Intracellular pH Regulation in Cultured Embryonic Chick Heart Cells

Na^+-dependent Cl^-/HCO_3^- Exchange

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ABSTRACT The contribution of Cl^-/HCO_3^- exchange to intracellular pH (pH_i) regulation in cultured chick heart cells was evaluated using ion-selective microelectrodes to monitor pH_i, Na^+ (a^i_{Na}), and Cl^- (a^i_{Cl}) activity. In (HCO_3^- + CO_2)–buffered solution steady-state pH_i was 7.12. Removing (HCO_3^- + CO_2) buffer caused a SITS (0.1 mM)–sensitive alkalinization and countergradient increase in a^i_{Cl} along with a transient DIDS-sensitive countergradient decrease in a^i_{Na}. SITS had no effect on the rate of pH_i recovery from alkalinization. When (HCO_3^- + CO_2) was reintroduced the cells rapidly acidified, a^i_{Na} increased, a^i_{Cl} decreased, and pH_i recovered. The decrease in a^i_{Cl} and the pH_i recovery were SITS sensitive. Cells exposed to 10 mM NH_4Cl became transiently alkaline concomitant with an increase in a^i_{Cl} and a decrease in a^i_{Na}. The intracellular acidification induced by NH_4Cl removal was accompanied by a decrease in a^i_{Cl} and an increase in a^i_{Na} that led to the recovery of pH_i. In the presence of (HCO_3^- + CO_2), addition of either amiloride (1 mM) or DIDS (1 mM) partially reduced pH_i recovery, whereas application of amiloride plus DIDS completely inhibited the pH_i recovery and the decrease in a^i_{Cl}. Therefore, after an acid load pH_i recovery is HCO_{3o}^- and Na_o^- dependent and DIDS sensitive (but not Ca_o^{2+} dependent). Furthermore, SITS inhibition of Na^+-dependent Cl^-/HCO_3^- exchange caused an increase in a^i_{Cl} and a decrease in the ^{36}Cl efflux rate constant and pH_i. In (HCO_3^- + CO_2)–free solution, amiloride completely blocked the pH_i recovery from acidification that was induced by removal of NH_4Cl. Thus, both Na^+/H^+ and Na^+-dependent Cl^-/HCO_3^- exchange are involved in pH_i regulation from acidification. When the cells became alkaline upon removal of (HCO_3^- + CO_2), a SITS-sensitive increase in pH_i and a^i_{Cl} was accompanied by a

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decrease of a^i_{Na}, suggesting that the HCO_3^- efflux, which can attenuate initial alkalinization, is via a Na^+-dependent Cl^-/HCO_3^- exchange. However, the mechanism involved in pH_i regulation from alkalinization is yet to be established. In conclusion, in cultured chick heart cells the Na^+-dependent Cl^-/HCO_3^- exchange regulates pH_i response to acidification and is involved in the steady-state maintenance of pH_i.

INTRODUCTION

Direct measurement of intracellular pH (pH_i) with pH-sensitive microelectrodes has confirmed that pH_i in cardiac muscle cells is more alkaline than predicted for passive transmembrane H^+ distribution (Ellis and Thomas, 1976; Deitmer and Ellis, 1980; Ellis and MacLeod, 1985; Kaila and Vaughan-Jones, 1987). Mechanisms that could be involved in maintaining low $[H^+]$ are (*a*) net H^+ efflux, (*b*) net OH^- (HCO_3^-) influx, or (*c*) both. Na^+/H^+ exchange has been implicated in the steady-state maintenance and regulation of pH_i in both cultured chick heart cells (Frelin et al., 1985; Piwnica-Worms et al., 1985) and Purkinje fibers (Kaila and Vaughan-Jones, 1987). However, in the presence of amiloride or its derivatives the small change in Na_i and pH_i from steady state indicates that the contribution of Na^+/H^+ exchange to physiological pH_i maintenance may be small (Deitmer and Ellis, 1980; Frelin et al., 1985; Piwnica-Worms et al., 1985; Kaila and Vaughan-Jones, 1987). Alternatively, Cl^-/HCO_3^- exchange can provide a net HCO_3^- influx and hence result in an intracellular alkalinization. Vaughan-Jones (1982) suggested that in sheep Purkinje fibers pH_i regulation via Cl^-/HCO_3^- exchange (the main mechanism for maintaining $[Cl^-]_i$) is operable only under intracellular alkaline conditions. Interestingly, little is known about the physiological role of Cl^-/HCO_3^- exchange in pH_i regulation of cardiac muscle cells because most studies have been pursued in a HEPES-buffered solution that is free of ($HCO_3^- + CO_2$).

pH_i recovery from intracellular acidosis may reveal whether Na^+/H^+ exchange, Cl^-/HCO_3^- exchange, or both mechanisms are responsible for maintaining pH_i. In Purkinje fibers (Deitmer and Ellis, 1980; Ellis and MacLeod, 1985; Kaila and Vaughan-Jones, 1987) and in cultured chick heart cells (Frelin et al., 1985) Na^+/H^+ exchange is thought to be the mechanism underlying the recovery of pH_i after an acid load in HCO_3^--free solution. However, the conditions of these studies probably prevented the detection of Cl^-/HCO_3^- exchange in pH_i regulation because (*a*) absence of the HCO_3^- substrate inactivates Cl^-/HCO_3^- exchange, and (*b*) the concentration of SITS used in these experiments may have been too low to inhibit Cl^-/HCO_3^- exchange. Although our earlier findings in HCO_3^--buffered solution indicated that Na^+/H^+ exchange is important in pH_i regulation from an acid load (Piwnica-Worms et al., 1985), Na-dependent Cl^-/HCO_3^- exchange was not eliminated because of the limitation of the pH_i measurements and the difficulties in resolving Na_i^+ from measurements using atomic absorption analysis (see Discussion).

By incorporating ion-selective microelectrode (ISME) techniques to obtain continuous and direct measurements of pH_i, a^i_{Cl}, and a^i_{Na}, we have been able to characterize the Cl^-/HCO_3^- exchange mechanism and examine its possible role in pH_i regulation. We find that cultured chick heart cells possess a Na_o-dependent Cl^-/HCO_3^- exchange (($Na^+ + HCO_3^-/Cl^- + H^-$) exchange) that regulates pH_i in conjunction

with Na^+/H^+ exchange in response to intracellular acidification, and may also participate in maintaining physiological pH_i. This study confirms the statement of Thomas (1989) that in evaluating the mechanisms involved in pH_i regulation, experiments must be carried out in the presence of a HCO_3^--buffered solution. Preliminary results have been presented in abstract form (Lieberman and Liu, 1988; Liu et al., 1988).

METHODS

Preparations

Experiments were performed with growth-oriented, tissue-cultured heart cells (polystrands) from 11-d-old chick embryos. The techniques for producing spontaneously beating strands have been described in detail (Horres et al., 1977). The original method of cell growth orientation was modified such that the substrate supporting the nylon monofilament to which the cells attached consisted of a U-shaped stainless steel wire (0.6 mm in diameter) that was wrapped with a continuous winding of nylon (20 μm in diameter). The nylon containing support was autoclaved and placed in the center of a 35-mm culture dish (Falcon Labware, Oxnard, CA). A sterile, silicone disc with a U-shaped groove around a central opening (3 mm wide, 10 mm long) was seated over the support and was held firmly in place by a light coating of silicone grease on its underside. 0.1 ml culture medium was added into the central well, and the culture dish was placed overnight in an incubator at 37°C in a 95% air–5% CO_2 atmosphere before seeding the cells.

The culture procedure used to produce spontaneously beating strands of muscle (o.d. $\approx$ 100 μm and <3 mm in length) was similar to that reported by Horres et al. (1977). Briefly, hearts were disaggregated by serial exposure to 0.05% trypsin (Gibco Laboratories, Grand Island, NY). The proportion of muscle cells was increased by using a differential cell attachment technique for 1 h. An aliquot of cells was suspended in medium 199 with a base of Earle's solution, 5% fetal bovine serum (HyClone Laboratories, Sterile Systems, Inc., Logan, UT), and 2% chick embryo extract. The cells were seeded in growth chambers and maintained at 37°C in a 95% air–5% CO_2 atmosphere for 3 d. During this time the cells formed spontaneously beating strands of muscle around the nylon cores. However, the reduced dimension of the nylon substrate required the seeding of fewer cells (1.25×10^5 cells/dish).

