Intracellular pH Regulation in Cultured Embryonic Chick Heart Cells

**Na⁺-dependent Cl⁻/HCO₃⁻ Exchange**

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ABSTRACT The contribution of Cl⁻/HCO₃⁻ exchange to intracellular pH (pHi) regulation in cultured chick heart cells was evaluated using ion-selective microelectrodes to monitor pHi, Na⁺ (aNa), and Cl⁻ (aCl) activity. In (HCO₃⁻ + CO₂)-buffered solution steady-state pHi was 7.12. Removing (HCO₃⁻ + CO₂) buffer caused a SITS (0.1 mM)-sensitive alkalinization and countergradient increase in aCl along with a transient DIDS-sensitive countergradient decrease in aNa. SITS had no effect on the rate of pHi recovery from alkalinization. When (HCO₃⁻ + CO₂) was reintroduced the cells rapidly acidified, aNa increased, aCl decreased, and pHi recovered. The decrease in aCl and the pHi recovery were SITS sensitive. Cells exposed to 10 mM NH₄Cl became transiently alkaline concomitant with an increase in aCl and a decrease in aNa. The intracellular acidification induced by NH₄Cl removal was accompanied by a decrease in aCl and an increase in aNa that led to the recovery of pHi. In the presence of (HCO₃⁻ + CO₂), addition of either amiloride (1 mM) or DIDS (1 mM) partially reduced pHi recovery, whereas application of amiloride plus DIDS completely inhibited the pHi recovery and the decrease in aCl. Therefore, after an acid load pHi recovery is HCO₃⁻ and Na⁺ dependent and DIDS sensitive (but not Ca²⁺ dependent). Furthermore, SITS inhibition of Na⁺-dependent Cl⁻/HCO₃⁻ exchange caused an increase in aCl and a decrease in the ³⁶Cl efflux rate constant and pHi. In (HCO₃⁻ + CO₂)-free solution, amiloride completely blocked the pHi recovery from acidification that was induced by removal of NH₄Cl. Thus, both Na⁺/H⁺ and Na⁺-dependent Cl⁻/HCO₃⁻ exchange are involved in pHi regulation from acidification. When the cells became alkaline upon removal of (HCO₃⁻ + CO₂), a SITS-sensitive increase in pHi and aCl was accompanied by a
decrease of $a_{\text{Na}}$, 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 pHi 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$_i$, $a_{\text{Cl}}$, and $a_{\text{Na}}$, 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$_i$-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 pHₙ. This study confirms the statement of Thomas (1989) that in evaluating the mechanisms involved in pHₙ regulation, experiments must be carried out in the presence of a HCO₃⁻-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% CO₂ atmosphere before seeding the cells.

The culture procedure used to produce spontaneously beating strands of muscle (o.d. = 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% CO₂ 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 × 10⁵ 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). HCO₃⁻-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 NaHCO₃ in HCO₃⁻-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 NH₄Cl prepulse experiments the osmolality of the solutions was adjusted to match that of NH₄Cl containing solution by addition of 10 mM TMACl (295 mosM for HCO₃⁻-buffered solutions; 305 mosM for HEPES/Tris solutions). DIDS (4,4-diisothiocyanato-2,2'-disulfonic acid stilbene; Pierce Chemical Co., Rockford, IL or Fluka Chemical, Hauppaugsage, NY) or SITS (4-acetamido-4'-isothio-cyanostilbene-2',2'-disulfonic acid; ICN Biomedical, 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$_{Na}$ and a$_{Cl}$) of polystrands were measured as described previously (Liu et al., 1987). The reported values of a$_{Cl}$ were uncorrected for residual a$_{Na}$ whereas values used to calculate the thermodynamic driving force were corrected by subtracting 10 mM from apparent a$_{Cl}$ (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 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**

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; 

| TABLE I |
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| **Composition of Solutions** |
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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$_2$PO$_4$, 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 × 10$^{-10}$ Ω. The tips of the microelectrodes were then immersed into the filling solution for ~1 h before calibration. $E_m$ and pH 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, multiplied by an assumed constant intracellular buffering capacity ($\beta_i$) (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⁻/HCO₃⁻-Exchange

To determine the existence of a Cl⁻/HCO₃⁻ exchange in cultured chick heart cells, we manipulated the HCO₃⁻ or Cl⁻ gradient and monitored pHᵢ and aCl or aCl⁻ content. In HCO₃⁻-buffered control solution, steady-state pHᵢ at 37°C was 7.12 ± 0.01 (n = 46), and apparent aCl was 36.4 ± 0.8 mM (n = 63) (maximal diastolic potential ~ -75 mV).

