Characterization of acid extrusion mechanisms in cultured fetal rat hippocampal neurones

Keith A. Baxter and John Church*

Department of Anatomy, University of British Columbia, 2177 Wesbrook Mall, Vancouver, British Columbia, Canada V6T 1Z3

1. We investigated the mechanisms regulating acid extrusion in cultured fetal rat hippocampal neurones loaded with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein.

2. In the absence of HCO_3^-, removal of external Na^+ by substitution with *N*-methyl-D-glucamine caused a sustained intracellular acidification that was not observed when Na^+ was replaced by Li^+, but neither steady-state intracellular pH (pH_i) nor the rate of pH_i recovery from an imposed acid load were influenced by amiloride analogues or HOE 694, inhibitors of Na^+–H^+ exchange in other cell types. In the presence of HCO_3^-, removal of external Na^+ or Cl^- evoked an intracellular acidification and a 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid-sensitive (DIDS-sensitive) intracellular alkalinization, respectively. Applied alone, however, DIDS elicited a fall in steady-state pH_i at room temperature but not at 37 °C. The DIDS-evoked fall in steady-state pH_i and the 0 Cl^--evoked intracellular alkalinization observed in the presence of HCO_3^- at room temperature were dependent on external Na^+.

3. At room temperature (18–22 °C), but not at 37 °C, the transition from HCO_3^--free to HCO_3^--containing medium at a constant pH_o produced a net alkalinization that was dependent on external Na^+ and was inhibited by DIDS or the depletion of internal Cl^-.

4. Recovery of pH_i from an acid load imposed in the absence of HCO_3^- was dependent on external Na^+. Addition of HCO_3^- to the perfusion medium increased the rate of pH_i recovery from an acid load at room temperature but not at 37 °C. In the presence of HCO_3^-, DIDS slowed the rate of recovery of pH_i from an acid load at both room temperature and at 37 °C.

5. Recovery of pH_i following an imposed intracellular acidification to pH < 6·5 could occur in the absence of external Na^+, providing that HCO_3^- was present in the perfusate. This slow, Na^+-independent recovery of pH_i from very low levels of intracellular pH was sensitive to DIDS.

6. The results indicate that acid extrusion in cultured fetal rat hippocampal neurones involves primarily two Na^+-dependent mechanisms, one HCO_3^- dependent (a HCO_3^-–Cl^- exchanger) and the other HCO_3^- independent (possibly a Na^+–H^+ exchanger). Although both mechanisms participate in the maintenance of steady-state pH_i at room temperature, only the HCO_3^--independent mechanism does so at 37 °C.

The mechanisms regulating intracellular pH (pH_i) have been extensively documented in invertebrate neuronal and vertebrate non-neuronal cells (Roos & Boron, 1981; Thomas, 1984; Frelin, Vigne, Ladoux & Lazdunski, 1988) and, more recently, similar studies in mammalian and other vertebrate (e.g. Chesler, 1986) neurones have emerged. In cultured rat sympathetic neurones (Tolkovsky & Richards, 1987), rat brain synaptosomes (Jean, Frelin, Vigne, Barbry & Lazdunski, 1985; Nachshen & Drapeau, 1988; Sánchez-Armass, Martínez-Zaguilán, Martínez & Gillies, 1994), cultured rat cerebellar Purkinje cells (Gaillard & Dupont, 1990) and cultured rat neocortical neurones (Ou-yang, Mellergård & Siesjö, 1993), a Na^+–H^+ exchanger, either wholly or partially sensitive to amiloride and its analogues, appears to be the dominant acid extrusion mechanism. In contrast, in CA1 hippocampal pyramidal neurones acutely dissociated from 4- to 14-day-old rats, Na^+-dependent HCO_3^-–Cl^- exchange dominates acid extrusion and the

* To whom correspondence should be addressed.

maintenance of resting pH_i, with amiloride-insensitive Na^+–H^+ exchange making a minor contribution (Schwiening & Boron, 1994). Although a Na^+- and HCO_3^--dependent mechanism has been reported to participate in the recovery of pH_i from acid loading in cultured fetal rat hippocampal neurones, the dependence of this mechanism on Cl^- is unknown, and resting pH_i in these cells appears to be determined largely by an amiloride-insensitive Na^+–H^+ exchanger (Raley-Susman, Cragoe, Sapolsky & Kopito, 1991). Furthermore, anion exchange activity (assayed by observing the intracellular alkalinization consequent upon the acute removal of external Cl^-) was reported by Raley-Susman, Sapolsky & Kopito (1993) in acutely dissociated adult, but not fetal, rat hippocampal neurones, despite the fact that mRNA for the anion exchanger AE3 (which catalyses the reversible exchange of Cl^- for HCO_3^- in a Na^+-independent manner; see Kopito, 1990) is expressed in both cell types (Raley-Susman *et al.* 1993).

In the present study we have characterized the mechanisms participating in both acid extrusion and the maintenance of steady-state pH_i in cultured fetal rat hippocampal neurones, paying particular regard, in light of the studies outlined above, to the role of HCO_3^--dependent processes. Experiments were performed at both 37 °C and room temperature, in the knowledge that temperature can affect the activities of pH_i-regulating mechanisms (e.g. Aickin & Thomas, 1977; Roos & Boron, 1981).

METHODS

Cell preparation

Primary cultures of fetal rat hippocampal neurones were prepared according to Banker & Cowan (1977) with minor modifications (Brewer & Cotman, 1989). Eighteen-day pregnant Wistar rats were anaesthetized with pentobarbitone (50 mg kg^{-1} I.P.) and, following removal of the fetuses, were killed with an intracardiac injection of pentobarbitone. Fetuses were decapitated, hippocampi were removed and, following enzymatic and mechanical dissociation, cells were plated onto glass coverslips coated with poly-D-lysine and laminin at a density of 1×10^5 cells cm^{-2}. Cultures were treated with 5-fluorodeoxyuridine to arrest glial cell proliferation and maintained in a 5% CO_2 atmosphere at 35 °C in serum-free, N2-supplemented Dulbecco's modified Eagle's medium containing 22 mM $NaHCO_3$ (Life Technologies, Burlington, ON, Canada). Neurones were used 6–14 days after plating.

Solutions and chemicals

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 $MgSO_4$, 10 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 26 mM (room temperature) or 20 mM (at 37 °C) $NaHCO_3$, by equimolar substitution for NaCl, together with the constituents listed above. All HCO_3^-/CO_2-buffered media were equilibrated with 5% CO_2–95% air; final pH values were 7·34 ± 0·01 at room temperature (mean ± S.E.M.; $n = 22$) and 7·36 ± 0·01 at 37 °C ($n = 19$). When external Na^+ was omitted, *N*-methyl-D-glucamine ($NMDG^+$) and choline were employed as substitutes in Hepes-buffered and HCO_3^-/CO_2-buffered media, respectively. When external Cl^- was omitted, the corresponding gluconate salts were substituted. The HCO_3^-/CO_2-buffered medium lacking both Na^+ and Cl^- contained the same elements as Cl^--free medium, except for the equimolar replacements of $NaHCO_3$ with choline bicarbonate, sodium gluconate with choline base and gluconic acid, and the omission of NaH_2PO_4. Ultrapure water (Milli-Q UF Plus, Millipore Ltd, Mississauga, ON, Canada) was used for all experimental media. The pH of each solution was re-checked at the appropriate temperature following every experiment.

5-(*N*-ethyl, *N*-isopropyl)-Amiloride (EIPA; Sigma) and 5-(*N*-methyl, *N*-guanidinocarbonylmethyl)-amiloride (MGCMA; supplied by Dr V. Palatý, Department of Anatomy, University of British Columbia) were prepared as 50 mM stock solutions in dimethylsulphoxide (DMSO). 4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; Sigma) was prepared as a 100 mM stock solution in DMSO and (3-methylsulphonyl-4-piperidinobenzoyl) guanidine methanesulphonate (HOE 694; a gift from Hoechst A.G., Frankfurt, Germany) was prepared as a 10 mM stock solution in distilled water. The final concentration of DMSO in the experimental solutions never exceeded 0·3% which, in control experiments, had no effect on steady-state pH_i or on the rate of pH_i recovery from an imposed intracellular acidification (data not shown). Perfusion lines were replaced following each experiment.

pH_i measurement

2′,7′-Bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Rink, Tsien & Pozzan, 1982) was obtained from Molecular Probes Inc. (Eugene, OR, USA). The loading medium contained the same elements as the standard HCO_3^-/CO_2-free, Hepes-buffered solution with the isosmotic addition of 3 mM $NaHCO_3$ in place of NaCl. Coverslips plated with neurones were placed in loading medium containing 2 μM BCECF-AM for 30 min at room temperature and then mounted in a temperature-controlled perfusion chamber so as to form the base of the chamber. Neurones were then superfused at a rate of 2·4 ml min^{-1} for 15 min with the initial experimental solution at the appropriate temperature prior to the start of an experiment. During perfusion with HCO_3^-/CO_2-buffered media, the atmosphere in the recording chamber consisted of 5% CO_2–95% air. Experiments were performed at room temperature (18–22 °C) and at 37 °C.

pH_i was measured using the dual-excitation fluorescence ratio method, employing an Attofluor Digital Fluorescence Microscopy System (Atto Instruments Inc., Rockville, MD, USA; Carl Zeiss Canada Ltd, Don Mills, ON, Canada). Using excitation wavelengths of 460 and 488 nm, fluorescence emissions (at 520 nm) were obtained from multiple neurone somata simultaneously. Analysis was restricted to those neurones able to retain the fluorescent indicator (as judged by raw emission intensity values) throughout the entire course of the experiment (see Schwiening & Boron, 1994). Raw intensity data at each excitation wavelength were corrected for background fluorescence prior to calculation of the ratio. The ratios of background-corrected emission intensities (I_{488}/I_{460}) were transformed into pH_i values using the equation

$$\mathrm{pH} = \log\left(\frac{R_\mathrm{n} - R_{\mathrm{n(min)}}}{R_{\mathrm{n(max)}} - R_\mathrm{n}}\right) + \mathrm{p}K_\mathrm{a}, \quad (1)$$

where R_n is the background-subtracted ratio normalized to pH 7·0, $R_{n(min)}$ and $R_{n(max)}$ are the minimum and maximum obtainable values for the normalized ratio and pK_a is the –log of the dissociation constant of the fluorophore (see Boyarsky, Ganz,

Sterzel & Boron, 1988*a*). The parameters fitting eqn (1) were derived from *in situ* calibration experiments in which neurones were exposed to a 10 μM nigericin-containing high-K^+ solution at a variety of pH values (Fig. 1; see Boyarsky *et al.* 1988*a*). The high-K^+ solution contained the same constituents as the standard Hepes-buffered medium except for the isosmotic substitution of potassium gluconate for NaCl and KCl; pH was adjusted with 10 M KOH. For the ten calibration experiments used in analysing all experiments, the values of pK_a, $R_{n(min)}$ and $R_{n(max)}$ were $7{\cdot}00 \pm 0{\cdot}02$, $0{\cdot}51 \pm 0{\cdot}01$ and $1{\cdot}51 \pm 0{\cdot}02$, respectively (means $\pm$ S.E.M.). These values were not dependent on the temperature at which the calibration was conducted (data not shown).