Solutions

The solutions used were modified Earle's balanced salt solutions (MEBSS) as described in Table I. Each solution also contained 1.0 g/liter bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO). HCO_3-free MEBSS contained 5.4 mM HEPES (Research Organics Inc., Cleveland, OH) and 4.6 mM Tris(hydroxy-methyl)aminomethane (Sigma Chemical Co.). Tetramethylammonium chloride (TMACl; ICN Pharmaceuticals, Inc., Plainview, NY) was used to replace Na and balance the osmolality as required (Jacob et al., 1987). Choline bicarbonate was also used to replace $NaHCO_3$ in HCO_3-buffered MEBSS. Cl-free solutions were made by equimolar substitution of the impermeant anion, methanesulfonate (Eastman Kodak Corp., Rochester, NY) (Piwnica-Worms et al., 1985). In NH_4Cl prepulse experiments the osmolality of the solutions was adjusted to match that of NH_4Cl containing solution by addition of 10 mM TMACl (295 mosM for HCO_3^--buffered solutions; 305 mosM for HEPES/Tris solutions). DIDS (4,4-diisothiocyano-2,2′-disulfonic acid stilbene; Pierce Chemical Co., Rockford, IL or Fluka Chemical, Hauppauge, NY) or SITS (4-acetamido-4′-isothio-cyanostilbene-2′,2′-disulfonic acid; ICN Biomedicals, Inc., K+K Labs, Plainview, NY) and amiloride (Sigma Chemical Co.) were added directly as powder to solutions before each

experiment. In experiments when both 1 mM DIDS and 1 mM amiloride were used, we found that DIDS (Fluka) caused precipitation in an amiloride-containing solution, whereas DIDS (Pierce) did not. Unless otherwise stated, all experiments were done in the presence of (HCO_3^- + CO_2) buffer

Electrophysiological Methods

Transmembrane potentials (E_m) and intracellular Na and Cl activities (a^i_{Na} and a^i_{Cl}) of polystrands were measured as described previously (Liu et al., 1987). The reported values of a^i_{Cl} were uncorrected for residual a^i_{Cl}, whereas values used to calculate the thermodynamic driving force were corrected by subtracting 10 mM from apparent a^i_{Cl} (Liu et al., 1987). With a perfusion rate of 4 ml/min the solution completely cleared the chamber in 5 s as measured with ISMEs. E_m was recorded with conventional 3 M KCl-filled glass microelectrodes (15–20

TABLE I

Composition of Solutions

	Control	Na^+-free	NH_4Cl	Cl^--free	Ca^{2+}-free	HCO_3^--free
Na^+	143.4	—	143.4	143.4	143.4	143.4
K^+	5.4	5.4	5.4	5.4	5.4	5.4
Ca^{2+}	1.0	1.0	1.0	1.0	—	1.0
Mg^{2+}	0.8	0.8	0.8	0.8	0.8	0.8
TMA^+	—	127.2	—	—	—	—
$Choline^+$	—	26.2	—	—	—	—
NH_4^+	—	—	10.0	—	—	—
Cl^-	123.8	123.8	133.8	—	123.8	150.0
HCO_3^-	26.2	26.2	26.2	26.2	26.2	—
$H_2PO_4^-$	0.8	0.8	0.8	0.8	0.8	0.8
SO_4^{2-}	0.8	0.8	0.8	0.8	0.8	0.8
MSA^-	—	—	—	133.8	—	—
HEPES	—	—	—	—	—	5.4
Tris	—	—	—	—	—	4.6
Glucose	5.6	5.6	5.6	5.6	5.6	5.6
Osmolality, mosM	275	275	295	275	275	285

Compositions are given in millimolar.

The pH of solutions was 7.39 ± 0.02; HCO_3^--buffered solution was gassed with 5% CO_2/95% air.

MΩ). A reference electrode containing a Ag–AgCl pellet (Fisher Scientific Co., Pittsburgh, PA) in 3 M KCl was connected to ground via a 3% (wt/vol) 3 M KCl-agar bridge positioned close to the outlet of the perfusion chamber.

Measurement of pH_i

pH_i was monitored by either a single-barreled or a concentric double-barreled pH-selective microelectrode. Single-barreled pH-selective microelectrodes were made by the same method described previously for Na- and Cl-selective microelectrodes (Liu et al., 1987). Concentric double-barreled ISME were made by a modification of the method described by Thomas (1985). Briefly, an Omega Dot borosilicate glass tube (o.d., 1.0 mm; i.d., 0.5 mm; 13 cm in length; Glass Co. of America, Bargaintown, NJ) was affixed with epoxy to the inner wall of an aluminosilicate glass tube (Omega Dot, #1724, o.d., 2.0 mm; i.d., 1.5 mm, 10 cm in length;

Glass Co. of America). Two pipettes were pulled with either a vertical electrode puller (model 700C; David Kopf Instruments, Tujunga, CA) or a horizontal microelectrode puller (Brown-Flaming, P77; Sutter Instruments Co., San Francisco, CA) and then dried in an oven at 200°C for 15 min. The open end of the longer inner barrels of double-barreled micropipettes was then rapidly touched to hexamethyldisilazane that was loaded in a separate tube. The coated micropipettes were cured in an oven at 200°C for another 30 min. The outer barrels (reference electrode) were backfilled with 3 M KCl for E_m measurements. The silanized micropipettes were backfilled with ~5 μl of the modified Fluka proton cocktail (a gift from Prof. D. Ammann, ETH, Switzerland) and left in a petri dish in air for 2 h. A solution consisting of 40 mM KH_2PO_4, 23 mM NaOH, and 15 mM NaCl buffered to pH 7.0 was also backfilled (Ammann et al., 1981). The pH-selective microelectrode was then connected via a Ag-AgCl wire to a high input impedance preamplifier (AD515; Analog Devices, Inc., Norwood, MA), and referred to ground through a reference electrode. The tips were slightly beveled using an alumina-KCl slurry (Lederer et al., 1979) to reduce the electrical resistance to $0.5–2 \times 10^{10}$ Ω. The tips of the microelectrodes were then immersed into the filling solution for ~1 h before calibration. E_m and pH_i changes measured with a double-barreled pH-selective microelectrode were compatible with those recorded with a conventional voltage microelectrode and a single-barreled pH-selective microelectrode (cf. Fig. 2).

The pH-selective microelectrodes were calibrated over the range of 6.0–8.5 in HEPES-Tris–buffered solutions containing either 140 mM K and 10 mM Na or 140 mM Na, 5.4 mM K, and 1.0 mM Ca. The response of single-barreled pH-selective microelectrodes to pH changes was linear with a slope of 59–61 mV/pH unit at 37°C, as compared with a slope of 50–60 mV for double-barreled pH-selective microelectrodes (the yield of double-barreled pH-selective microelectrodes was <20%). Suitable electrodes were those with a slope >50 mV, potential drift of ~1 mV/h, offset changes <3 mV, and a 90% chemical response time <5 s. Signals were observed with an oscilloscope (model 5111; Tektronix, Inc., Beaverton, OR) and recorded using both a magnetic tape recorder (model 4DS; Racal Recorders Inc., Sarasota, FL) and a chart recorder (Brush 220; Gould Inc., Cleveland, OH). Data were acquired through a computer interface (AXOLAB-1; Axon Instruments, Inc., Foster City, CA) and stored for analysis with a personal computer (COMPAQ 286, Houston, TX).

Flux and Chloride Content Measurement

Methods to determine the rate constant for ^{36}Cl efflux and cellular Cl content have been described in detail previously (Piwnica-Worms et al., 1983). Na influx and Cl efflux were also determined by multiplying the initial rate of change in ion activity (within 20 s) by the volume/surface area ratio (V/A, 1.06×10^{-4} cm; Horres et al., 1977). Since most manipulations in this study were not expected to cause an immediate change in cell volume, V/A was assumed to be constant. We calculated relative changes in cell volume by measuring the outer diameter of a single strand from the video signal of a television-microscopy system (Desai et al., 1985). Acid extrusion (or H^+ efflux) in HCO_3^--buffered MEBSS was determined by the rate of change in pH_i multiplied by an assumed constant intracellular buffering capacity (β_T) (see Discussion).

Statistics

Values are presented as means ± SEM. Statistical significance was evaluated by the two-tailed paired Student's t test. Data obtained from the same preparation were used to express the results in terms of percentages.

RESULTS

I. Evidence for Cl^-/HCO_3^--Exchange

To determine the existence of a Cl^-/HCO_3^- exchange in cultured chick heart cells, we manipulated the HCO_3^- or Cl^- gradient and monitored pH_i and a^i_{Cl} or Cl_i^- content. In HCO_3^--buffered control solution, steady-state pH_i at 37°C was 7.12 ± 0.01 ($n = 46$), and apparent a^i_{Cl} was 36.4 ± 0.8 mM ($n = 63$) (maximal diastolic potential ~ −75 mV).