![Figure 1](image-url)  
**FIGURE 1.** Removal of (HCO₃⁻ + CO₂) affects pHᵢ and aCl⁻. (A) Polystrands were superfused with HCO₃⁻ + CO₂-buffered MEBSS and pHᵢ was monitored with pH-selective microelectrodes. A transient alkalinization (from control pHᵢ 7.1 to 7.6) occurred when the solution was switched to (HCO₃⁻ + CO₂)-free solution. Then pHᵢ 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 (HCO₃⁻ + CO₂)-buffered solution caused a transient acidification to pHᵢ 6.85 that regulated to approach control level. (B) Intracellular Cl activity (aCl⁻) was monitored with a Cl-selective microelectrode in a separate preparation. Removal of (HCO₃⁻ + CO₂) caused an increase in aCl⁻. Upon restoring (HCO₃⁻ + CO₂), aCl⁻ decreased and recovered to control level.

Effect of HCO₃⁻ gradient on pHᵢ, aCl⁻, and aCl₆

**Alkalization.** Polystrands were perfused with (HCO₃⁻ + CO₂) control solution and then exposed to (HCO₃⁻ + CO₂)-free (HEPES/Tris buffer) solution. This maneuver initiated a rapid efflux of CO₂ and a concomitant increase in pHᵢ of 0.38 ± 0.03 (n = 15), which in 15 min regulated toward a new steady state at a pHᵢ of 7.23 ± 0.03 (n = 17); maximal diastolic potential changed less than 1.0 mV. A representative recording of the changes in pHᵢ is shown in Fig. 1 A. Following the same protocol with different preparations. Fig. 1 B shows that removal of HCO₃⁻ caused a countergradient increase in aCl⁻ to a new steady-state level and a countergra-
dient transient decrease in $a_{\text{Na}}$ by $2.1 \pm 0.3$ mM ($n = 14$). Steady-state $a_{\text{cl}}$ in HCO$_3^-$-free solution was $43.7 \pm 0.9$ 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 ~25% faster than that in the absence of SITS. Addition of SITS also revealed a greater alkalinization by $0.08 \pm 0.02$ pH units ($n = 6, P < 0.01$), and also reduced the increase of $a_{\text{cl}}$ by $1.6 \pm 0.5$ mM ($n = 4$). 0.3 mM DIDS completely inhibited the transient decrease in $a_{\text{Na}}$ ($n = 1$). 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 $0.22 \pm 0.02$, $n = 15$) that regulated to control pH$_i$ (see also Fig. 1 A) and a transient increase in $a_{\text{Na}}$ (3.3 ± 0.5 mM, $n = 8$). The increase in $a_{\text{Na}}$ 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) pH 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, $0.08 \pm 0.02$ pH/min, $n = 5$; with SITS, $0.02 \pm 0.01$ pH/min, $n = 5$, $P < 0.05$). In Fig. 1 B $a_{\text{cl}}$ rapidly decreased to control value upon restoring HCO$_3^-$. SITS inhibited both the magnitude and rate of decrease in $a_{\text{cl}}$ induced by restoring HCO$_3^-$ (see also Table II). In Cl$^-$-free solution (10 min) pH$_i$ increased by $-0.07$ ($n = 2$), indicating that Cl$^-$ removal induced a HCO$_3^-$ influx (i.e., Cl$^-$ in exchange for HCO$_3^-$).

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

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 $0.61 \pm 0.02$ to $0.51 \pm 0.02$ ($n = 3$) ($P < 0.05$).