At the end of every experiment, neurones were exposed to a high-K^+ solution (pH 7·0) containing 10 μM nigericin (see Fig. 3*A* and *D*). The resulting background-corrected ratio at pH_i 7·00 was used as a normalization factor for that particular experiment. As outlined by Boyarsky *et al.* (1988*a*), the advantage of this normalization step is that it allows a one-point calibration for each neurone studied. After dividing experimentally derived ratios by the determined normalization factor, each R_n was converted into a pH_i value using eqn (1) and the appropriate fitted calibration parameters employing Visual Basic macros running in Excel v. 5.0 (Microsoft Corp.). For analysis of the rate of pH_i recovery from an imposed intracellular acid load, the recovery portion of the experiment was fitted to a single exponential function (SigmaPlot v. 1.02, Jandel Scientific, San Rafael, CA, USA) and the first derivative of this function was used to determine the rate of pH_i change as a function of time (dpH_i/dt). Instantaneous recovery rates were determined at 10 s after the peak acidification (the 'initial rate') and at t_{50}, defined as the time at which pH_i recovered to 50% of the difference between the steady-state pH_i value immediately prior to the induced intracellular acid load and the minimum pH_i observed during the acid load.

Statistical comparisons were carried out using Student's two-tailed t test with a 95% confidence limit. In all cases unpaired t values were calculated with supplemental paired data added when appropriate. Errors are expressed as S.E.M., with the accompanying n value referring to the number of cell populations (i.e. number of coverslips) analysed. The number of neurones from which data were obtained in all experiments was 2492.

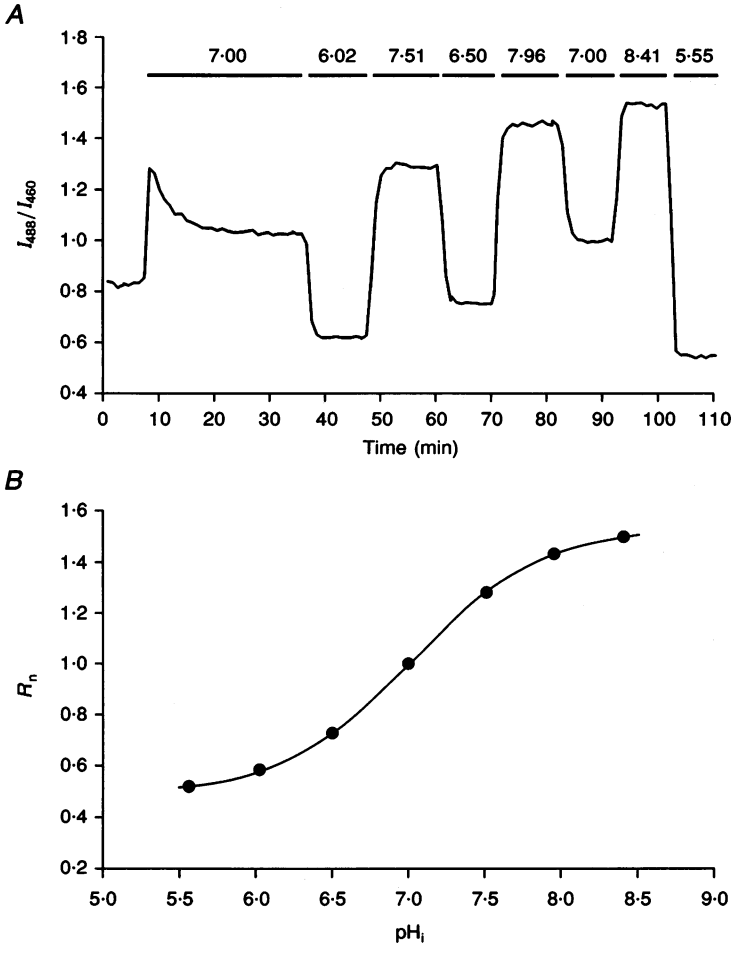

Figure 1. Sample calibration plot for BCECF

A, a calibration experiment in which 29 neurones were exposed to 10 μM nigericin-containing high K^+ solutions at the pH values shown above the trace. The resulting background-subtracted ratios (I_{488}/I_{460}) were normalized to 1·00 at pH_i 7·00. *B*, plot of pH_i *versus* the resulting normalized ratio (R_n). Error bars are contained within each datum point ($n = 3$). The curve is the result of a non-linear least squares regression fit to eqn (1). For this particular calibration, the values of $R_{n(max)}$, $R_{n(min)}$ and pK_a were 1·542, 0·491 and 7·027, respectively.

Periodically, brief fluctuations in the incident radiation from the photon source (a 100 W mercury arc burner) produced variations in emission intensities. In order to smooth the graphical representation of the pH_i *versus* time records, a moving average (period = 3) was applied to all traces shown, except those in Figs 1 and 8*A* (see Boyarsky *et al.* 1988*a*).

RESULTS

pH_i regulation at 37 °C

Steady-state pH_i. In nominally HCO_3^--free medium, steady-state pH_i was 7·23 ± 0·03 ($n = 29$; Fig. 2*A*). The removal of extracellular Na^+ (by substitution with $NMDG^+$) evoked a rapid and sustained 0·53 ± 0·05 pH unit fall in pH_i ($n = 5$; Fig. 3*A*), suggesting that a Na^+-dependent acid extrusion mechanism contributes to the maintenance of steady-state pH_i under HCO_3^--free conditions at 37 °C. The application of the amiloride analogues EIPA (50 μM) and MGCMA (100 μM), pharmacological inhibitors of Na^+–H^+ exchange in a wide variety of cell types (see Clark & Limbird, 1991), did not alter resting pH_i ($n = 3$ in each case). HOE 694, a novel inhibitor of Na^+–H^+ exchange in peripheral cell types (Counillon, Scholz, Lang & Pouysségur, 1993), was applied at 100 μM but also had no effect on steady-state pH_i ($n = 3$). However, as shown in Fig. 3*B*, the replacement of external Na^+ with Li^+, though marked by an initial brief acidification, did not result in a net change in pH_i (a 0·02 ± 0·02 pH unit increase measured at 10 min following the introduction of the Li^+-substituted medium, $n = 3$). As Li^+ is an alternative substrate for the Na^+–H^+ exchanger (see Aronson, 1985; Jean *et al.* 1985; Busch, Burckhardt & Siffert, 1995), the result suggests that the Na^+-dependent acid extrusion mechanism may be a variant of the Na^+–H^+ exchanger that is insensitive to known pharmacological inhibitors of the exchanger in other cell types. Under nominally HCO_3^--free conditions, the removal of external Cl^- did not alter resting pH_i ($n = 3$; Fig. 3*C*), suggesting that Cl^--dependent, HCO_3^--independent pH_i-regulating mechanisms (e.g. Cl^-–H^+ cotransport; Martínez-Zaguilán, Gillies & Sánchez-Armass, 1994) are not active at steady-state pH_i values in cultured fetal rat hippocampal neurones at 37 °C.

Steady-state pH_i in HCO_3^-/CO_2-buffered medium at 37 °C was 7·13 ± 0·01 ($n = 44$), which is lower than the value recorded under HCO_3^--free conditions at the same temperature (Fig. 2*A*). Removal of Na^+ from the perfusion medium in the presence of HCO_3^- caused a 0·65 ± 0·04 pH unit fall in pH_i ($n = 8$; Fig. 3*D*), similar to the change observed under HCO_3^--free conditions. Replacing external Cl^- with gluconate in the presence of HCO_3^- evoked a gradual pH_i increase of 0·19 ± 0·01 pH units ($n = 5$; Fig. 3*E*), which reversed upon the re-introduction of Cl^-. This 0 Cl^--induced alkalinization, which was not observed in the absence of HCO_3^- (see Fig. 3*C*), was abolished by 200 μM DIDS ($n = 2$; Fig. 3*E*), consistent with its mediation by a carrier coupling HCO_3^- and Cl^- fluxes. Applied alone, however, DIDS did not significantly alter steady-state pH_i at 37 °C (a 0·01 ± 0·01 pH unit fall in pH_i; $n = 3$; Fig. 3*F*).