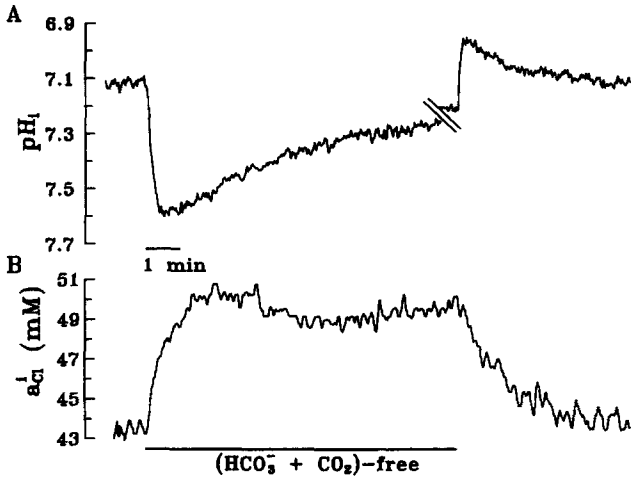

FIGURE 1. Removal of (HCO_3^- + CO_2) affects pH_i and a^i_{Cl}. (*A*) Polystrands were superfused with HCO_3 + CO_2–buffered MEBSS and pH_i was monitored with pH-selective microelectrodes. A transient alkalinization (from control pH_i 7.1 to 7.6) occurred when the solution was switched to (HCO_3^- + CO_2)–free solution. Then pH_i slowly regulated toward the control level and stabilized within 15 min at a level slightly more alkaline than control (the break of trace represents ~4 min). Reintroducing (HCO_3^- + CO_2)–buffered solution caused a transient acidification to pH_i 6.85 that regulated to approach control level. (*B*) Intracellular Cl activity (a^i_{Cl}) was monitored with a Cl-selective microelectrode in a separate preparation. Removal of (HCO_3^- + CO_2) caused an increase in a^i_{Cl}. Upon restoring (HCO_3^- + CO_2), a^i_{Cl} decreased and recovered to control level.

Effect of HCO_3^- gradient on pH_i, a^i_{Cl}, and a^i_{Na}

Alkalinization. Polystrands were perfused with (HCO_3^- + CO_2) control solution and then exposed to (HCO_3^- + CO_2)–free (HEPES/Tris buffer) solution. This maneuver initiated a rapid efflux of CO_2 and a concomitant increase in pH_i of 0.38 ± 0.03 ($n = 15$), which in 15 min regulated toward a new steady state at a pH_i of 7.23 ± 0.03 ($n = 17$); maximal diastolic potential changed less than 1.0 mV. A representative recording of the changes in pH_i is shown in Fig. 1 *A*. Following the same protocol with different preparations. Fig. 1 *B* shows that removal of HCO_3^- caused a countergradient increase in a^i_{Cl} to a new steady-state level and a countergra-

dient transient decrease in a^i_{Na} by 2.1 ± 0.3 mM ($n = 14$). Steady-state a^i_{Cl} in HCO_3^--free solution was 43.7 ± 0.9 mM ($n = 44$). The effect of adding 0.1 mM SITS simultaneously with the removal of ($HCO_3^- + CO_2$) buffer is summarized in Table II; the rapid initial rate of alkalinization is ~26% faster than that in the absence of SITS. Addition of SITS also revealed a greater alkalinization by 0.08 ± 0.02 pH units ($n = 6$, $P < 0.01$), and also reduced the increase of a^i_{Cl} by 1.6 ± 0.5 mM ($n = 4$). 0.3 mM DIDS completely inhibited the transient decrease in a^i_{Na} ($n = 1$). The results suggest that HCO_3^- efflux via Cl^-/HCO_3^- exchange can attenuate alkalinization. The absence of an effect of SITS on pH_i recovery from alkalinization because Cl^-/HCO_3^- exchange is inactivated in ($HCO_3^- + CO_2$)–free solution (see Discussion).

Acidification. Reintroducing ($HCO_3^- + CO_2$) resulted in a transient acidification (pH_i decreased by 0.22 ± 0.02, $n = 15$) that regulated to control pH_i (see also Fig. 1 *A*) and a transient increase in a^i_{Na} (3.3 ± 0.5 mM, $n = 8$). The increase in a^i_H by 50 nM is similar to the decrease by 53 nM that occurred upon removing ($HCO_3^- + CO_2$). However, the decrease in pH_i is smaller than the increase in pH_i, because (*a*) a^i_H change is plotted as pH_i, which is a nonlinear scale; (*b*) the acidification could be attenuated resulting from an increased intracellular buffering capacity; and (*c*) CO_2-induced acidification turns on regulating ion transport mechanisms such as Na^+/H^+ exchange. Application of 0.1 mM SITS did not significantly affect the CO_2-induced acidification, but attenuated the rate of regulation from acidification (without SITS, 0.08 ± 0.02 pH/min, $n = 5$; with SITS, 0.02 ± 0.01 pH/min, $n = 5$, $P < 0.05$). In Fig. 1 *B* a^i_{Cl} rapidly decreased to control value upon restoring HCO_3^-. SITS inhibited both the magnitude and rate of decrease in a^i_{Cl} induced by restoring HCO_3^- (see also Table II). In Cl^-_o-free solution (10 min) pH_i increased by ~0.07 ($n = 2$), indicating that Cl^-_o removal induced a HCO_3^- influx (i.e., Cl^-_i in exchange for HCO^-_{3o}.

Steady-state ^{36}Cl efflux and Cl^-_i

Strands were equilibrated with ^{36}Cl in control solution for 25 min, and the rate constant for ^{36}Cl efflux in the presence of 0.1 mM SITS was shown to decline from 0.61 ± 0.02 to 0.51 ± 0.02 ($n = 3$) ($P < 0.05$).

As mentioned above, in HCO_3^--buffered solution the apparent steady-state a^i_{Cl} significantly increased within 10 min in HCO_3^--free solutions ($P < 0.001$). Similar results were obtained from Cl^-_i content measurements. In HCO_3^--buffered solution 0.1 mM SITS or DIDS significantly increased a^i_{Cl} by 0.7 ± 0.1 mM ($n = 6$, $P < 0.001$) and decreased pH_i by 0.05 ± 0.01 ($n = 10$, $P < 0.001$) within 10 min. As expected, in HCO_3^--free solutions SITS did not significantly change a^i_{Cl} (−0.07 ± 0.10 mM, $n = 7$).

These results indicate that in cultured heart cells Cl^-/HCO_3^- exchange can regulate pH_i after acidification, and can provide a net Cl^- efflux and HCO_3^- influx in physiological conditions.

II. Cl^-/HCO_3^- Exchange and pH_i Regulation

A well-established method of studying the mechanism responsible for pH_i regulation involves the NH_4^+ prepulse protocol (Roos and Boron, 1981), by which cells are

TABLE II

Change in HCO_3^- Gradient Affects pH_i and a^i_{Cl}: Influence of 0.1 mM SITS

	(HCO_3^- + CO_2) removal				(HCO_3^- + CO_2) addition			
	ΔpH_i	$d(pH_i)/dt$	Δa^i_{Cl}	$d(a^i_{Cl})/dt$	ΔpH_i	$d(pH_i)/dt$	Δa^i_{Cl}	$d(a^i_{Cl})/dt$
Control	0.36 ± 0.05	0.95 ± 0.18	5.4 ± 0.6	5.0 ± 1.0	−0.22 ± 0.05	−0.81 ± 0.25	−5.1 ± 0.4	−3.7 ± 0.6
+SITS	0.44 ± 0.05*	1.19 ± 0.21*	3.8 ± 0.4*	2.8 ± 0.4‡	−0.18 ± 0.01	−0.77 ± 0.21	−2.3 ± 0.6‡	−1.6 ± 0.0‡
	(6)	(5)	(4)	(4)	(4)	(4)	(3)	(3)

Values are mean ± SE; number in parentheses indicates paired measurements.

ΔpH_i and Δa^i_{Cl} were defined as the peak change in pH_i and a^i_{Cl} in response to change in (HCO_3^- + CO_2) buffer. (Positive values for ΔpH_i: alkalinization; negative values: acidification. Positive values for Δa^i_{Cl}: influx; negative values: efflux.)

$d(pH_i)/dt$, initial rate (pH/minute) of change in pH_i.

$d(a^i_{Cl})/dt$, initial rate (millimolar/minute) of change in a^i_{Cl}.

*Comparison with control group, $P < 0.01$ (paired Student's t test).

‡Comparison with control group, $P < 0.02$ (paired Student's t test).

exposed to NH_4^+-salt solution for a period of time and then returned to control solution. To examine the possible role of Cl^-/HCO_3^- exchange in pH_i regulation, we followed a standard protocol in which cells were exposed to 10 mM NH_4Cl for 5 min and then returned to control solution. We monitored pH_i, a^i_{Cl}, and Cl_i^- content during this procedure.