As mentioned above, in HCO$_3^-$-buffered solution the apparent steady-state $a_{\text{cl}}$ significantly increased within 10 min in HCO$_3^-$-free solutions ($P < 0.001$). Similar results were obtained from Cl$^-$ content measurements. In HCO$_3^-$-buffered solution 0.1 mM SITS or DIDS significantly increased $a_{\text{cl}}$ by $0.7 \pm 0.1$ mM ($n = 6, P < 0.001$) and decreased pH$_i$ by $0.05 \pm 0.01$ ($n = 10, P < 0.001$) within 10 min. As expected, in HCO$_3^-$-free solutions SITS did not significantly change $a_{\text{cl}}$ ($-0.07 \pm 0.10$ 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
|                | (HCO₃⁻ + CO₂) removal |                    | (HCO₃⁻ + CO₂) addition |                    |
|----------------|-----------------------|-------------------|------------------------|-------------------|
|                | ΔpHᵢ                  | d(pHᵢ)/dt         | Δaᵢₑ                    | d(aᵢₑ)/dt         |
| Control        | 0.36 ± 0.05           | 0.95 ± 0.18       | 5.4 ± 0.6               | 5.0 ± 1.0         |
| + SITS         | 0.44 ± 0.05*          | 1.19 ± 0.21*      | 3.8 ± 0.4*              | 2.8 ± 0.4*        |
|                | (6)                   | (5)               | (4)                     | (4)               |

|                | ΔpHᵢ                  | d(pHᵢ)/dt         | Δaᵢₑ                    | d(aᵢₑ)/dt         |
| + SITS         | 0.44 ± 0.05*          | 1.19 ± 0.21*      | 3.8 ± 0.4*              | 2.8 ± 0.4*        |
|                | (4)                   | (4)               | (3)                     | (3)               |

Values are mean ± SE; number in parentheses indicates paired measurements. ΔpHᵢ and Δaᵢₑ were defined as the peak change in pHᵢ and aᵢₑ in response to change in (HCO₃⁻ + CO₂) buffer. (Positive values for ΔpHᵢ: alkalinization; negative values: acidification. Positive values for Δaᵢₑ: influx; negative values: efflux.)
d(pHᵢ)/dt, initial rate (pH/minute) of change in pHᵢ.
d(aᵢₑ)/dt, initial rate (millimolar/minute) of change in aᵢₑ.

*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$_4$Cl for 5 min and then returned to control solution. We monitored pH$_i$, $a_{Cl}^-$, and Cl$^-$ 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$_4$Cl increased pH$_i$ by 0.20 ± 0.01 pH units ($n = 51$), which then returned close to the control level. Removal of NH$_4$Cl 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 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).

**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$_4$Cl (HCO$_3^-$-buffered solution) for 5 min underwent a transient alkalinization followed by a slow recovery. Removal of NH$_4$Cl induced a transient acidification that regulated to control level in 5 min. B shows a recording obtained from cells exposed to 10 mM NH$_4$Cl. After 3 min, removal of (HCO$_3^-$ + CO$_2$) simultaneously with the addition of 1 mM amiloride caused a transient alkalinization (see also Fig. 1A). Removal of NH$_4$Cl 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$_i$ 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-
Inhibition of Na⁺/H⁺ exchange with amiloride affects pHᵢ in HCO₃⁻-buffered solution. A shows a control change in pHᵢ (lower trace) and Eₘ (upper trace) in response to NH₄⁺ prepulse. In B, cells were first exposed to 10 mM NH₄Cl for 5 min; addition of 1 mM amiloride simultaneously with removal of NH₄Cl enhanced the magnitude of acidification and slowed the pHᵢ recovery.

Another using cultured chick heart cells (Piwnica-Worms et al., 1985). The protocol in Fig. 2B also confirms this finding: at the third minute of a NH₄Cl pulse, removal of (HCO₃⁻ + CO₂) buffer and addition of 1 mM amiloride caused a transient alkalinization. Under this condition, removal of NH₄Cl intensified the acidification and amiloride inhibited pHᵢ recovery. Washout of amiloride led to a complete recovery of pHᵢ.