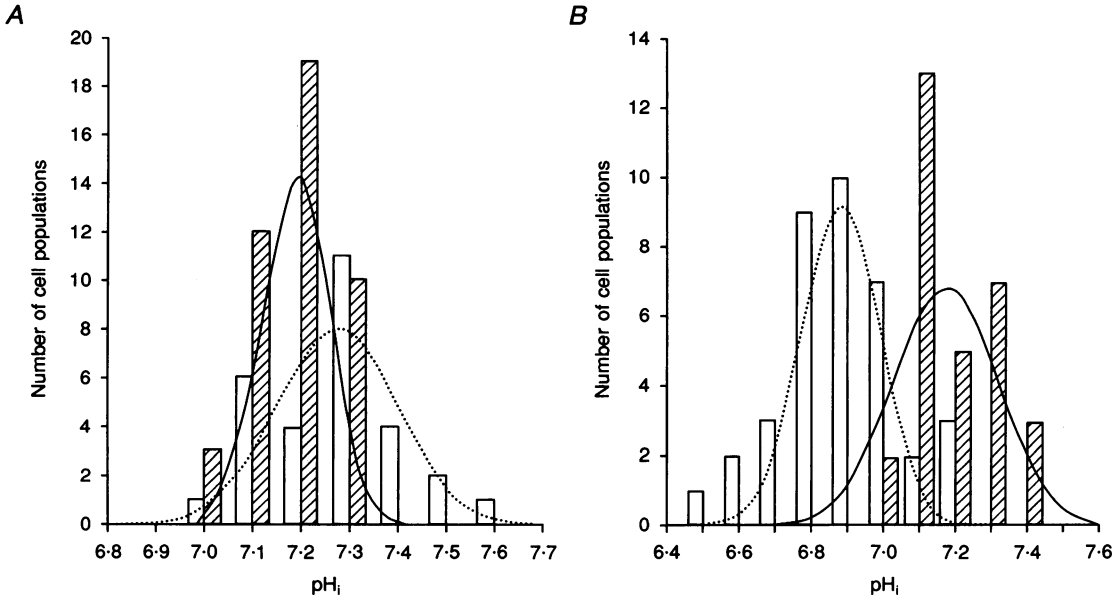

Figure 2. Distribution of steady-state pH_i

A, the distribution of steady-state pH_i at 37 °C ($pH_o = 7·36$). In the absence of HCO_3^- (□), the mean resting pH_i was 7·23 ± 0·03 ($n = 29$), whereas in the presence of HCO_3^- (▨), the mean resting pH_i was 7·13 ± 0·01 ($n = 44$). *B*, the distribution of steady-state pH_i at room temperature ($pH_o = 7·34$). The mean steady-state pH_i in the absence of HCO_3^- (□) was 6·84 ± 0·03 ($n = 37$), which increased to 7·14 ± 0·02 ($n = 30$) in the presence of HCO_3^- (▨). In both *A* and *B*, the interrupted and continuous curves represent the least squares Gaussian fits to the data in the absence and presence of HCO_3^-, respectively.

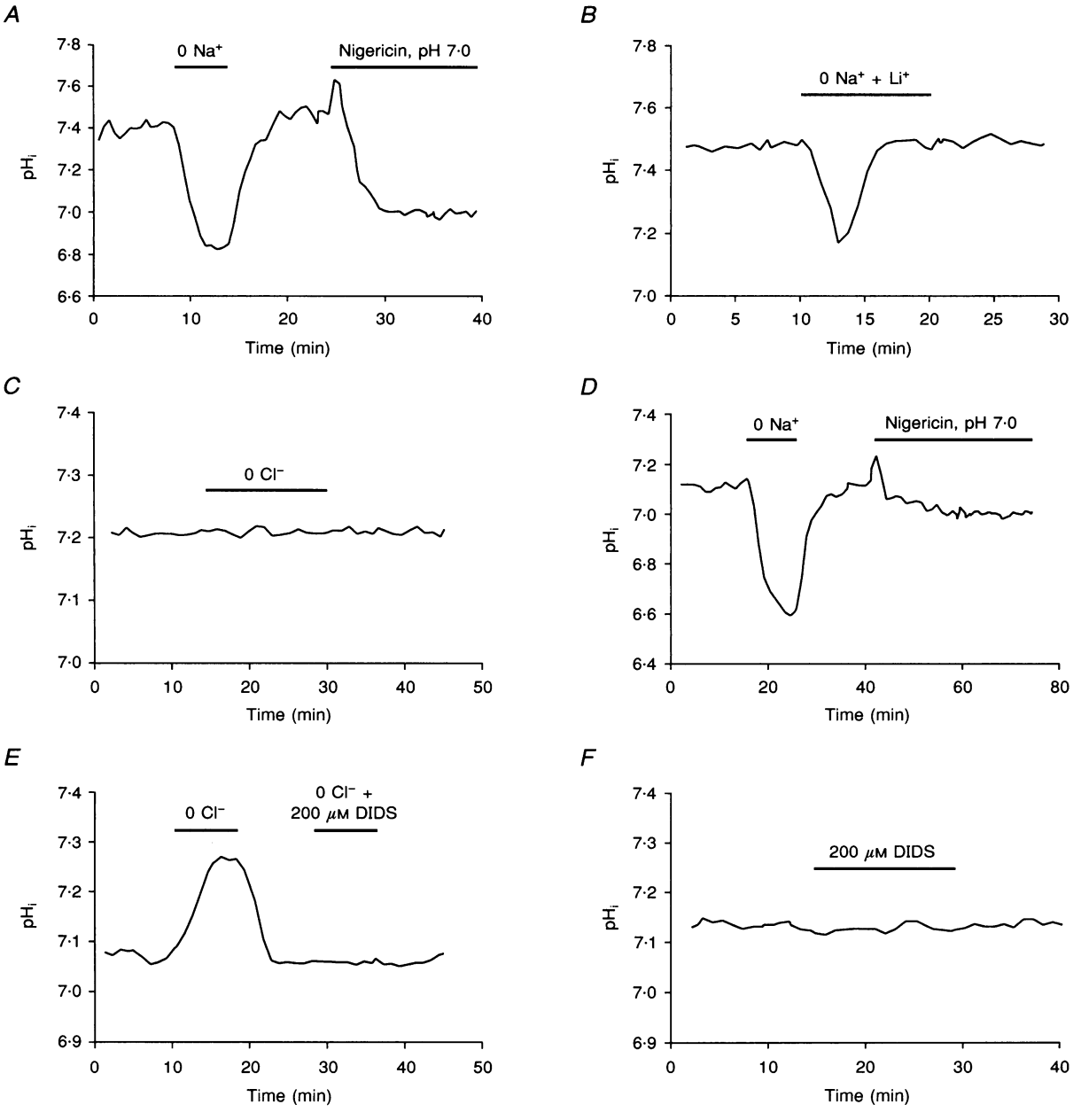

Figure 3. Steady-state pH_i in the absence or presence of HCO_3^- at 37 °C

Records shown in *A*, *B* and *C* were obtained under HCO_3^-/CO_2-free, Hepes-buffered conditions, whereas those in *D*, *E* and *F* were obtained in the presence of HCO_3^-/CO_2. *A*, the removal of external Na^+ (replacement with $NMDG^+$) for the period indicated by the bar above the trace caused an ~0·6 pH unit fall in pH_i. The reintroduction of Na^+ led to a rapid return to steady-state pH_i levels. Also shown is a one point calibration with 10 μM nigericin at pH 7·0. *B*, the replacement of external Na^+ with Li^+ evoked a transient acidification followed by a normalization of pH_i, despite the continued absence of Na^+. *C*, the acute removal of external Cl^- in the absence of HCO_3^- did not change steady-state pH_i; cf. the similar experiment performed in the presence of HCO_3^- at 37 °C (*E*). *D*, the removal of external Na^+ (replacement with choline) in the presence of HCO_3^- caused an intracellular acidification of ~0·5 pH units, similar to that observed in the absence of HCO_3^- at 37 °C (*A*). pH_i recovered to a normal resting level upon the reintroduction of Na^+. Also shown is a one point calibration with 10 μM nigericin at pH 7·0. *E*, the removal of external Cl^- in the presence of HCO_3^- evoked an ~0·2 pH unit alkalinization that was abolished by the co-application of 200 μM DIDS. *F*, 200 μM DIDS, applied in the presence of HCO_3^-, did not alter steady-state pH_i. This result differs from that observed at room temperature (see Fig. 5*A*). Records are means of data obtained simultaneously from 6 cells in *A*, 5 cells in *B*, 26 cells in *C* and *D*, 16 cells in *E* and 44 cells in *F*, with each experiment performed on a separate coverslip.

Table 1. The recovery of pH_i from NH_4^+-induced intracellular acidifications

Solution	Temp.	pH_i increase (pH units)	pH_i decrease (pH units)	Initial rate ($\times 10^{-3}$ pH units s^{-1})	t_{50} rate ($\times 10^{-3}$ pH units s^{-1})	n
HCO_3^- free	37 °C	0·27 ± 0·03	0·62 ± 0·03	5·56 ± 1·04	3·43 ± 0·66	12
+ 50 μM EIPA	37 °C			5·64 ± 0·45	3·51 ± 0·29	6
HCO_3^- containing	37 °C	0·11 ± 0·01	0·43 ± 0·03	5·01 ± 0·51	2·90 ± 0·29	19
+ 0 Na^+	37 °C			0·17 ± 0·03		3
HCO_3^- free	Room	0·52 ± 0·05	0·68 ± 0·04	1·31 ± 0·33	0·73 ± 0·16	6
HCO_3^- containing	Room	0·38 ± 0·01	0·50 ± 0·01	2·55 ± 0·17	1·38 ± 0·09	7
+ 200 μM DIDS	Room			0·95 ± 0·04	0·63 ± 0·03	3

Rates of pH_i recovery were determined at room temperature (18–22 °C) and at 37 °C under the conditions listed. Values are reported as the means of n coverslips (i.e. cell populations) ± S.E.M. The 'pH_i increase' is the increase in pH_i caused by a 3 min application of NH_4^+. The 'pH_i decrease' is the difference between the steady-state pH_i immediately prior to the application of NH_4^+ and the minimum pH_i attained during the induced intracellular acidification. Indicated rates are the instantaneous change in pH_i per unit time (dpH_i/dt) determined from the derivatives of exponential fit curves for the pH_i *versus* time records (see Methods).

pH_i recovery from an acid load. Intracellular acid transients at a constant pH_o were produced using the NH_4^+-prepulse technique. The results are summarized in Table 1. As an indication of the apparent intracellular buffering power, the increase in pH_i caused by a 3 min exposure to 20 mM NH_4Cl (by isosmotic substitution for NaCl) was also quantified by taking the difference between the steady-state pH_i immediately prior to the application of NH_4^+ and the pH_i immediately prior to its removal. In cells exposed to a nominally HCO_3^--free solution at 37 °C, a 3 min application of NH_4^+ evoked a 0·27 ± 0·03 pH unit rise ($n = 12$), whereas pH_i increased by 0·11 ± 0·01 pH units ($n = 19$) in the presence of HCO_3^-. These increases are smaller than the respective changes that were observed at room temperature (Table 1).

At 37 °C and in the absence of HCO_3^-, neurones recovered from an imposed intracellular acid load at an initial rate of $(5{\cdot}56 \pm 1{\cdot}04) \times 10^{-3}$ pH units s^{-1} ($n = 12$; Table 1). As shown in Fig. 4*A*, pH_i recovery was abolished when Na^+ was removed from the perfusate ($n = 3$). The application of 50 μM EIPA did not influence the rate of pH_i recovery from an imposed acidification (Fig. 4*B*; Table 1). Similarly, 100 μM MGCMA and 100 μM HOE 694 were without effect ($n = 3$ in each case; data not shown).