The data in Fig. 2 were obtained with a concentric double-barreled pH-sensitive microelectrode and illustrate a typical recording of changes in pH_i induced by the NH_4^+ prepulse. In HCO_3^--buffered solution, exposure of cells to 10 mM NH_4Cl increased pH_i by 0.20 ± 0.01 pH units (n = 51), which then returned close to the control level. Removal of NH_4Cl induced a rapid acidification (a decrease of pH_i by 0.30 ± 0.09, n = 70) followed by a rapid regulation at an initial rate of 0.32 ± 0.03

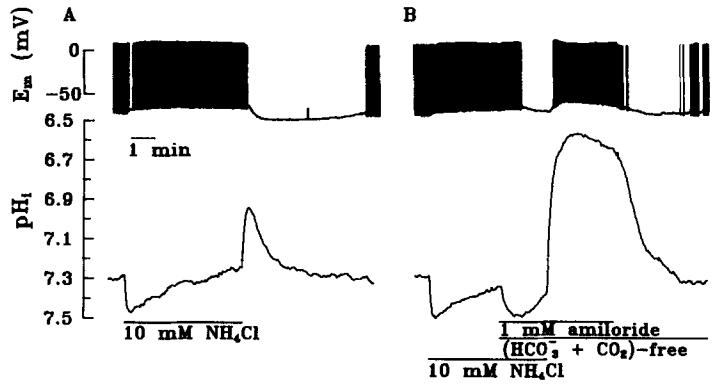

FIGURE 2. Inhibition of Na^+/H^+ exchange with amiloride affects pH_i in HCO_3^--free solution. *A* shows a representative recording of a change in pH_i (lower trace) and E_m (upper trace) in response to NH_4^+ prepulse. Cells exposed to 10 mM NH_4Cl (HCO_3^--buffered solution) for 5 min underwent a transient alkalinization followed by a slow recovery. Removal of NH_4Cl induced a transient acidification that regulated to control level in 5 min. *B* shows a recording obtained from cells exposed to 10 mM NH_4Cl. After 3 min, removal of (HCO_3^- + CO_2) simultaneously with the addition of 1 mM amiloride caused a transient alkalinization (see also Fig. 1 *A*). Removal of NH_4Cl then caused a substantial decrease in pH_i that was maintained until the washout of amiloride, at which time pH_i recovered to control.

pH units/min (n = 65). pH_i recovery from acidification was accompanied by a membrane hyperpolarization, presumably due to stimulation of the Na^+/K^+ pump (Piwnica-Worms et al., 1985).

pH_i regulation in HCO_3^--free solution

pH_i regulation in cardiac muscle has been studied extensively in HCO_3^--free solution, a condition that inactivates Cl^-/HCO_3^- exchange. Thus, when pH_i recovery was attenuated in the presence of amiloride, the conclusion was proposed that Na^+/H^+ exchange is the main mechanism regulating pH_i from an acid load (Deitmer and Ellis, 1980; Ellis and MacLeod, 1985; Frelin et al., 1985; Kaila and Vaughan-Jones, 1987). Similar conclusions were proposed from data obtained in our labora-

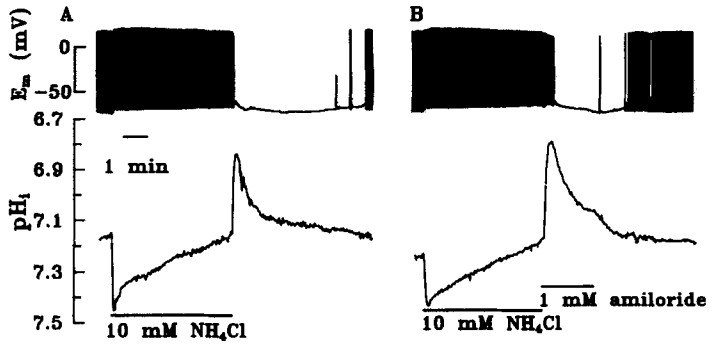

FIGURE 3. Inhibition of Na^+/H^+ exchange with amiloride affects pH_i in HCO_3^--buffered solution. *A* shows a control change in pH_i (lower trace) and E_m (upper trace) in response to NH_4^+ prepulse. In *B*, cells were first exposed to 10 mM NH_4Cl for 5 min; addition of 1 mM amiloride simultaneously with removal of NH_4Cl enhanced the magnitude of acidification and slowed the pH_i recovery.

tory using cultured chick heart cells (Piwnica-Worms et al., 1985). The protocol in Fig. 2 *B* also confirms this finding: at the third minute of a NH_4Cl pulse, removal of (HCO_3^- + CO_2) buffer and addition of 1 mM amiloride caused a transient alkalinization. Under this condition, removal of NH_4Cl intensified the acidification and amiloride inhibited pH_i recovery. Washout of amiloride led to a complete recovery of pH_i.

Inhibition of Na^+/H^+ exchange on pH_i recovery in HCO_3^--buffered solution

When Na^+/H^+ exchange was identified as an important pH_i-regulating mechanism after an acid load in cultured chick heart cells (Piwnica-Worms et al., 1985), no

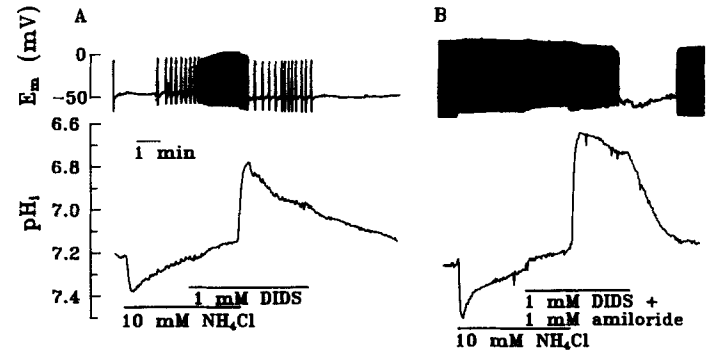

FIGURE 4. Inhibition of Cl^-/HCO_3^- exchange with DIDS affects pH_i. (*A*) Polystrands were exposed to 10 mM NH_4Cl; after 3 min, 1 mM DIDS was added to the NH_4Cl solution. Upon removal of NH_4Cl the sustained presence of DIDS promoted intracellular acidification that recovered very slowly toward control. Upon washout of DIDS, pH_i recovery was delayed because DIDS dissociates slowly from membrane binding sites. (*B*) Similarly, in another experiment, at the third minute of a 5-min exposure to 10 mM NH_4Cl solution, 1 mM DIDS and 1 mM amiloride were added. Removal of NH_4Cl now resulted in a sustained acidification which returned to control level only after washout of amiloride and DIDS.

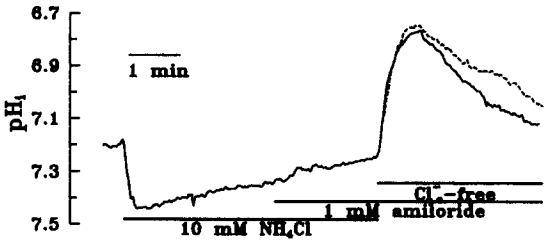

FIGURE 5. Cl_o^- removal affects pH_i recovery during inhibition of Na^+/H^+ exchange. Polystrand was first exposed to 10 mM NH_4Cl, and 1 mM amiloride was added after 3 min. Removing Cl_o^- in NH_4^+-free solution accelerated pH_i recovery. The superimposed trace (dashed line) shows the change in pH_i that occurred upon removal of NH_4Cl in 134 mM $[Cl^-]_o$.

direct quantification of pH_i was made because amiloride interfered with the optical response of the pH-sensitive dye, 6-carboxy-fluorescein. To circumvent this problem we used pH-selective microelectrodes to examine further the contribution of Na^+/H^+ exchange to pH_i regulation. Amiloride was added either 2 min before or simultaneously with removal of NH_4Cl (Fig. 3 *B*); acidification was augmented by 0.11 ± 0.02 pH units ($n = 13$, $P < 0.01$) and reduced the rate of pH_i recovery by 27.1 ± 4.3% ($n = 12$). These results suggest that pH_i regulation from an acid load does not involve only Na^+/H^+ exchange.

Inhibition of Cl^-/HCO_3^- exchange affects pH_i recovery

Based on experiments such as those described in Fig. 2, Cl^-/HCO_3^- exchange may also regulate pH_i after acidification. Thus, we tested the effect of DIDS, a Cl^-/HCO_3^- exchange inhibitor, on pH_i regulation. After exposure to NH_4Cl, 0.1 mM DIDS added either before or simultaneously with NH_4Cl removal augmented acidification by 0.05 ± 0.01 pH units ($n = 12$) and attenuated the rate of pH_i recovery by 34.9 ± 4.7% ($n = 9$). Increasing the concentration of DIDS to 1 mM (Fig. 4 *A*) caused a further decrease of the rate of pH_i recovery by 46.6 ± 11.2% ($n = 4$).

Application of 1 mM DIDS plus 1 mM amiloride during the NH_4Cl pulse slightly decreased pH_i (Fig. 4 *B*). Upon removal of NH_4Cl the acidification response was

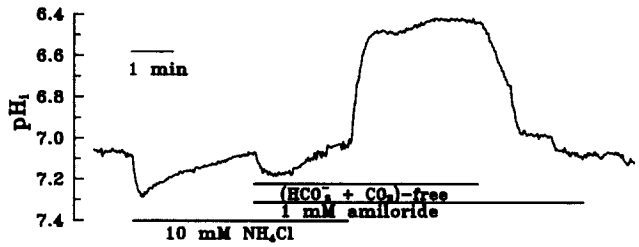

FIGURE 6. pH_i recovery is HCO_3^- dependent during inhibition of Na^+/H^+ exchange. After the third minute of a 10-mM NH_4Cl pulse the polystrand was exposed to HCO_3^--free plus 1 mM amiloride solution. Upon washout of NH_4Cl, the continuing absence of HCO_3^- caused a significant maintained acidification. Reintroducing HCO_3^- (in the presence of amiloride) caused pH_i to recover rapidly to the control level.

magnified by 0.34 pH units (control, -0.26 ± 0.06 pH, $n = 5$; with DIDS plus amiloride, -0.60 ± 0.03 pH, $n = 5$) and pH_i recovery was almost completely blocked. From these results we conclude that a DIDS-sensitive Cl^-/HCO_3^- exchange can regulate pH_i after intracellular acidification. Furthermore, instead of causing a hyperpolarization DIDS plus amiloride depolarized E_m (Fig. 4 *B*) (see Discussion).