Inhibition of Na⁺/H⁺ exchange on pHᵢ recovery in HCO₃⁻-buffered solution

When Na⁺/H⁺ exchange was identified as an important pHᵢ-regulating mechanism after an acid load in cultured chick heart cells (Piwnica-Worms et al., 1985), no
FIGURE 5. Chase removal affects pH recovery during inhibition of Na\(^+\)/H\(^+\) exchange. Polystrand was first exposed to 10 mM NH\(_4\)Cl, and 1 mM amiloride was added after 3 min. Removing Cl\(^-\) in NH\(_4\)\(^+\)-free solution accelerated pH recovery. The superimposed trace (dashed line) shows the change in pH that occurred upon removal of NH\(_4\)Cl in 134 mM [Cl\(^-\)].

direct quantification of pH 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 regulation. Amiloride was added either 2 min before or simultaneously with removal of NH\(_4\)Cl (Fig. 3 B); acidification was augmented by 0.11 \(\pm\) 0.02 pH units (\(n = 13\), \(P < 0.01\)) and reduced the rate of pH recovery by 27.1 \(\pm\) 4.3% (\(n = 12\)). These results suggest that pH regulation from an acid load does not involve only Na\(^+\)/H\(^+\) exchange.

Inhibition of Cl\(^-\)/HCO\(_3\)\(^-\) exchange affects pH recovery

Based on experiments such as those described in Fig. 2, Cl\(^-\)/HCO\(_3\)\(^-\) exchange may also regulate pH after acidification. Thus, we tested the effect of DIDS, a Cl\(^-\)/HCO\(_3\)\(^-\) exchange inhibitor, on pH regulation. After exposure to NH\(_4\)Cl, 0.1 mM DIDS added either before or simultaneously with NH\(_4\)Cl removal augmented acidification by 0.05 \(\pm\) 0.01 pH units (\(n = 12\)) and attenuated the rate of pH recovery by 34.9 \(\pm\) 4.7% (\(n = 9\)). Increasing the concentration of DIDS to 1 mM (Fig. 4 A) caused a further decrease of the rate of pH recovery by 46.6 \(\pm\) 11.2% (\(n = 4\)).

Application of 1 mM DIDS plus 1 mM amiloride during the NH\(_4\)Cl pulse slightly decreased pH (Fig. 4 B). Upon removal of NH\(_4\)Cl the acidification response was

FIGURE 6. pH recovery is HCO\(_3\)\(^-\) dependent during inhibition of Na\(^+\)/H\(^+\) exchange. After the third minute of a 10-mM NH\(_4\)Cl pulse the polystrand was exposed to HCO\(_3\)\(^-\)-free plus 1 mM amiloride solution. Upon washout of NH\(_4\)Cl, the continuing absence of HCO\(_3\)\(^-\) caused a significant maintained acidification. Reintroducing HCO\(_3\)\(^-\) (in the presence of amiloride) caused pH to recover rapidly to the control level.
magnified by 0.34 pH units (control, \(-0.26 \pm 0.06\) pH, \(n = 5\); with DIDS plus amiloride, \(-0.60 \pm 0.03\) 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\(^-\) removal affects pH\(_i\) recovery**

From a thermodynamic standpoint, in the presence of a Cl\(^-\)/HCO\(_3^-\) exchange removing Cl\(^-\) should increase the driving force for net HCO\(_3^-\) influx and accelerate pH\(_i\) recovery from acidification. Fig. 5 shows that inhibition of Na\(^+\)/H\(^+\) exchange coupled with removal of Cl\(^-\) (solid line) indeed facilitates the rate of pH\(_i\) recovery in paired experiments by 50 \(\pm\) 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\(_3^-\) dependent

If at the third minute of the NH\(_4^+\) Cl pulse cells were exposed to HCO\(_3^-\)-free solution containing 1 mM amiloride, and the subsequent removal of NH\(_4^+\) Cl resulted in a substantial maintained acidification and a complete inhibition of pH\(_i\) recovery (Fig.
6. Even with the continued inhibition of Na\(^+\)/H\(^+\) exchange, reintroduction of [HCO\(_3\)^-] still led to a rapid recovery of pH\(_i\).