Rates of pH_i recovery from acid loads imposed in the presence of HCO_3^- at 37 °C were not significantly different ($P > 0{\cdot}6$) from those observed in the absence of HCO_3^- at the same temperature (Fig. 4*C*; Table 1; see also Fig. 8*A* and *B*). Because intracellular buffering power is greater in the presence than in the absence of HCO_3^- (see above), the nearly equivalent rates of pH_i recovery under both conditions indicate a greater acid extrusion rate in the presence than in the absence of HCO_3^- (see Boyarsky *et al.* 1988*a*). Consistent with the possibility that a HCO_3^--dependent process contributes to acid extrusion at 37 °C, the ability of the neurones to recover from an imposed acidification in HCO_3^--containing media was inhibited by 200 μM DIDS ($n = 5$; Fig. 4*D*). In three paired experiments of the type illustrated in Fig. 7*B* (but conducted at 37 °C), 200 μM DIDS reduced the rate of pH_i recovery from $(3{\cdot}62 \pm 0{\cdot}16) \times 10^{-3}$ pH units s^{-1} under control conditions to $(1{\cdot}12 \pm 0{\cdot}16) \times 10^{-3}$ pH units s^{-1}. In similar experiments conducted in the absence of HCO_3^-/CO_2, 200 μM DIDS failed to reduce the rate of pH_i recovery from an imposed acid load ($n = 3$; data not shown).

The recovery of pH_i from an imposed acid load at 37 °C was greatly diminished by the removal of external Na^+ (Fig. 4*E*; Table 1). However, as can be seen in Fig. 4*E*, slow pH_i recovery was observed even in the absence of external Na^+, providing that HCO_3^- was present in the perfusate. Because this recovery was not observed under Na^+-free, HCO_3^--free conditions (see Fig. 4*A*), it might reflect the activity of a Na^+-independent HCO_3^-–Cl^- exchanger operating in reverse mode during extreme intracellular acidosis (see below). The possible presence of a Na^+-independent HCO_3^-–Cl^- exchange mechanism was also suggested by the fact that, in the presence of HCO_3^-, the acute removal of external Cl^- in the absence of external Na^+ elicited an intracellular alkalinization of 0·17 ± 0·03 pH units ($n = 3$; Fig. 4*F*).

pH_i regulation at room temperature

Steady-state pH_i. In HCO_3^--free medium, steady-state pH_i rested at 6·84 ± 0·03 ($n = 37$; Fig. 2*B*), a value lower than that observed under the same conditions at 37 °C. The removal of external Na^+ (replaced with $NMDG^+$) under HCO_3^--free conditions caused a sustained intracellular acidification of 0·51 ± 0·05 pH units ($n = 8$), a fall similar in magnitude to that observed at 37 °C. Thus a Na^+-dependent, HCO_3^--independent acid extrusion mechanism contributes to the maintenance of steady-state pH_i at room

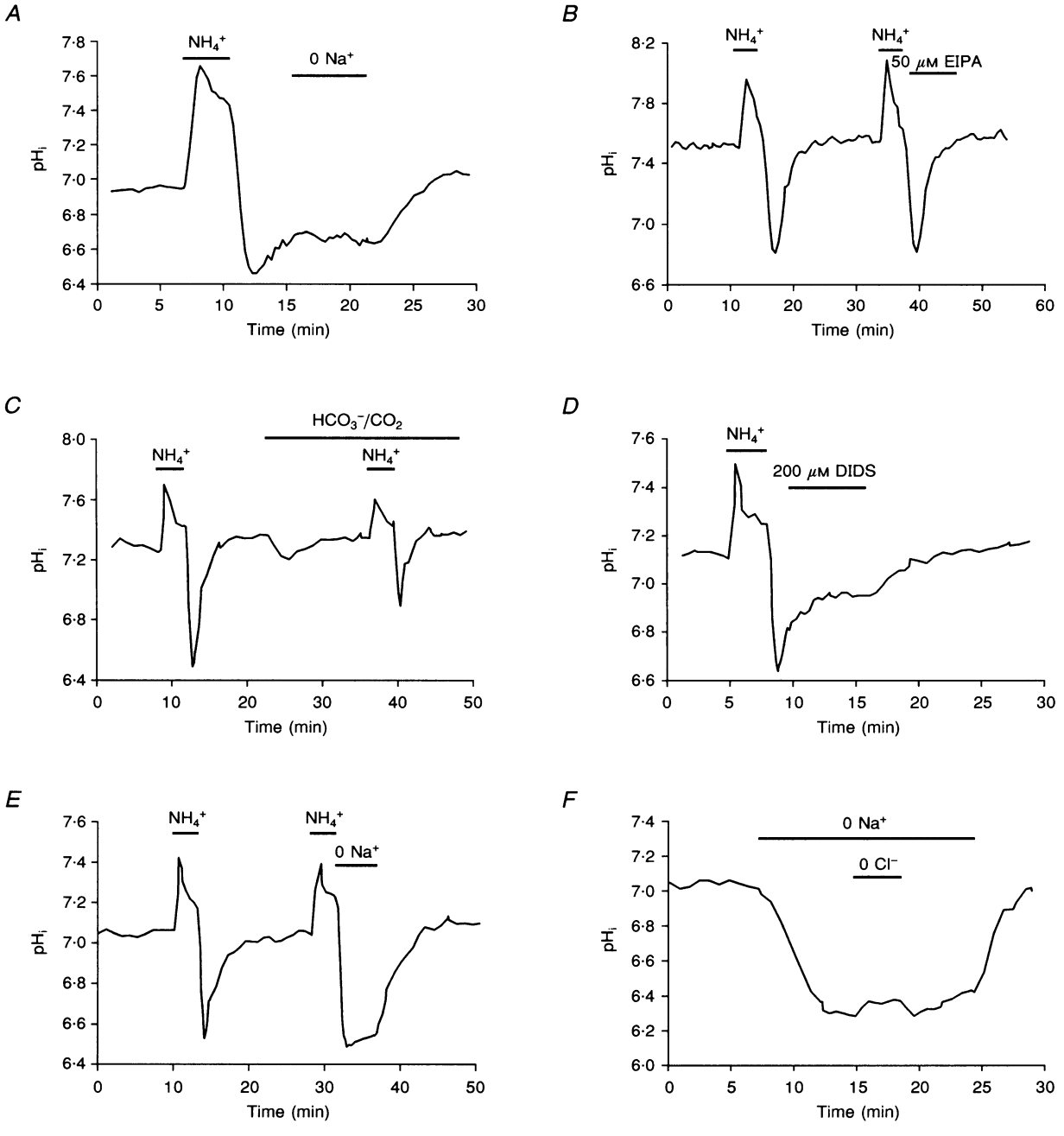

Figure 4. Recovery of pH_i from imposed intracellular acid loads at 37 °C

A, the removal of external Na^+ (replacement with $NMDG^+$) in the absence of HCO_3^- abolished pH_i recovery from an NH_4^+-induced acid load. Upon the reintroduction of Na^+, pH_i increased to normal resting levels. *B*, the addition of 50 μM EIPA, applied at the point of peak acidification following an NH_4^+ prepulse conducted in the absence of HCO_3^-, did not affect the rate of pH_i recovery. *C*, an initial acid load was imposed under HCO_3^--free conditions. The addition of HCO_3^-/CO_2 to the perfusion medium evoked a transient acidification but did not affect the rate of pH_i recovery following the second NH_4^+ prepulse (see also Fig. 8*B*). *D*, in an experiment conducted entirely in the presence of HCO_3^-, the application of 200 μM DIDS reduced the rate of pH_i recovery from an imposed acid load. *E*, the removal of external Na^+ in the presence of HCO_3^- caused a marked, but incomplete, inhibition of pH_i recovery from an imposed acid load. The readdition of Na^+ resulted in a rapid restoration of pH_i to steady-state levels. *F*, the removal of external Na^+ (in the presence of HCO_3^-) produced a fall in steady-state pH_i. The removal of external Cl^- in the continued absence of Na^+ evoked a fully reversible rise in pH_i. Records are means of data obtained simultaneously from 10 cells in *A*, 30 cells in *B*, 9 cells in *C*, 31 cells in *D*, 37 cells in *E* and 8 cells in *F*, each experiment performed on a separate coverslip.

temperature. At the same temperature but in the presence of HCO_3^-, steady-state pH_i resided at the substantially higher level of $7{\cdot}14 \pm 0{\cdot}02$ ($n = 30$; Fig. 2*B*) suggesting that, in contrast to findings at 37 °C, HCO_3^--dependent mechanism(s) also contribute to the maintenance of steady-state pH_i at room temperature. The equimolar replacement of constituent ions in the perfusion media and the application of DIDS were employed to investigate this HCO_3^--dependent mechanism. The application of 200 μM DIDS caused a reversible reduction in steady-state pH_i of $0{\cdot}10 \pm 0{\cdot}03$ pH units ($n = 4$; Fig. 5*A*). Conversely, the acute removal of external Cl^- evoked a reversible pH_i increase of $0{\cdot}25 \pm 0{\cdot}01$ pH units ($n = 9$; see Fig. 5*C*). This 0 Cl^--induced intracellular alkalinization was reduced to a $0{\cdot}04 \pm 0{\cdot}04$ pH unit rise ($n = 3$) in pH_i by 200 μM DIDS. The results indicate that, in the presence of HCO_3^-, a DIDS-sensitive anion exchange mechanism participates in the maintenance of steady-state pH_i at room temperature.

In order to determine whether the activity of the DIDS-sensitive anion exchanger was dependent on external Na^+, additional experiments were conducted under Na^+-free conditions. As illustrated in Fig. 5*B* and *C*, the removal of external Na^+ in the presence of HCO_3^- at room temperature evoked a $0{\cdot}52 \pm 0{\cdot}04$ pH unit fall ($n = 6$) in pH_i after 10 min. In order to raise pH_i back to values typically observed in the presence of external Na^+, $(NH_4)_2SO_4$ or trimethylamine HCl (each at a concentration of 30 mM by substitution for choline chloride) were applied in the continued absence of Na^+. The application of 200 μM DIDS under these conditions, where $pH_i = 7{\cdot}21 \pm 0{\cdot}10$ ($n = 3$), failed to evoke the intracellular acidification seen in the presence of external Na^+ (Fig. 5*B*). Furthermore, the 0 Cl^--induced alkalinization observed in the presence of external Na^+ was reduced under Na^+-free conditions to $0{\cdot}03 \pm 0{\cdot}02$ pH units at a pH_i of $7{\cdot}16 \pm 0{\cdot}01$ ($n = 3$; Fig. 5*C*). The results suggest that the DIDS-sensitive HCO_3^-–Cl^- exchanger active at physiological levels of pH_i at room temperature is Na^+ dependent.