Cl_o^- removal affects pH_i recovery

From a thermodynamic standpoint, in the presence of a Cl^-/HCO_3^- exchange removing Cl_o^- should increase the driving force for net HCO_3^- influx and accelerate

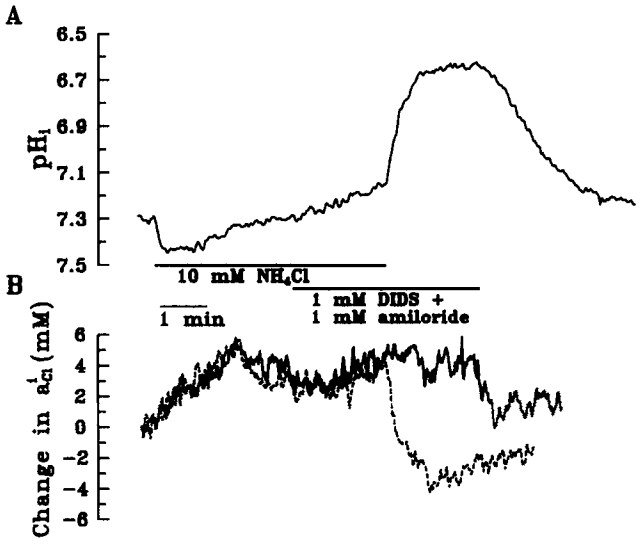

FIGURE 7. Inhibition of pH_i recovery affects a_{Cl}^i. Polystrand was exposed to 10 mM NH_4Cl in the absence or presence of 1 mM amiloride plus 1 mM DIDS; pH_i (*A*) and a_{Cl}^i (*B*) were monitored simultaneously with a double-barreled pH-selective microelectrode and a single-barreled Cl-selective microelectrode. In the absence of amiloride and DIDS, NH_4Cl pulse induced an increase in a_{Cl}^i. Removing NH_4Cl resulted in a rapid decrease in a_{Cl}^i (dashed line in *B*). If 1 mM amiloride plus 1 mM DIDS were added at the third minute of a 5-min NH_4Cl pulse, removal of NH_4Cl did not lead to a decrease of a_{Cl}^i (*B*) or a recovery of pH_i (*A*). Washout of DIDS and amiloride caused a decrease in a_{Cl}^i (*B*) and pH_i recovery (*A*).

pH_i recovery from acidification. Fig. 5 shows that inhibition of Na^+/H^+ exchange coupled with removal of Cl_o^- (solid line) indeed facilitates the rate of pH_i recovery in paired experiments by 50 ± 21% ($n = 3$) as compared with that in 134 mM $[Cl^-]_o$ plus amiloride (dashed line).

pH_i recovery in the presence of amiloride is HCO_{3o}^- dependent

If at the third minute of the NH_4Cl pulse cells were exposed to HCO_3^--free solution containing 1 mM amiloride, and the subsequent removal of NH_4Cl resulted in a substantial maintained acidification and a complete inhibition of pH_i recovery (Fig.

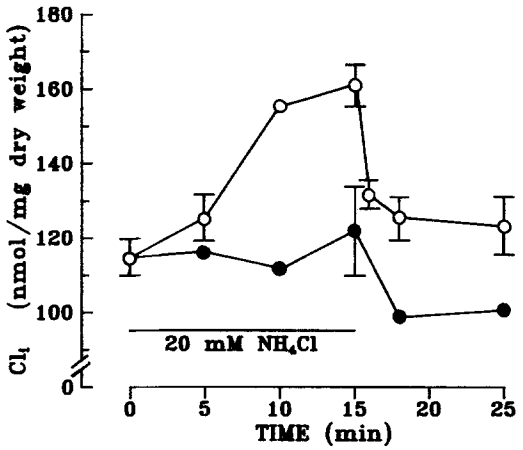

FIGURE 8. Inhibition of Cl^-/HCO_3^- exchange with DIDS affects Cl_i^- content. Exposure to 20 mM NH_4Cl for 15 min caused an increase in Cl_i^- content; removal of NH_4Cl induced a rapid decrease in Cl_i^- content (open circles). When 0.1 mM DIDS was present throughout the experiment, the changes in Cl_i^- were markedly attenuated (filled circles).

6). Even with the continued inhibition of Na^+/H^+ exchange, reintroduction of $[HCO_3^-]_o$ still led to a rapid recovery of pH_i.

Changes in pH_i affects Cl_i^-

Fig. 7 illustrates simultaneously obtained measurements of a^i_{Cl} and pH_i during and after the NH_4Cl pulse. Cells exposed to 10 mM NH_4Cl increased a^i_{Cl} by 3.7 ± 0.3 mM (n = 16), a Cl^- influx occurring against Cl^- electrochemical gradient. After 5 min a^i_{Cl} stabilized at a level 2.5 ± 0.4 mM (n = 10) above control. Removing NH_4Cl resulted in a rapid decrease in a^i_{Cl} (lower trace) at an initial rate of 0.25 ± 0.05 mM/s (n = 9). 1 mM DIDS coupled with 1 mM amiloride completely inhibited the decrease of a^i_{Cl} (Fig. 7 *B*, upper trace) as well as pH_i recovery from acidification (Fig.

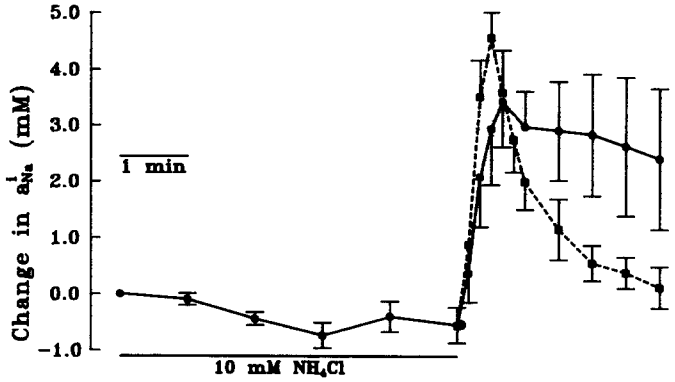

FIGURE 9. Amiloride affects a^i_{Na} during pH_i recovery. Exposure to 10 mM NH_4Cl caused a small decrease in a^i_{Na}. Removing NH_4Cl induced a rapid increase in a^i_{Na} that gradually regulated to the control value (dashed line). 1 mM amiloride, which was added either before or simultaneously with removal of NH_4Cl, slightly reduced the rate of the increase in a^i_{Na} and blocked the a^i_{Na} recovery (solid line). Data presented are means ± SE from five paired measurements.

7 *A*). Washout of DIDS and amiloride led to the resumption of Cl efflux and pH_i recovery.

When the Cl^- coulometric titration method was used to monitor changes in Cl_i^- content after a 15-min exposure to 20 mM NH_4Cl, we observed an increase in Cl_i^- content by ~50%; removal of NH_4Cl resulted in a decrease of Cl_i^- (Fig. 8). The higher concentration and longer exposure to NH_4Cl in this protocol may have contributed to greater net Cl movements detected by content measurements compared with the results obtained with ISME. After a similar protocol in 20 mM NH_4Cl, its removal increased the rate constant of ^{36}Cl efflux from 0.70 ± 0.01 (n = 3) to 0.82 ± 0.05 min^{-1} (n = 3). Fig. 8 (lower trace) shows that the presence of 0.1 mM DIDS throughout the NH_4Cl prepulse experiment suppressed net countergradient Cl^- uptake (~70%) during exposure to NH_4Cl and net Cl^- washout upon NH_4Cl removal. Furthermore, if the NH_4Cl prepulse experiment was carried out in HCO_3^--free solution, no significant change in Cl_i^- content was detected. The findings

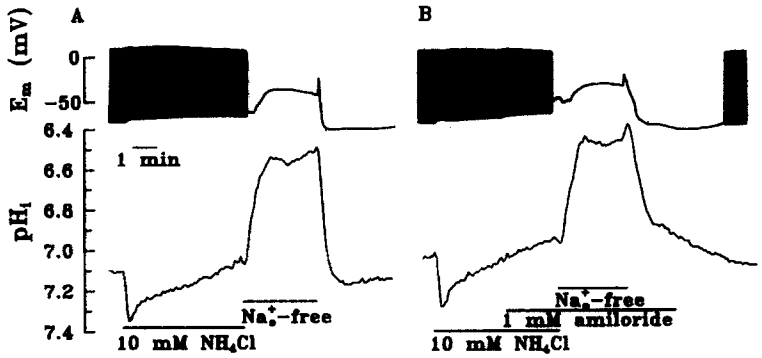

FIGURE 10. pH_i recovery is Na_o^+ dependent. In *A*, after a 5-min exposure to 10 mM NH_4Cl, simultaneous removal of Na_o^+ and NH_4Cl resulted in a substantial maintained acidification that rapidly regulated to control level upon reintroducing Na_o^+. In *B*, after inhibition of Na^+/H^+ exchange by the addition of 1 mM amiloride, removal of Na_o^+ and NH_4Cl in the presence of amiloride still resulted in a sustained acidification. Restoring Na_o^+ regulates pH_i to the control level even during maintained inhibition of Na^+/H^+ exchange.

thus support the concept that Cl^-/HCO_3^- exchange can regulate pH_i in response to intracellular alkalinization and acidification.

pH_i regulation and a_{Na}^i

Alkalinization. Exposure of cells to NH_4Cl caused a decrease in a_{Na}^i by 1.5 ± 0.2 mM (n = 19) accompanied by an increase in pH_i, indicating that net outward Na^+ movement is against its electrochemical gradient.