Changes in pH\(_i\) affects Cl\(^-\)

Fig. 7 illustrates simultaneously obtained measurements of a\(_{c0}\) and pH\(_i\) during and after the NH\(_4\)Cl pulse. Cells exposed to 10 mM NH\(_4\)Cl increased a\(_{c0}\) by 3.7 ± 0.3 mM (n = 16), a Cl\(^-\) influx occurring against Cl\(^-\) electrochemical gradient. After 5 min a\(_{c0}\) stabilized at a level 2.5 ± 0.4 mM (n = 10) above control. Removing NH\(_4\)Cl resulted in a rapid decrease in a\(_{c0}\) (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\(_{c0}\) (Fig. 7 B, upper trace) as well as pH\(_i\) recovery from acidification (Fig. 8). The inhibition of Cl\(^-\)/HCO\(_3\)^- exchange with DIDS affects Cl\(^-\) content. Exposure to 20 mM NH\(_4\)Cl for 15 min caused an increase in Cl\(^-\) content; removal of NH\(_4\)Cl induced a rapid decrease in Cl\(^-\) content (open circles). When 0.1 mM DIDS was present throughout the experiment, the changes in Cl\(^-\) were markedly attenuated (filled circles).

Fig. 9. Amiloride affects a\(_{in}\) during pH\(_i\) recovery. Exposure to 10 mM NH\(_4\)Cl caused a small decrease in a\(_{in}\). Removing NH\(_4\)Cl induced a rapid increase in a\(_{in}\) that gradually regulated to the control value (dashed line). 1 mM amiloride, which was added either before or simultaneously with removal of NH\(_4\)Cl, slightly reduced the rate of the increase in a\(_{in}\) and blocked the a\(_{in}\) recovery (solid line). Data presented are means ± SE from five paired measurements.
Washout of DIDS and amiloride led to the resumption of Cl efflux and pH recovery. When the Cl\textsuperscript{−} coulometric titration method was used to monitor changes in Cl\textsuperscript{−} content after a 15-min exposure to 20 mM NH\textsubscript{4}Cl, we observed an increase in Cl\textsuperscript{−} content by ~50%; removal of NH\textsubscript{4}Cl resulted in a decrease of Cl\textsuperscript{−} (Fig. 8). The higher concentration and longer exposure to NH\textsubscript{4}Cl 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\textsubscript{4}Cl, its removal increased the rate constant of 36Cl efflux from 0.70 ± 0.01 (n = 3) to 0.82 ± 0.05 min\textsuperscript{−1} (n = 3). Fig. 8 (lower trace) shows that the presence of 0.1 mM DIDS throughout the NH\textsubscript{4}Cl prepulse experiment suppressed net counter-gradient Cl\textsuperscript{−} uptake (~70%) during exposure to NH\textsubscript{4}Cl and net Cl\textsuperscript{−} washout upon NH\textsubscript{4}Cl removal. Furthermore, if the NH\textsubscript{4}Cl prepulse experiment was carried out in HCO\textsubscript{3}−-free solution, no significant change in Cl\textsuperscript{−} content was detected. The findings thus support the concept that Cl\textsuperscript{−}/HCO\textsubscript{3}− exchange can regulate pH\textsubscript{i} in response to intracellular alkalization and acidification.

**pH\textsubscript{i} regulation and a\textsubscript{Na\textsuperscript{+}}**

**Alkalization.** Exposure of cells to NH\textsubscript{4}Cl caused a decrease in a\textsubscript{Na\textsuperscript{+}} by 1.5 ± 0.2 mM (n = 19) accompanied by an increase in pH\textsubscript{i}, indicating that net outward Na\textsuperscript{+} movement is against its electrochemical gradient.

**Acidification.** Removing NH\textsubscript{4}Cl increased a\textsubscript{Na\textsuperscript{+}} 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\textsubscript{Na\textsuperscript{+}} then returned relatively slowly to the control level (Fig. 9). Removal of NH\textsubscript{4}Cl in the presence of 1 mM amiloride reduced the rate of increase in a\textsubscript{Na\textsuperscript{+}} by 30.3 ± 13.4% (n = 8) but did not significantly inhibit the augmented level of a\textsubscript{Na\textsuperscript{+}}. Amiloride also appeared to markedly attenuate the recovery rate of a\textsubscript{Na\textsuperscript{+}} in six of eight experiments.
Na⁺ dependence of Cl⁻/HCO₃⁻ exchange in pHᵢ regulation