To investigate further the role of HCO_3^--dependent mechanisms in maintaining steady-state pH_i at room temperature, the next series of experiments explored the effect on pH_i of the transition from HCO_3^--free to HCO_3^--containing media at a constant pH_o. As shown in Fig. 6*A*, this manoeuvre was marked by an initial acidification but, in contrast to similar experiments performed at 37 °C, this brief fall in pH_i was followed by a sustained alkalinization, presumably due to the activation of HCO_3^--dependent acid extrusion mechanism(s). The transition back into HCO_3^--free medium resulted in a transient increase in pH_i, after which pH_i fell to the normal resting value observed under HCO_3^--free conditions. The net alkaline shift in pH_i evoked by exposure to HCO_3^--containing media was inhibited by 200 μM DIDS ($n = 5$; Fig. 6*B*) and did not occur in neurones depleted of intracellular Cl^- by exposure to Cl^--

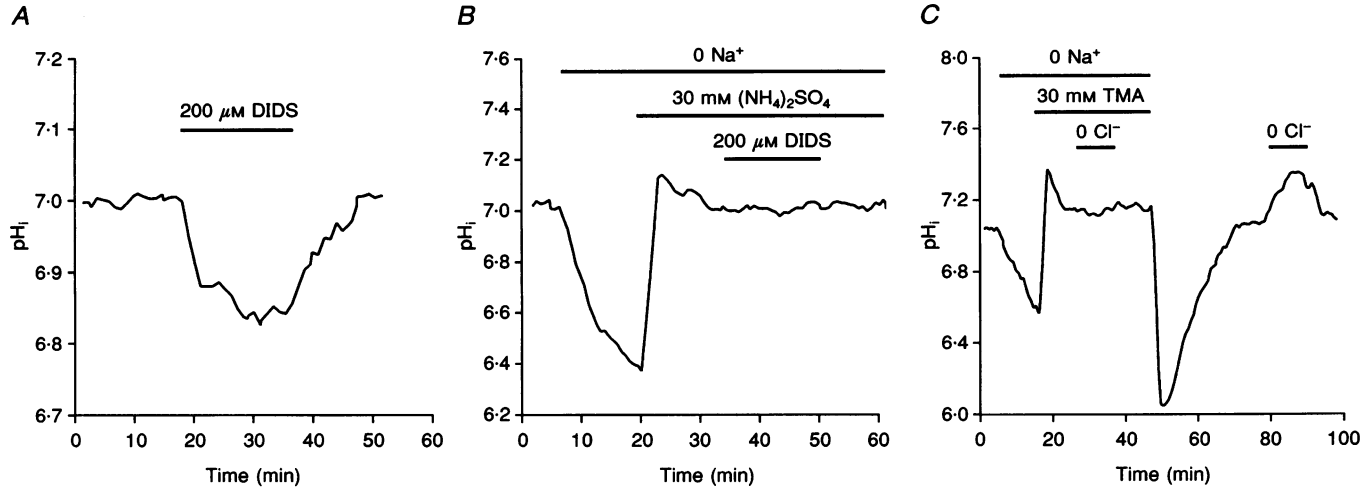

Figure 5. Steady-state pH_i in the presence of HCO_3^- at room temperature

A, the addition of 200 μM DIDS to HCO_3^--containing medium evoked an ~0·15 pH unit intracellular acidification, an effect not observed in a similar experiment performed at 37 °C (Fig. 3*F*). pH_i returned to normal levels upon removal of DIDS. *B*, in the presence of HCO_3^- the removal of external Na^+ caused an ~0·6 pH unit acidification. $(NH_4)_2SO_4$ (30 mM) was then applied to raise pH_i to the level observed prior to the removal of external Na^+. Under these Na^+-free conditions, 200 μM DIDS failed to change pH_i. *C*, the removal of external Na^+ in the presence of HCO_3^- produced an ~0·5 pH unit acidification. Upon application of 30 mM trimethylamine (TMA), pH_i rose to ~7·1 and the removal of external Cl^- under these Na^+-free conditions failed to change pH_i. TMA was then withdrawn and external Na^+ was reintroduced. Once pH_i had stabilized, the removal of external Cl^- evoked an intracellular alkalinization that reversed upon the reintroduction of Cl^-. Records are means of data obtained simultaneously from 30 cells in *A*, 4 cells in *B* and 6 cells in *C*, each experiment performed on a separate coverslip.

free medium for 1·5 h prior to the start of the experiment ($n = 4$; Fig. 6*C*; see Boyarsky, Ganz, Sterzel & Boron, 1988*b*). In the experiment shown in Fig. 6*C*, exposure to HCO_3^--containing medium following the reintroduction of Cl^- once again evoked a rapid intracellular alkalinization. The HCO_3^--evoked alkalinization was also dependent on external Na^+. In the experiment illustrated in Fig. 6*D*, exposure to 0 Na^+ (in the absence of HCO_3^-) produced an intracellular acidification that was compensated for by the addition of 5 mM trimethylamine to the Na^+-free perfusate. Under these conditions, where $pH_i = 6{\cdot}85 \pm 0{\cdot}05$ ($n = 3$), the transition from HCO_3^--free to HCO_3^--containing medium at a constant pH_o failed to evoke an intracellular alkalinization. The results indicate that the more alkaline steady-state pH_i observed in the presence, compared with the absence, of HCO_3^- at room temperature (Fig. 2*B*) reflects the activity of a DIDS-sensitive, Na^+-dependent HCO_3^-–Cl^- exchange mechanism. The fact that depletion of internal Cl^- inhibited the intracellular alkalinization resulting from the addition of HCO_3^- also suggests that Na^+–HCO_3^- cotransport does not contribute to the regulation of steady-state pH_i in cultured fetal rat hippocampal neurones.

pH_i recovery from an acid load

At room temperature in the absence of HCO_3^-, neurones recovered from an NH_4^+-induced intracellular acidification at an initial rate of $(1{\cdot}31 \pm 0{\cdot}33) \times 10^{-3}$ pH units s^{-1} ($n = 6$; Table 1). In contrast to experiments performed at 37 °C, the rate of pH_i recovery from an imposed acid load increased significantly ($P < 0{\cdot}005$) in the presence of

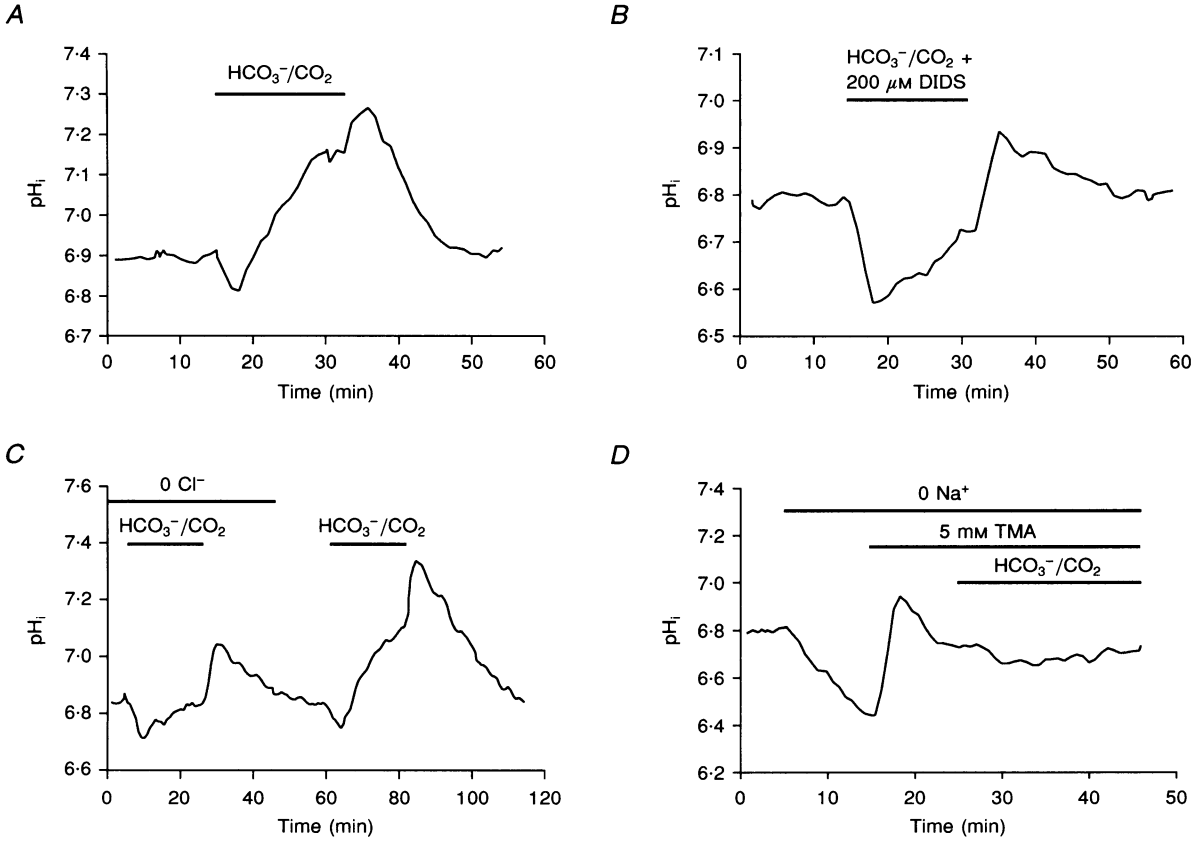

Figure 6. The effect on pH_i of the transition from HCO_3^--free to HCO_3^--containing media at room temperature

A, the transition from Hepes-buffered medium to HCO_3^-/CO_2-buffered medium at a constant pH_o evoked a brief acidification followed by a net alkalinization of ~0·25 pH units. The transition back into Hepes-buffered medium was marked by a transient alkalinization followed by a fall in pH_i to the normal resting level found in the absence of HCO_3^-. The net alkalinization evoked by the transition into HCO_3^-/CO_2-buffered medium was inhibited by 200 μM DIDS (*B*) or depleting intracellular Cl^- (*C*) by incubating the neurones in Cl^--free medium for 1·5 h prior to the start of the experiment. In *C*, a HCO_3^-/CO_2-induced net alkalinization was observed following the reintroduction of Cl^-. *D*, the dependence of the HCO_3^--evoked intracellular alkalinization on external Na^+ was examined by removing Na^+ from a Hepes-buffered medium. This produced a fall in pH_i that was returned to normal levels by the addition of 5 mM trimethylamine (TMA). Under these conditions, exposure of the neurones to HCO_3^--containing medium failed to elicit an intracellular alkalinization. Records are means of data obtained simultaneously from 10 cells in *A* and *B*, 21 cells in *C* and 8 cells in *D*, with each experiment performed on a separate coverslip.