Acidification. Removing NH_4Cl increased a_{Na}^i by 5.5 ± 0.5 mM (n = 12) at an initial rate of 0.24 ± 0.03 mM/s (n = 12). The increase in a_{Na}^i then returned relatively slowly to the control level (Fig. 9). Removal of NH_4Cl in the presence of 1 mM amiloride reduced the rate of increase in a_{Na}^i by 30.3 ± 13.4% (n = 8) but did not significantly inhibit the augmented level of a_{Na}^i. Amiloride also appeared to markedly attenuate the recovery rate of a_{Na}^i in six of eight experiments.

Na_o^+ dependence of Cl^-/HCO_3^- exchange in pH_i regulation

Removal of Na_o^+ resulted in an amiloride-sensitive acidification (Piwnica-Worms et al., 1985). We applied the NH_4Cl prepulse protocol to determine the mechanism by which removal of Na_o^+ affects pH_i regulation. Fig. 10 *A* shows that the simultaneous removal of Na_o^+ with NH_4Cl resulted in a substantial maintained acidification. In Figs. 4 and 7 we showed that in HCO_3^--buffered solution both amiloride-sensitive Na^+/H^+ exchange and DIDS-sensitive Cl^-/HCO_3^- exchange are involved in pH_i regulation after an acid load. pH_i recovery can occur after inhibition of Na^+/H^+ exchange by the action of the Cl^-/HCO_3^- exchange. If the sustained acidification in Na_o^+-free solution is due only to inhibition or reversal of Na^+/H^+ exchange, then acidification induced by NH_4^+ removal should be reversed by Cl^-/HCO_3^- exchange. However, as shown in Fig. 10 *A*, in Na_o^+-free solution (HCO_3^- buffer), pH_i did not

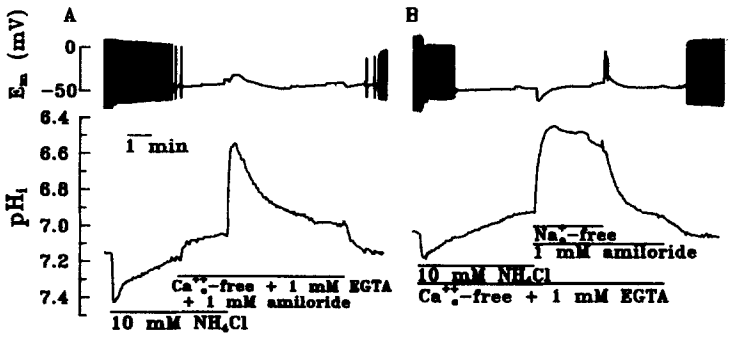

FIGURE 11. Inhibition of pH_i recovery by Na_o^+ removal is not affected by Ca_o^{2+}. In *A*, cells were incubated for 3 min in 10 mM NH_4Cl and then exposed to Ca_o^{2+} -free solution plus 1 mM EGTA and 1 mM amiloride. Removing NH_4Cl in this Ca_o^{2+} -free solution induced a transient acidification that still regulated toward a level slightly more acidic than control pH_i (lower trace). Return to control solution led to a complete recovery of pH_i. *B* shows that cells were exposed for 5 min to a Ca_o^{2+}-free EGTA (1 mM) solution containing 10 mM NH_4Cl, and simultaneous removal of Na_o^+ and NH_4Cl in the presence of 1 mM amiloride caused a sustained acidification. Reintroducing Na_o^+ regulated pH_i to a level slightly more acidic than control; pH_i completely recovered upon returning to control solution.

recover upon removing NH_4Cl until Na_o^+ was restored. We then tested whether removal of Na_o^+ is still capable of affecting pH_i recovery after amiloride inhibition of Na^+/H^+ exchange. Fig. 10 *B* shows that in the presence of amiloride the simultaneous removal of Na_o^+ and NH_4Cl augmented the acidification (normal Na_o^+, -0.35 ± 0.01 pH units; Na_o^+ free, -0.49 ± 0.03 pH units, $n = 5$) and completely blocked pH_i regulation. Since the reintroduction of Na_o^+ led to the return of pH_i to control levels, these findings suggest that Cl^-/HCO_3^- exchange is Na_o^+ dependent.

Inhibition of pH_i recovery by Na_o^+ removal is not affected by removal of Ca_o^{2+}

In this study we have shown that in the presence of amiloride removal of Na_o^+ caused a sustained acidification by inhibiting the Cl^-/HCO_3^- exchange. In previous studies

we also showed that Na_o removal reverses Na^+/Ca^{2+} exchange to cause an increase in Ca_i^{2+} (Jacob et al., 1987), which in turn can decrease pH_i via a Ca^{2+}-H^+ interaction (Liu and Lieberman, 1989). Therefore, we attempted to determine if the sustained acidification in Na_o^+-free plus amiloride solution could relate to a change in Ca_i^{2+}. When the NH_4Cl prepulse experiments were carried out in a Ca_o^{2+}-free solution containing 1 mM EGTA and 1 mM amiloride, we found that acidification induced by NH_4Cl removal recovered to a level slightly more acidic than control pH_i (Fig. 11 *A*). If Na_o^+ and NH_4Cl were removed simultaneously in the presence of 1 mM amiloride and the absence of Ca_o^{2+}, a sustained acidification occurred which then returned to control level upon reintroducing Na_o^+ (Fig. 11 *B*). This finding shows that (*a*) Ca_o^{2+} does not affect the Na_o^+ requirement of Cl^-/HCO_3^- exchange in regulating pH_i, and (*b*) the sustained acidification resulting from inhibition of pH_i recovery in Na_o^+-free solution cannot be attributed to Na^+/Ca^{2+} exchange and Ca^{2+}-H^+ interaction.

DISCUSSION

pH_i and Buffering Capacity

pH_i of cultured chick heart cells, measured in HCO_3^--buffered solution at 37°C with pH-selective microelectrodes, is 7.12 ± 0.01 ($n = 46$) in agreement with values of pH_i reported from other cardiac muscle cells (Ellis and Thomas, 1976; reviewed by Roos and Boron, 1981). Although most measurements in this study were made with single-barreled pH microelectrodes in combination with conventional voltage microelectrodes, the values of pH_i do not differ from those measured with double-barreled pH microelectrodes.

The total intracellular buffering capacity (β_T) can be described by the sum of intrinsic buffering power (β_I) and that attributed to ($HCO_3^- + CO_2$) buffer (β_{CO_2}) (Roos and Boron, 1981), the latter determined by multiplying $[HCO_3^-]_i$ by the factor 2.3. According to the Henderson-Hasselbalch equation, $[HCO_3^-]_i$ is calculated to be 13.0 mM at a pH_i of 7.12, assuming that (*a*) $[HCO_3^-]_i$ depends only on pH_i, $[CO_2]_i$ and the partial pressure of CO_2, and (*b*) the carbonic acid dissociation constant and the solubility coefficient for CO_2 are identical on either side of the cell membrane. The calculated value of β_{CO_2} for cultured chick heart cells is 29.9 mM/pH unit. β_I, which can be estimated by dividing the change in $[HCO_3^-]_i$ by the associated change in pH_i upon removing $[HCO_3^-]_o$ (as shown in Fig. 1), is 59.4 mM/pH unit, compatible with the values reported for other cardiac tissues (for review, see Roos and Boron, 1981). Therefore, β_T of cultured heart cells is 89.3 mM/pH unit in ($HCO_3^- + CO_2$) solutions.

Na_o^+- dependent Cl^-/HCO_3^- Exchange vs. Cl^-/HCO_3^- Exchange

We first demonstrated a DIDS- or SITS-sensitive Cl^-/HCO_3^- exchange in cultured chick heart cells by showing that (*a*) removing ($HCO_3^- + CO_2$) buffer results in an increase in pH_i and a_{Cl}^i (SITS accentuates the alkalinization and attenuates the increase of a_{Cl}^i), (*b*) reintroducing ($HCO_3^- + CO_2$) buffer results in a SITS-sensitive pH_i recovery from acidification and a decrease in a_{Cl}^i, and (*c*) removing Cl_o causes a

countergradient increase in HCO_{3i}^-.[1] The SITS-sensitive changes in pH_i and a_{Cl}^i that occur when the HCO_3^- gradient is manipulated suggest that Cl^-/HCO_3^- exchange can regulate pH_i after cell acidification and can cause cell alkalinization. In contrast, the suggestion proposed for sheep Purkinje fibers is that Cl^-/HCO_3^- exchange appears to regulate pH_i only after alkalinization (Vaughan-Jones, 1982). Interestingly, a_{Cl}^i decreased when sheep Purkinje fibers were exposed to HCO_3^--free solution (Vaughan-Jones, 1979), a finding contrary to what would be expected based on the results of the Cl^-/HCO_3^- exchanger in cultured chick heart cells.

