductive pathway is the fact that Zn^{2+}-sensitive increases in pH_i after anoxia could occur even when the proton gradient across the plasma membrane was not apparently outwardly directed (i.e., $pH_i > pH_o$). However, this observation is tempered by the facts that a marked membrane depolarization occurs during and following anoxia (Tanaka et al. 1997) and that the local $[H^+]$ in the vicinity of presumed H^+-conducting channels may greatly exceed that monitored in bulk cytoplasm. Indeed, as noted by DeCoursey and Cherny (1994b) "... spatial or temporal pH fluctuations may activate the g_{H^+} in situations not predictable from time-averaged, bulk pH measurements, for example, by fluorescent dyes."

Summary and functional implications

In summary, acutely isolated adult rat hippocampal CA1 neurons typically respond to anoxia with a triphasic pattern of pH_i changes. The increase in pH_i during anoxia is mediated, at least in part, by a Zn^{2+}-sensitive alkalinizing mechanism, possibly a g_{H^+} activated as a consequence of membrane depolarization, although this does not preclude contributions from other mechanism(s), such as a decreased rate of internal acid loading following the onset of anoxic depolarization (e.g., see Erecinska et al. 1991; Sánchez-Armass et al. 1994). The fact that voltage-gated H^+ channels are activated by depolarization, are selective for H^+ ions, and do not incur an energy cost to the cell (DeCoursey and Cherny 1994a, 2000), would make them very suited to the rapid alleviation of the potentially detrimental decreases in pH_i imposed by anoxia and ischemia in mammalian central neurons (see Siesjö et al. 1996). This may particularly be the case during anoxia or ischemia in vivo, where low pH_o conditions will limit the contribution of forward Na^+/H^+ exchange to acid extrusion and, depending on the magnitude of the rise in $[Na^+]_i$, may even favor reverse-mode (i.e., H^+-loading) Na^+/H^+ exchange activity. It is also noteworthy that g_{H^+}s can couple to Na^+/H^+ exchange (see DeCoursey and Cherny 1994b; Demaurex et al. 1995), such that the activation of a g_{H^+} during anoxia would act as an "acid-relief valve" to limit the potentially detrimental activation of forward Na^+/H^+ exchange that may occur in the immediate postanoxic period (Vornov et al. 1996). This raises the interesting possibility that inhibition of g_{H^+}s may contribute to the neurotoxic effects of high micromolar concentrations of Zn^{2+} (Lipton 1999; Weiss et al. 2000) by promoting a marked activation of Na^+/H^+ exchange on reoxygenation.

In contrast to the rise in pH_i during anoxia, both a presumed g_{H^+} and Na^+/H^+ exchange contribute to the increase in pH_i observed following the return to normoxia. Activation of Na^+/H^+ exchange, possibly mediated by an anoxia-induced activation of the cAMP/PKA second-messenger pathway, may

increase the internal Na^+ load in the period immediately after anoxia and thereby, for example, worsen cellular energy state (Chinopoulos et al. 2000; Fried et al. 1995), potentiate NMDA receptor-mediated responses (Yu and Salter 1998), promote Ca^{2+} efflux from mitochondria (Zhang and Lipton 1999), and/or promote the reversal of plasmalemmal Na^+/Ca^{2+} exchange and $[Ca^{2+}]_i$ overload (Kiedrowski et al. 1994). In these and, possibly, other ways, Na^+/H^+ exchange may also contribute to the neurotoxic effects associated with activation of the cAMP/PKA pathway in the context of cerebral ischemia (Shibata et al. 1992; Small et al. 1996). There is a growing body of evidence that pharmacological inhibition of Na^+/H^+ exchange in the immediate postanoxic period effectively protects against anoxia- and ischemia-induced neuronal injury (e.g., Kuribayashi et al. 1999; Phillis et al. 1999; Vornov et al. 1996). Whether a similar benefit might be conferred in mature rat hippocampal CA1 pyramidal neurons awaits the identification of pharmacological inhibitors of Na^+/H^+ exchange in this highly vulnerable cell type.

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