HCO_3^- ($n = 7$; Fig. 7*A*; Table 1). Taking changes in internal buffering power into consideration (see Table 1), this suggests that the HCO_3^--dependent mechanism that contributed to pH_i recovery from acid loading at 37 °C plays a greater relative role at room temperature. In the presence of HCO_3^-, the rate of pH_i recovery from an imposed acid load was reduced by 200 μM DIDS ($n = 3$; Fig. 7*B*; Table 1). Diagrammatic representations of the effects of changes in perfusate composition and temperature on rates of pH_i recovery following NH_4^+-induced intracellular acidifications are presented in Fig. 8.

We attempted to determine the Na^+ dependence of the HCO_3^--dependent, DIDS-sensitive mechanism participating in the recovery of pH_i from an acid load by removing external Na^+. As shown in Fig. 9*A*, pH_i recovery following an NH_4^+-induced acid load was completely blocked when external Na^+ was removed in the absence of HCO_3^-. However, when HCO_3^- was added to the perfusion medium in the continued absence of external Na^+, pH_i began to recover, albeit slowly ($n = 2$; Fig. 9*A*). A slow HCO_3^--dependent, Na^+-independent recovery was also observed following a profound fall in steady-state pH_i induced by the removal of external Na^+ ($n = 6$; Fig. 9*B*). Figure 9*C* illustrates that this HCO_3^--dependent, Na^+-independent alkalinizing tendency was blocked by 300 μM DIDS ($n = 3$). Furthermore, the acute removal of external Cl^- in the absence of Na^+ evoked a rise in steady-state pH_i of $0{\cdot}30 \pm 0{\cdot}01$ pH units ($n = 3$; Fig. 9*D*). These data suggest that a Na^+-independent HCO_3^-–Cl^- exchanger contributes to acid extrusion at very low levels of pH_i. However, this Na^+-independent mechanism appears unlikely to be able to account fully for the marked increase in the rate of pH_i recovery from an acid load observed upon the addition of HCO_3^- at room temperature (see Fig. 8*A* and *C*). Thus the rate of pH_i recovery from an acid load imposed in the presence of HCO_3^- and in the absence of Na^+ was $(0{\cdot}44 \pm 0{\cdot}04) \times 10^{-3}$ pH units s^{-1} ($n = 8$). This value can be compared with the respective recovery rates obtained from paired experiments ($n = 3$) in the presence of external Na^+ under HCO_3^--free ($(0{\cdot}68 \pm 0{\cdot}15) \times 10^{-3}$ pH units s^{-1}) and HCO_3^--containing ($(2{\cdot}91 \pm 0{\cdot}16) \times 10^{-3}$ pH units s^{-1}) conditions; the difference between these values ($(2{\cdot}23 \pm 0{\cdot}03) \times 10^{-3}$ pH units s^{-1}) presumably reflects the total contribution of HCO_3^--dependent mechanisms, whether Na^+ dependent or independent, to pH_i recovery from an imposed intracellular acid load. This difference is significantly ($P \leqslant 0{\cdot}001$) greater than the rate of pH_i recovery seen in the presence of HCO_3^- and in the absence of external Na^+. Therefore, the Na^+-dependent HCO_3^-–Cl^- exchanger identified in the steady-state experiments (see above) contributes to pH_i recovery from an imposed intracellular acid load at room temperature.

DISCUSSION

Acid extrusion in the presence of external Na^+

Our results, which are in general agreement with those of Raley-Susman *et al.* (1991), indicate that acid extrusion in cultured fetal rat hippocampal neurones is governed primarily by two Na^+-dependent processes. The first of these is a HCO_3^--independent mechanism that contributes to the maintenance of steady-state pH_i at both room temperature and 37 °C. Although this mechanism was insensitive to amiloride analogues and HOE 694, replacement of external Na^+ with Li^+ did not jeopardize the ability of the neurones to maintain their resting pH_i, suggesting that the Na^+-dependent, HCO_3^--independent

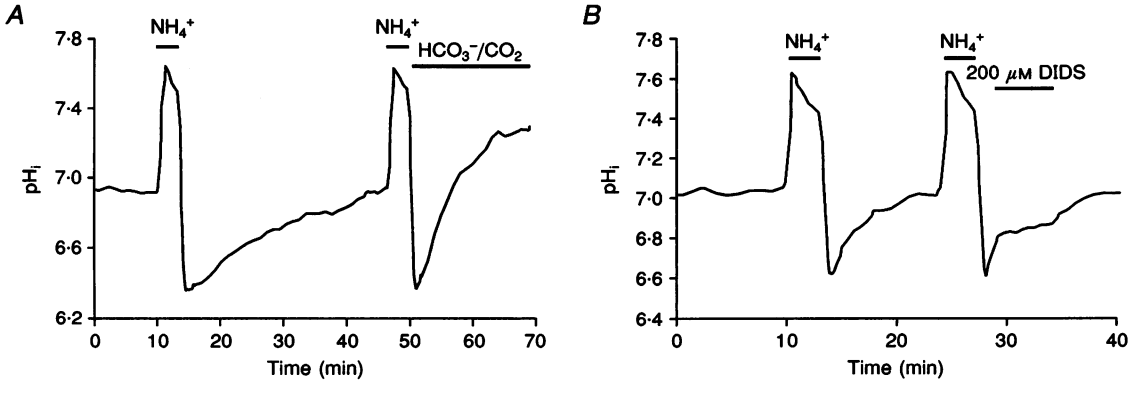

Figure 7. Recovery of pH_i from imposed intracellular acid loads at room temperature

A, following the first NH_4^+-induced intracellular acid load, pH_i was allowed to recover in the absence of HCO_3^-. The addition of HCO_3^- at the point of peak acidification following the second NH_4^+ prepulse increased the rate of pH_i recovery. In the presence of HCO_3^-, pH_i recovered to a higher steady-state level than that prevailing under HCO_3^--free conditions. *B*, an initial acid load was conducted under HCO_3^--containing conditions. A second acid load was then performed and DIDS, applied for the period indicated by the bar above the trace, slowed pH_i recovery from the imposed acid load. Records are means of data obtained simultaneously from 4 cells in *A* and 22 cells in *B*, with each experiment performed on a separate coverslip.

mechanism may be a Na^+–H^+ exchanger (Aronson, 1985; Busch *et al.* 1995). Measurements of $[Na^+]_i$ would be required to substantiate this possibility (e.g. Aickin & Thomas, 1977; Thomas, 1977). We were unable to confirm the possibility (Raley-Susman *et al.* 1991) that harmaline might be an inhibitor of the Na^+–H^+ exchange mechanism because, in agreement with Schwiening & Boron (1994), the compound interfered to a marked extent with the BCECF signal.

The second Na^+-dependent process that contributes to acid extrusion in cultured fetal rat hippocampal neurones is

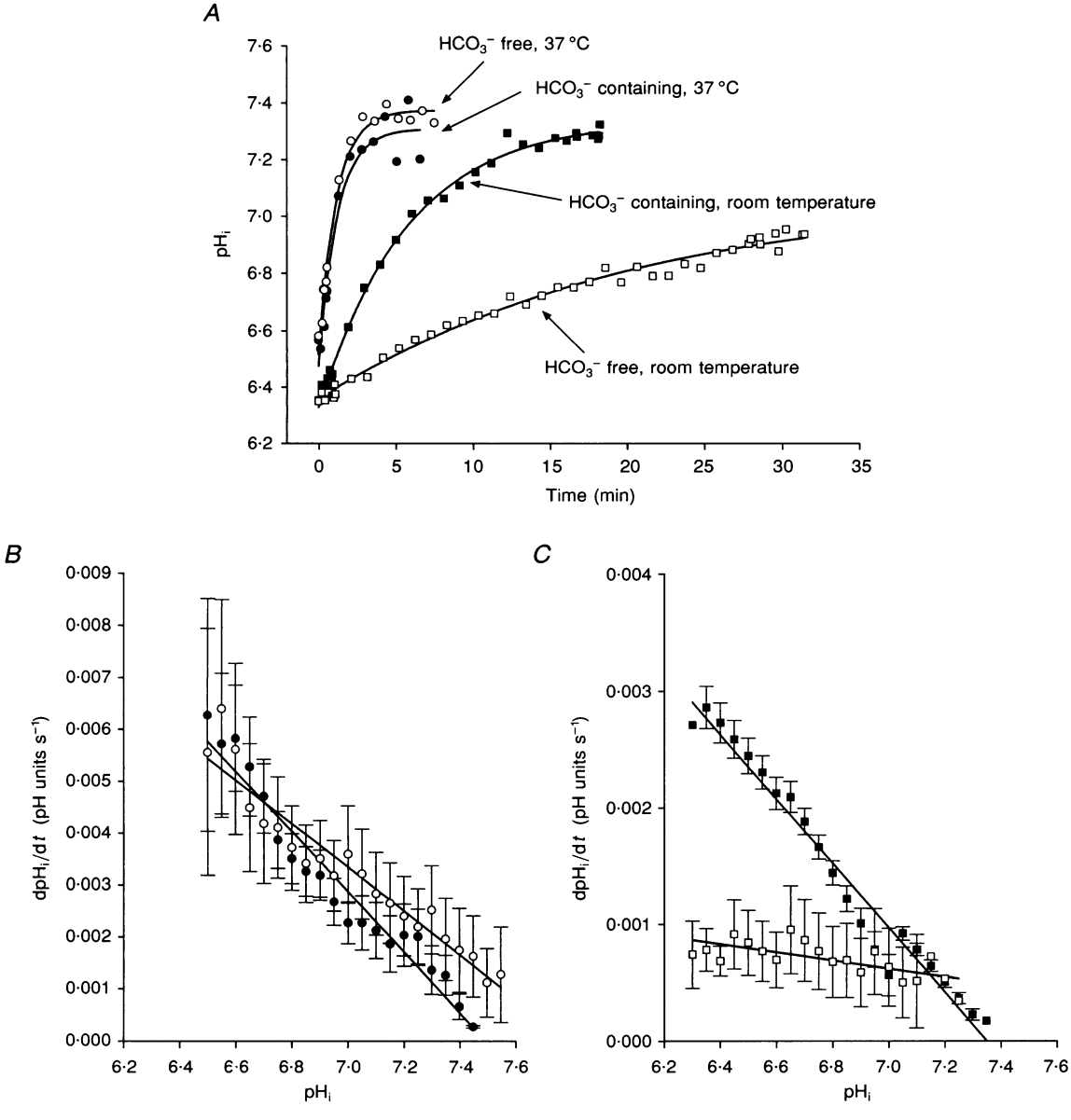

Figure 8. Effects of changes in perfusate composition and temperature on rates of pH_i recovery from imposed acid loads