We showed that under physiological conditions application of SITS or DIDS increases a_{Cl}^i, reduces the rate constant of steady-state ^{36}Cl efflux, and decreases pH_i, suggesting that Cl^-/HCO_3^- exchange provides a steady-state Cl^- efflux and HCO_3^- influx. Heinemeyer and Bay (1987) similarly reported that 0.5 mM SITS acidified pH_i in mammalian ventricle. If measurements obtained from our data are used to calculate the thermodynamic driving force for a putative Cl^-/HCO_3^- exchange that is solely dependent on the Cl^- and HCO_3^- gradient, one would expect a net Cl^- influx and HCO_3^- efflux. This being contrary to our experimental observations, we thus propose that the stilbene derivative–sensitive Cl^-/HCO_3^- exchange in cultured chick heart cells must be coupled to an additional ion gradient to reverse the net direction.

Removal of (HCO_3^- + CO_2) causes a transient alkalinization (due to rapid CO_2 efflux) that is accompanied by a countergradient increase of a_{Cl}^i and a countergradient decrease of a_{Na}^i . As shown in Table II, inhibition of Cl^-/HCO_3^- exchange in the presence of SITS or DIDS enhances the rate and magnitude of alkalinization, attenuates the increase in a_{Cl}^i, and inhibits the transient decrease of a_{Na}^i. These findings demonstrate that upon removing (HCO_3^- + CO_2) buffer HCO_3^- efflux via Cl^-/HCO_3^- exchange attenuates cell alkalinization induced by a rapid CO_2 efflux. SITS has no effect on the rate of pH_i recovery from alkalinization because this pH_i is rather due to dissipation of CO_2 and HCO_3^- gradients. Conversely, the introduction of (HCO_3^- + CO_2) results in a transient acidification (due to rapid CO_2 influx) followed by a SITS-sensitive pH_i recovery that is accompanied by a transient increase in a_{Na}^i and a decrease in a_{Cl}^i. Similar changes in ion content occurred on exposure to NH_4Cl (alkalinization) and on its removal (acidification). Consequently, Na^+ transport must be coupled with HCO_3^- movement in exchange for Cl^-, supporting the hypothesis that manipulating the HCO_3^- gradient can change a_{Na}^i, a_{Cl}^i, and pH_i via a Na^+-dependent Cl^-/HCO_3^- exchange mechanism.

Using the NH_4^+ prepulse method, we presented evidence in Fig. 10 showing that the induced acid load cannot be regulated in Na_o^+-free solution, thus rendering unlikely the possibility that Na^+-independent Cl^-/HCO_3^- exchange is involved in pH_i regulation. Therefore, under acid conditions pH_i regulation only involves a Na_o^+-dependent process. We also confirmed that the maintained acidification in Na_o^+-free (with or without Ca_o^{2+}-free) amiloride-containing solution cannot be attributed to the combined effect of Na_o^+ removal on Na^+/Ca^{2+} exchange and Ca^{2+}-H^+ interaction (Fig. 11). In addition, the presence of an inhibitor of (Na^+ + K^+ + $2Cl^-$)

[1] In Cl^--free medium, ~90% of the Cl^- loss is through (Na^+ + K^+ + $2Cl^-$) cotransport, the mechanism for maintaining high Cl_i^- in our preparations (Liu et al., 1987).

cotransport (0.1 mM bumetanide) throughout the NH_4Cl prepulse experiment does not affect the inhibition of pH_i recovery caused by removing Na_o^+. Furthermore, the decrease in a_{Cl}^i by <5 mM during a 5-min exposure to Na_o^+-free MEBSS causes an insignificant change to the calculated driving force of Cl^-/HCO_3^- exchange. Therefore, inhibition of pH_i recovery upon removal of Na_o^+ cannot be secondary to a decrease in Cl_i^- via ($Na^+ + K^+ + 2Cl^-$) cotransport.

From a thermodynamic standpoint, a Na_o^+-dependent Cl^-/HCO_3^- exchange should provide an inward transport of HCO_3^- in exchange for Cl_i^-. This would allow a complete recovery of pH_i to occur after removal of NH_4Cl in the presence of amiloride (Figs. 6 and 11). Under these conditions, the driving force for a putative (Na^+-independent) Cl^-/HCO_3^- exchange would allow pH_i to return only to ~6.8 (based on calculations from the values of a_{Cl}^i and $[HCO_3^-]_i$). In the absence of Na_o^+, the lack of pH_i recovery indicates that this stilbene-sensitive, HCO_3^--dependent Cl^- transport and acid extrusion (amiloride-insensitive) mechanism must be a Na_o^+-dependent Cl^-/HCO_3^- exchange.

Amiloride-insensitive Increase of a_{Na}^i

When NH_4Cl is removed cardiac cells rapidly take up Na^+, which is then expelled by a recovery process that is accompanied by membrane hyperpolarization, i.e., secondary stimulation of the Na^+/K^+ pump (Piwnica-Worms et al., 1985). Amiloride does not inhibit Na^+ uptake but attenuates Na_i^+ recovery so that the net effect is a gain of a_{Na}^i (Fig. 9). The relatively slow a_{Na}^i recovery indicates that the Na^+/K^+ pump is less active than in the absence of amiloride. This finding cannot be explained by a change in cell volume for the following reasons: (*a*) direct monitoring of the preparations did not reveal a detectable change in cell volume during the NH_4^+ prepulse experiment (see Methods), and (*b*) a_{Cl}^i and a_{Na}^i measurements are compatible with Cl_i^- and Na_i^+ content measurements, confirming that we are observing activity changes resulting from net transmembrane ion transport. Nevertheless, this amiloride-insensitive net Na^+ uptake is consistent with a Na^+ influx mediated by Na_o^+-dependent Cl^-/HCO_3^- exchange.

However, in one experiment 1 mM DIDS did not block, but rather augmented, the amiloride-insensitive gain of a_{Na}^i. This unexpected net a_{Na}^i gain may result from inhibition of the Na^+/K^+ pump by an increase in a_{Ca}^i (>1 μM; Turi and Somogyi, 1988) and by intracellular acidification (Breitwieser et al., 1987), as well as stimulation of Na^+/Ca^{2+} exchange by an increase of a_{Ca}^i caused by an intracellular acidification and a rapid depolarization. In the absence of amiloride or DIDS, removal of NH_4Cl caused increases both in a_{Ca}^i in sheep Purkinje fibers (Bers and Ellis, 1982) and in free Ca_i^{2+} (<1 μM) as recently measured with fura-2 in cultured chick heart cells (Freudenrich, C. C., personal communication). The augmented and sustained acidification (pH_i 6.5) that was accompanied by E_m depolarization in the presence of amiloride and DIDS would be expected to increase Ca_i^{2+} to a greater degree than in the absence of these agents (Figs. 2 *B*, 4 *B*, 6, and 7 *A*). The observed membrane depolarization supports the hypothesis that amiloride- and DIDS-insensitive gain of Na^+ activity results from inhibition of the Na^+/K^+ pump and stimulation of Na^+/Ca^{2+} exchange.

Na_o^+-dependent Cl^-/HCO_3^- Exchange vs. Na^+/H^+ Exchange

We demonstrated that pH_i recovery from acidification involves both Na^+/H^+ exchange and Na_o^+-dependent Cl^-/HCO_3^- exchange. Inhibition of both mechanisms leads to a complete block of pH_i recovery after an acid load. Although caution must be used in applying pharmacological agents to quantitatively define a transport mechanism, Na^+/H^+ exchange seems slightly more sensitive to acidification than Na_o^+-dependent Cl^-/HCO_3^- exchange. Upon removal of NH_4Cl, our findings show a greater acidification in 1 mM amiloride than in 1 mM DIDS. However, the rate of pH_i recovery is reduced by 35% and 47% in 0.1 and 1 mM DIDS, respectively, whereas 1 mM amiloride only reduces the rate of pH_i recovery by 27%. In addition, the amiloride-sensitive H^+ efflux is 16.9 ± 4.0 pmol · cm^{-2} · s^{-1} as compared with the DIDS-sensitive H^+ efflux of 28.2 ± 4.5 pmol · cm^{-2} · s^{-1}. Under these conditions, Na_o^+-dependent Cl^-/HCO_3^- exchange is more efficient than Na^+/H^+ exchange in regulating pH_i from an acid load.