A, pH_i recoveries from NH_4^+-induced intracellular acidifications in the absence of HCO_3^- at room temperature (□), in the presence of HCO_3^- at room temperature (■), in the presence of HCO_3^- at 37 °C (●) and in the absence of HCO_3^- at 37 °C (○). The continuous lines represent least squares exponential best fits to the data points indicated, which were obtained in four separate representative experiments under the conditions specified. Lines begin at the minimum pH_i evoked by the NH_4^+ prepulse and represent the trend found in all experiments conducted under similar conditions. *B*, average rates of pH_i recoveries from acid loads in the presence (●, $n = 19$) and absence (○, $n = 12$) of HCO_3^- at 37 °C, at pH_i values shown on the abscissa. *C*, average rates of pH_i recoveries from acid loads in the presence (■, $n = 7$) and absence (□, $n = 6$) of HCO_3^- at 20 °C, at pH_i values shown on the abscissa. In *B* and *C*, rates were evaluated at 0·05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition.

HCO_3^- dependent. This mechanism appears to play a greater relative role in acid extrusion at room temperature than at 37 °C and, in addition, is appreciably involved in the maintenance of steady-state pH_i only at room temperature. Thus in contrast to observations made at 37 °C, the addition of HCO_3^- to the perfusion medium at room temperature caused steady-state pH_i to rise suggesting that, at room temperature, a HCO_3^--dependent acid extrusion mechanism contributes to the maintenance of steady-state pH_i. Furthermore, although the application of DIDS (in the presence of HCO_3^-) reduced the rate of pH_i recovery from an imposed acid load at both room temperature and at 37 °C, only at room temperature did DIDS reduce resting pH_i. The net alkalinization evoked by the transition from HCO_3^--free to HCO_3^--containing medium at room temperature was inhibited by the application of DIDS or the depletion of intracellular Cl^- and, in experiments carried out at room temperature in the absence of external Na^+ but at pH_i levels similar to those observed in the presence of Na^+, the application of HCO_3^- failed to evoke an increase in steady-state pH_i. Thus the Na^+- and HCO_3^--dependent acid extrusion mechanism which participates in the maintenance of steady-state pH_i at room temperature is Cl^- dependent and DIDS sensitive.

Acid extrusion in the absence of external Na^+

At very low levels of pH_i ($< 6{\cdot}5$), Na^+-independent HCO_3^-–Cl^- exchange also participates in acid-equivalent extrusion in cultured fetal rat hippocampal neurones under our experimental conditions. This mechanism is active at room temperature and 37 °C. Following an imposed intracellular acidification (induced by the NH_4^+-prepulse technique or by the removal of external Na^+) pH_i recovered slowly even in the absence of external Na^+, providing that HCO_3^- was present in the perfusate. This HCO_3^--

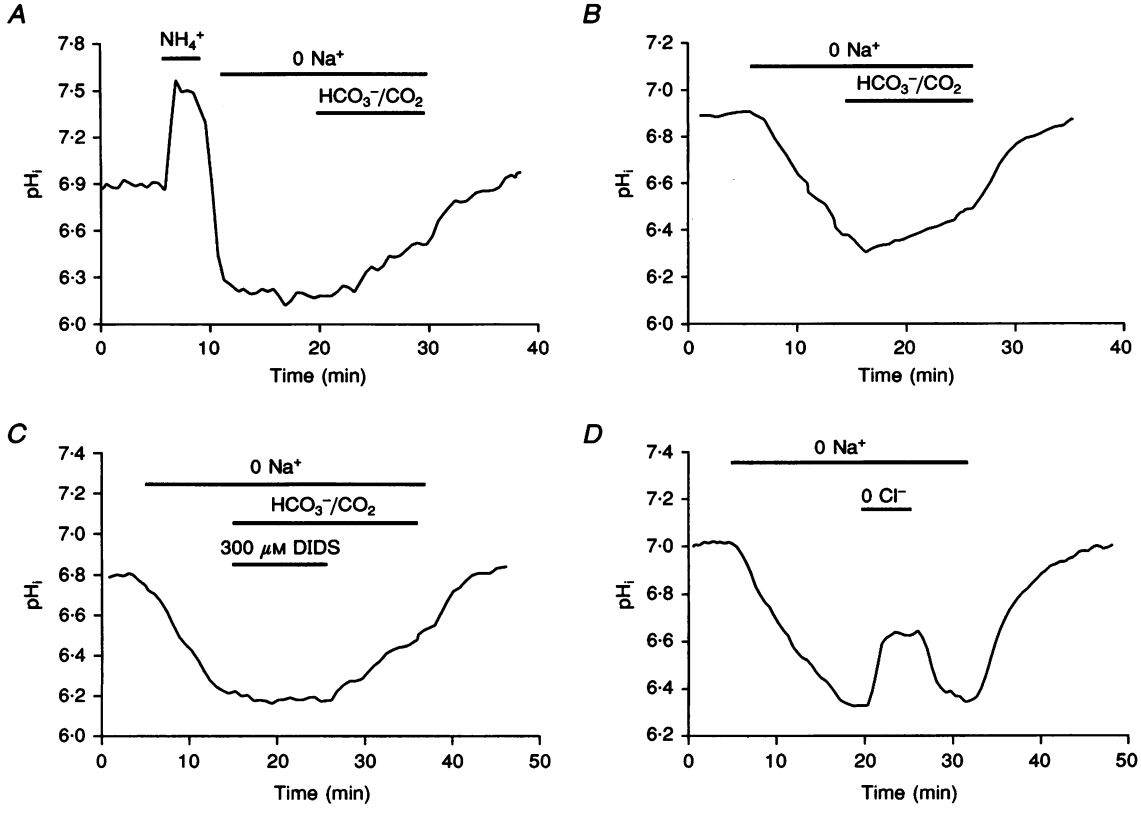

Figure 9. Acid extrusion in the absence of external Na^+ at room temperature

A, pH_i recovery from an intracellular acid load imposed in the absence of HCO_3^- was blocked by the removal of external Na^+ (replacement with $NMDG^+$). The subsequent introduction of HCO_3^- in the continued absence of Na^+ allowed recovery to resume at a slow rate. Recovery was further increased upon the reintroduction of Na^+. *B*, under steady-state conditions the removal of external Na^+ from HCO_3^--free medium evoked a fall in pH_i. The introduction of HCO_3^- in the continued absence of Na^+ led to a slow rise in pH_i which, as shown in *C*, was sensitive to the application of 300 μM DIDS. *D*, the removal of external Na^+ in the presence of HCO_3^- produced a fall in steady-state pH_i. The removal of external Cl^- in the continued absence of Na^+ evoked a fully reversible rise in pH_i. Records are means of data obtained simultaneously from 4 cells in *A*, 34 cells in *B*, 7 cells in *C* and 20 cells in *D*, with each experiment performed on a separate coverslip.

dependent alkalinizing tendency was inhibited by DIDS. In addition, at low levels of pH_i and in the absence of external Na^+, the acute removal of external Cl^- (in the presence of HCO_3^-) evoked a rise in pH_i. At normal or elevated levels of pH_i, Na^+-independent HCO_3^-–Cl^- exchange mediates the loss of HCO_3^- from the cell and thus acts as an acidifying mechanism (e.g. Vaughan-Jones, 1982; Boyarsky *et al.* 1988*b*; Gaillard & Dupont, 1990; Ou-yang *et al.* 1993). At low levels of pH_i, however, this passive exchanger can reverse and couple the influx of HCO_3^- to the efflux of Cl^- (see Frelin *et al.* 1988). Operating in this mode, Na^+-independent HCO_3^-–Cl^- exchange has been reported to play a role in pH_i recovery from extreme acidosis in some preparations (e.g. Aickin & Thomas, 1977; Boron, 1983; Aickin, 1988). The gene for the anion exchanger AE3, which is believed to function as a Na^+-independent HCO_3^-–Cl^- exchanger, is expressed in mammalian central neurones (Kopito, 1990; Raley-Susman *et al.* 1993). Although the Na^+-independent, HCO_3^--dependent exchange mechanism present in our neurones could be inhibited by 300 μM DIDS, this concentration is far in excess of the reported IC_{50} value (0·43 μM) for inhibition by DIDS of the activity of AE3 (Lee, Gunn & Kopito, 1991). It is therefore unclear whether the Na^+-independent HCO_3^-–Cl^- exchange mechanism described in the present study is encoded by the AE3 gene or whether it might be an AE3 isoform that is relatively insensitive to DIDS (see Kopito, 1990).