Our results also show that on NH_4Cl removal, inhibition of Na^+/H^+ exchange augments the initial rate of decrease in a_{Cl}^i by 23.6 ± 10.4% ($n = 3$, $P < 0.05$), indicating an enhanced rate of Na_o^+-dependent Cl^-/HCO_3^- exchange. The other difference between Na^+/H^+ exchange and an amiloride-insensitive, Na_o^+-dependent Cl^-/HCO_3^- exchange is that Na^+/H^+ exchange is activated only after pH_i decreases beyond a threshold (Roos and Boron, 1981), whereas Na_o^+-dependent Cl^-/HCO_3^- exchange remains active under physiological conditions and is stimulated after acidification. Even though different modes of Cl^-/HCO_3^- exchange exist in a variety of cell preparations, kinetic studies consistently imply that Cl^-/HCO_3^- exchange is more sensitive to the HCO_3^- gradient, namely, pH gradient, than to the Cl gradient (Boron and Russell, 1983; Vaughan-Jones, 1986; Ahearn et al., 1987). This may account for the response of Na_o^+-dependent Cl^-/HCO_3^- exchange to pH_i change in cultured chick heart cells.

Na_o-dependent Cl^-/HCO_3^- Exchange vs. Other pH_i-regulating Systems

When NH_4Cl is removed, total acid extrusion rate (60.5 ± 4.9 pmol · cm^{-2} · s^{-1}, $n = 16$) induces a Na^+ influx (33.6 ± 5.7 pmol · cm^{-2} · s^{-1}, $n = 6$) and a Cl^- efflux (35.7 ± 6.9 pmol · cm^{-2} · s^{-1}, $n = 9$) that is calculated from the change in a_{Cl}^i and agrees with ^{36}Cl efflux measurements. After acidification, we observed an amiloride-insensitive Na^+ influx of 20.8 ± 7.0 pmol · cm^{-2} · s^{-1} ($n = 6$), a Cl^- efflux of 27.9 ± 4.1 pmol · cm^{-2} · s^{-1} ($n = 3$), and an acid extrusion of 38.6 ± 5.2 pmol · cm^{-2} · s^{-1} ($n = 12$). The ratio of acid extrusion, Na^+ influx, and Cl^- efflux is close to 2:1:1, in agreement with an electroneutral ($Na^+ + HCO_3/Cl^- + H^+$) exchange as reported in the snail neurone (Thomas, 1977), barnacle muscle (Boron et al., 1981), squid axon (Boron and Russell, 1983), basolateral membrane of the *Necturus* proximal tubule (Guggino et al., 1983), and leech neurone (Deitmer and Schlue, 1987). This Na^+-dependent Cl^-/HCO_3^- exchange system may be consistent with an ion pair model, i.e., $NaCO_3^-/Cl^-$ exchange (Becker and Duhm, 1978); however, this $NaCO_3^-/Cl^-$ exchange has not been shown to be reversible, i.e., inducing a Cl^- influx (Boron, 1986). This system may also be similar to an electrogenic Na^+-HCO_3^- cotransport (Fitz et al., 1989), which should cause change

in E_m by 10–20 mV in response to the HCO_3^- gradient change. However, we observed only a slight change in E_m (0–2 mV) (Fig. 2 *B*), and this change was not affected by DIDS or SITS. Furthermore, acidification induced by NH_4Cl removal activates Na^+-dependent anion exchange and results in a hyperpolarization of E_m which is inhibited by ouabain (Piwnica-Worms et al., 1985) but not DIDS. The estimate of stoichiometry lends further support to the hypothesis that Na^+-dependent Cl^-/HCO_3^- exchange is an electroneutral process and not an electrogenic Na^+-HCO_3^- cotransport.

In cultured chick heart cells, in addition to ($Na^+ + HCO_3/Cl^- + H^+$) exchange, our findings reveal an acidification-induced, amiloride-sensitive Na^+ uptake and acid extrusion. The similar dual pH_i-regulating system, i.e., Na^+/H^+ exchange and ($Na^+ + HCO_3/Cl^- + H^+$) exchange, is also found in crayfish neurones (Moody, 1981) and leech neurones (Schlue and Thomas, 1985; Deitmer and Schlue, 1987).

In Purkinje fibers Na^+/H^+ exchange appears to be the main pH_i-regulating mechanism after an acid load (Deitmer and Ellis, 1980; Vanheel et al., 1984). However, the results do not sufficiently rule out the involvement of Cl^-/HCO_3^- exchange because a controversy exists over the potency of anion exchanger inhibitors used to demonstrate Cl^-/HCO_3^- exchange. For example, Deitmer and Ellis (1980) showed that in the presence of 1 mM amiloride pH_i still recovers slowly in a HCO_3^--free 1-mM SITS solution. Perhaps 1 mM DIDS, a more potent inhibitor of Cl^-/HCO_3^- exchange, would have been more effective for such a study in HCO_3^--buffered solution. In addition, Vanheel et al. (1984) also demonstrated that 0.1 mM SITS and Cl^--free solutions do not impair pH_i recovery from acidification. However, Vaughan-Jones (1982) showed that in HCO_3^--buffered solution 0.18 mM DIDS inhibits the a_{Cl}^i changes in response to NH_4^+ prepulse and slightly reduces pH_i recovery, and attenuates pH_i recovery after an acid load induced by NH_4Cl (Vaughan-Jones, 1982, Fig. 6). These findings are, in fact, consistent with Cl^-/HCO_3^- exchange in pH_i regulation after alkalinization and acidification. If the experiments to examine the effect of DIDS were combined with the inhibition of Na^+/H^+ exchange, then the results would have more clearly identified the transport mechanisms that participate in pH_i recovery from an acid load in Purkinje fibers. Similarly, experimental conditions in a previous study from our laboratory (Piwnica-Worms et al., 1985) did not exclude the possible involvement of Cl^-/HCO_3^- exchange in pH_i recovery. First, DIDS (0.1 mM)-sensitive H^+ extrusion did not occur upon removal of NH_4Cl. However, the experiment was performed in HCO_3^--free solution so that Cl^-/HCO_3^- exchange was inactivated. Second, in HCO_3^--buffered solution, 0.1 mM DIDS only slightly decreased the acid-induced Na^+ uptake. We now show that pH_i recovery via Na^+/H^+ exchange can occur in the presence of DIDS even if the concentration of DIDS is raised to 1 mM. Therefore, to evaluate a DIDS-sensitive component of pH_i regulation, the effect of maximal inhibitory doses of DIDS on Na^+ uptake should be examined in the presence of amiloride.

Steady-state pH_i Maintenance and Physiological Importance of Na_o^+-dependent Cl^-/HCO_3^- Exchange

Steady-state pH_i of cultured heart cells is ~1 pH unit more alkaline than expected for H^+ electrochemical equilibrium. To maintain this gradient requires mechanisms

of either H^+ extrusion, e.g., Na^+/H^+ exchange, or HCO_3^- (OH^-) influx, e.g., Na_o^+-dependent Cl^-/HCO_3^- exchange, or both. In HCO_3^--buffered solution, inhibition of Na^+/H^+ exchange in the presence of 1 mM amiloride changes a_{Na}^i by 0.05 ± 0.06 mM ($n = 4$) and decreases pH_i by 0.18 ± 0.05 ($n = 5$) in 5 min. These results indicate that Na^+/H^+ exchange is not sufficiently active to contribute to maintenance of the physiological pH_i. Comparable results were found previously in cultured chick heart cells (Frelin et al., 1985; Piwnica-Worms et al., 1985) and in sheep Purkinje fibers (Deitmer and Ellis, 1980; Kaila and Vaughan-Jones, 1987).

Inhibition of Na_o^+-dependent Cl^-/HCO_3^- exchange after application of 0.1–1.0 mM SITS or DIDS (HCO_3^- buffer) for 5–10 min decreases pH_i and a_{Na}^i, and increases a_{Cl}^i. The stilbene-sensitive change in a_{Cl}^i is equivalent to a Cl^- efflux of 7.1 ± 1.0 pmol · cm^{-2} · s^{-1} ($n = 6$), comparable with a SITS-sensitive ^{36}Cl efflux of ~5.3 pmol · cm^{-2} · s^{-1}. These findings are consistent with the concept that a steady-state HCO_3^- influx in exchange for Cl_i^- via ($Na^+ + HCO_3/Cl^- + H^+$) exchange contributes to the observed low physiological H_i^+. This Cl^- efflux is counterbalanced by ($Na^+ + K^+ + 2Cl^-$) cotransport, the mechanism maintaining high Cl_i^- in cultured chick heart cells (Liu et al., 1987). Mechanisms responsible for pH_i maintenance other than Na^+/H^+ exchange and Na_o^+-dependent Cl^-/HCO_3^- exchange will require further investigation.

Conclusion

When cultured chick heart cells are acidified in HCO_3^--buffered solution, pH_i regulation occurs by stimulating a stilbene derivative–sensitive ($Na^+ + HCO_3/Cl^- + H^+$) exchange and an amiloride-sensitive Na^+/H^+ exchange. ($Na^+ + HCO_3^-/Cl^- + H^+$) exchange attenuates the initial phase of alkalinization due to ($HCO_3^- + CO_2$) removal and affects a_{Cl}^i and a_{Na}^i. Furthermore, under physiological conditions ($Na^+ + HCO_3/Cl^- + H^+$) exchange can maintain pH_i and, when combined with Na^+/H^+ exchange, may play an important role in regulating pH_i under pathophysiological conditions.

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