Comparison of pH_i regulation at 37 °C and room temperature

The present results confirm the novel finding of Schwiening & Boron (1994) that Na^+-dependent HCO_3^-–Cl^- exchange participates in the regulation of pH_i in rat hippocampal neurones. The essential difference between their results (obtained at 37 °C in neurones acutely dissociated from 4- to 14-day-old rats) and ours (obtained in fetal neurones) is the fact that only at room temperature could we demonstrate the active participation of this process in the maintenance of steady-state pH_i. Changes in intracellular buffering power appear unlikely to be able to account for the increased contribution of Na^+-dependent HCO_3^-–Cl^- exchange to acid extrusion as temperature falls. In the presence of HCO_3^-, a 3 min NH_4^+ application evoked a smaller pH_i increase at 37 °C than at room temperature (Table 1), suggesting the possibility that intracellular buffering power declines as temperature falls. In the absence of any changes in the activities of acid extrusion mechanisms, when intracellular buffering power is high (i.e. at 37 °C) then dpH_i/dt should be slow, whereas if intracellular buffering power is low (i.e. at room temperature) dpH_i/dt should be fast (Boron, 1989). However, as can be seen in Fig. 8 and Table 1, rates of pH_i recovery from imposed acid loads decreased as temperature declined, indicating that temperature-dependent changes in the activities of the acid extrusion mechanism(s) are likely to overshadow any effects on pH_i regulation of temperature-dependent changes in internal buffering power.

Although the mechanism(s) underlying the temperature-dependent changes in the activities of the acid-extruding transporters remain obscure, several possibilities exist. Firstly, it should be noted that solutions buffered with HCO_3^-/CO_2 contained 6 mM more HCO_3^- at room temperature than at 37 °C. This difference is, however, unlikely to underlie the apparent increase in Na^+-dependent HCO_3^-–Cl^- exchange activity as temperature is lowered from 37 to ~20 °C, given the low apparent K_m of the Na^+-dependent HCO_3^-–Cl^- exchanger for external HCO_3^- in other cell types (e.g. 2·3 mM in squid giant axon; Boron & Russell, 1983). Secondly, the activities of some HCO_3^- transport systems, including the Na^+-dependent HCO_3^-–Cl^- exchanger found in acutely dissociated adult rat hippocampal pyramidal neurones (Schwiening & Boron, 1994), are dependent on pH_i. In the present experiments, however, steady-state pH_i values at room temperature and 37 °C in the presence of HCO_3^- were almost identical, ruling out any effect of pH_i on the change in the activity of the Na^+-dependent HCO_3^-–Cl^- exchanger at the different temperatures. Thirdly, a reduction in the activity of Na^+–H^+ exchange as temperature declines may result in a relative increase in the observable contribution of Na^+-dependent HCO_3^-–Cl^- exchange to acid extrusion and the maintenance of steady-state pH_i, providing that anion exchange activity does not decline to the same extent. Such a mechanism may underlie the observations that, in the absence of HCO_3^-, the rate of pH_i recovery from an imposed acid load (mediated by Na^+–H^+ exchange) decreased about 4-fold upon lowering the perfusate temperature from 37 to ~20 °C, whereas in the presence of HCO_3^-, the rate of recovery only halved for the same reduction in temperature (Fig. 8; Table 1).

Conclusions

The present results indicate that, at both room temperature and 37 °C, acid extrusion in cultured fetal rat hippocampal neurones is dominated by two Na^+-dependent processes. The HCO_3^--independent mechanism, possibly an amiloride-insensitive variant of the Na^+–H^+ exchanger, is the major determinant of steady-state pH_i at 37 °C. The HCO_3^--dependent mechanism is a HCO_3^-–Cl^- exchanger. This mechanism is relatively more active at room temperature than at 37 °C and, furthermore, participates in the regulation of steady-state pH_i at room temperature but not at 37 °C. The results also reveal the presence of a Na^+-independent HCO_3^-–Cl^- exchange mechanism that may participate in acid extrusion during extreme intracellular acidosis at both room temperature and 37 °C.

AICKIN, C. C. (1988). Movement of acid equivalents across the mammalian smooth muscle cell membrane. In *Proton Passage Across Cell Membranes*, ed. BOCK, G. & MARSH, J., *CIBA Foundation Symposium*, vol. 139, pp. 3–22. Wiley, Chichester.

AICKIN, C. C. & THOMAS, R. C. (1977). An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *Journal of Physiology* **273**, 295–316.

ARONSON, P. S. (1985). Kinetic properties of the plasma membrane Na^+–H^+ exchanger. *Annual Review of Physiology* **47**, 545–560.

BANKER, G. A. & COWAN, W. M. (1977). Rat hippocampal neurons in dispersed cell culture. *Brain Research* **126**, 397–425.

BORON, W. F. (1983). Transport of H^+ and of ionic weak acids and bases. *Journal of Membrane Biology* **72**, 1–16.

BORON, W. F. (1989). Cellular buffering and intracellular pH. In *The Regulation of Acid–Base Balance*, ed. SELDIN, D. W. & GIEBISCH, G., pp. 33–56. Raven Press, New York.

BORON, W. F. & RUSSELL, J. M. (1983). Stoichiometry and ion dependencies of the intracellular-pH-regulating mechanism in squid giant axons. *Journal of General Physiology* **81**, 373–399.

BOYARSKY, G., GANZ, M. B., STERZEL, R. B. & BORON, W. F. (1988*a*). pH regulation in single glomerular mesangial cells I. Acid extrusion in absence and presence of HCO_3^-. *American Journal of Physiology* **255**, C844–856.

BOYARSKY, G., GANZ, M. B., STERZEL, R. B. & BORON, W. F. (1988*b*). pH regulation in single glomerular mesangial cells. II. Na^+-dependent and -independent Cl^-–HCO_3^- exchangers. *American Journal of Physiology* **255**, C857–869.

BREWER, G. J. & COTMAN, C. W. (1989). Survival and growth of hippocampal neurons in defined medium at low density: Advantages of a sandwich culture technique or low oxygen. *Brain Research* **494**, 65–74.

BUSCH, S., BURCKHARDT, B.-C. & SIFFERT, W. (1995). Expression of the human sodium/proton exchanger NHE-1 in *Xenopus laevis* oocytes enhances sodium/proton exchange activity and establishes sodium/lithium countertransport. *Pflügers Archiv* **429**, 859–869.

CHESLER, M. (1986). Regulation of intracellular pH in reticulospinal neurones of the lamprey, *Petromyzon marinus*. *Journal of Physiology* **381**, 241–261.

CLARK, J. D. & LIMBIRD, L. E. (1991). Na^+–H^+ exchanger subtypes: a predictive review. *American Journal of Physiology* **261**, C945–953.

COUNILLON, L., SCHOLZ, W., LANG, H. J. & POUYSSÉGUR, J. (1993). Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Molecular Pharmacology* **44**, 1041–1045.

FRELIN, C., VIGNE, P., LADOUX, A. & LAZDUNSKI, M. (1988). The regulation of the intracellular pH in cells from vertebrates. *European Journal of Biochemistry* **174**, 3–14.

GAILLARD, S. & DUPONT, J.-L. (1990). Ionic control of intracellular pH in rat cerebellar Purkinje cells maintained in culture. *Journal of Physiology* **425**, 71–83.

JEAN, T., FRELIN, C., VIGNE, P., BARBRY, P. & LAZDUNSKI, M. (1985). Biochemical properties of the Na^+/H^+ exchange system in rat brain synaptosomes. *Journal of Biological Chemistry* **260**, 9678–9684.

KOPITO, R. R. (1990). Molecular biology of the anion exchanger gene family. *International Review of Cytology* **123**, 177–199.

LEE, B. S., GUNN, R. B. & KOPITO, R. R. (1991). Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. *Journal of Biological Chemistry* **266**, 11448–11454.

MARTÍNEZ-ZAGUILÁN, R., GILLIES, R. J. & SÁNCHEZ-ARMASS, S. (1994). Regulation of pH in rat brain synaptosomes. II. Role of Cl^-. *Journal of Neurophysiology* **71**, 2249–2257.

NACHSHEN, D. A. & DRAPEAU, P. (1988). The regulation of cytosolic pH in isolated presynaptic nerve terminals from rat brain. *Journal of General Physiology* **91**, 289–303.

OU-YANG, Y., MELLERGÅRD, P. & SIESJÖ, B. K. (1993). Regulation of intracellular pH in single rat cortical neurons *in vitro*: a microspectrofluorometric study. *Journal of Cerebral Blood Flow and Metabolism* **13**, 827–840.

RALEY-SUSMAN, K. M., CRAGOE, E. J., SAPOLSKY, R. M. & KOPITO, R. R. (1991). Regulation of intracellular pH in cultured hippocampal neurons by an amiloride-insensitive Na^+/H^+ exchanger. *Journal of Biological Chemistry* **266**, 2739–2745.

RALEY-SUSMAN, K. M., SAPOLSKY, R. M. & KOPITO, R. R. (1993). Cl^-/HCO_3^- exchange function differs in adult and fetal rat hippocampal neurons. *Brain Research* **614**, 308–314.

RINK, T. J., TSIEN, R. Y. & POZZAN, T. (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. *Journal of Cell Biology* **95**, 189–196.

ROOS, A. & BORON, W. F. (1981). Intracellular pH. *Physiological Reviews* **61**, 296–434.

SÁNCHEZ-ARMASS, S., MARTÍNEZ-ZAGUILÁN, R., MARTÍNEZ, G. M. & GILLIES, R. J. (1994). Regulation of pH in rat brain synaptosomes. I. Role of sodium, bicarbonate, and potassium. *Journal of Neurophysiology* **71**, 2236–2248.

SCHWIENING, C. J. & BORON, W. F. (1994). Regulation of intracellular pH in pyramidal neurones from the rat hippocampus by Na^+-dependent Cl^-–HCO_3^- exchange. *Journal of Physiology* **475**, 59–67.

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *Journal of Physiology* **273**, 317–338.

THOMAS, R. C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. *Journal of Physiology* **354**, 3–22*P*.

TOLKOVSKY, A. M. & RICHARDS, C. D. (1987). Na^+/H^+ exchange is the major mechanism of pH regulation in cultured sympathetic neurons: Measurements in single cell bodies and neurites using a fluorescent pH indicator. *Neuroscience* **22**, 1093–1102.

VAUGHAN-JONES, R. D. (1982). Chloride–bicarbonate exchange in the sheep cardiac Purkinje fibre. In *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions*, ed. NUCCITELLI, R. & DEAMER, D. W., pp. 239–252. Alan R. Liss, New York.

Acknowledgements

We are grateful to Ms M. Grunert for the preparation of the neuronal cultures and to Dr V. Palatý for helpful discussions. Financial support was provided by an operating grant to J. C. from the Medical Research Council of Canada.

Author's email address

J. Church: jchurch@unixg.ubc.ca

Received 23 June 1995; accepted 15 January 1996.