Removal of Na⁺ resulted in an amiloride-sensitive acidification (Piwnica-Worms et al., 1985). We applied the NH₄Cl prepulse protocol to determine the mechanism by which removal of Na⁺ affects pHᵢ regulation. Fig. 10 A shows that the simultaneous removal of Na⁺ with NH₄Cl resulted in a substantial maintained acidification. In Figs. 4 and 7 we showed that in HCO₃⁻-buffered solution both amiloride-sensitive Na⁺/H⁺ exchange and DIDS-sensitive Cl⁻/HCO₃⁻ exchange are involved in pHᵢ regulation after an acid load. pHᵢ recovery can occur after inhibition of Na⁺/H⁺ exchange by the action of the Cl⁻/HCO₃⁻ exchange. If the sustained acidification in Na⁺-free solution is due only to inhibition or reversal of Na⁺/H⁺ exchange, then acidification induced by NH₄⁺ removal should be reversed by Cl⁻/HCO₃⁻ exchange. However, as shown in Fig. 10 A, in Na⁺-free solution (HCO₃⁻ buffer), pHᵢ did not recover upon removing NH₄Cl until Na⁺ was restored. We then tested whether removal of Na⁺ is still capable of affecting pHᵢ recovery after amiloride inhibition of Na⁺/H⁺ exchange. Fig. 10 B shows that in the presence of amiloride the simultaneous removal of Na⁺ and NH₄Cl augmented the acidification (normal Na⁺, -0.35 ± 0.01 pH units; Na⁺ free, -0.49 ± 0.03 pH units, n = 5) and completely blocked pHᵢ regulation. Since the reintroduction of Na⁺ led to the return of pHᵢ to control levels, these findings suggest that Cl⁻/HCO₃⁻ exchange is Na⁺ dependent.

Inhibition of pHᵢ recovery by Na⁺ removal is not affected by removal of Ca²⁺.

In this study we have shown that in the presence of amiloride removal of Na⁺ caused a sustained acidification by inhibiting the Cl⁻/HCO₃⁻ exchange. In previous studies...
we also showed that Na~ removal reverses Na+/Ca~ exchange to cause an increase in Ca~+. (Jacob et al., 1987), which in turn can decrease pH via a Ca~+H~ interaction (Liu and Lieberman, 1989). Therefore, we attempted to determine if the sustained acidification in Na~ free plus amiloride solution could relate to a change in Ca~+. When the NH4Cl prepulse experiments were carried out in a Ca~+ free solution containing 1 mM EGTA and 1 mM amiloride, we found that acidification induced by NH4Cl removal recovered to a level slightly more acidic than control pH (Fig. 14). If Na~ and NH4Cl were removed simultaneously in the presence of 1 mM amiloride and the absence of Ca~+, a sustained acidification occurred which then returned to control level upon reintroducing Na~ (Fig. 118). This finding shows that (a) Ca~+ does not affect the Na~ requirement of CI-/HCO~ exchange in regulating pH, and (b) the sustained acidification resulting from inhibition of pH recovery in Na~ free solution cannot be attributed to Na~ /Ca~ exchange and Ca~+H~ interaction.

DISCUSSION

pH~ and Buffering Capacity

pH~ of cultured chick heart cells, measured in HCO~ buffered solution at 37°C with pH-selective microelectrodes, is 7.12 ± 0.01 (n = 46) in agreement with values of pH~ 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~ do not differ from those measured with double-barreled pH microelectrodes.

The total intracellular buffering capacity (T) can be described by the sum of intrinsic buffering power (I) and that attributed to (HCO~ + CO2) buffer (CO2) (Roos and Boron, 1981), the latter determined by multiplying [HCO~] by the factor 2.3. According to the Henderson-Hasselbalch equation, [HCO~], is calculated to be 13.0 mM at a pH~ of 7.12, assuming that (a) [HCO~] depends only on pH, [CO2], and the partial pressure of CO2, and (b) the carbonic acid dissociation constant and the solubility coefficient for CO2 are identical on either side of the cell membrane. The calculated value of CO2 for cultured chick heart cells is 29.9 mM/pH unit. I, which can be estimated by dividing the change in [HCO~] by the associated change in pH upon removing [HCO~] (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, T of cultured heart cells is 89.3 mM/pH unit in (HCO~ + CO2) solutions.

Na~ dependent Cl~ /HCO~ Exchange vs. Cl~ /HCO~ Exchange

We first demonstrated a DIDS- or SITS-sensitive Cl~ /HCO~ exchange in cultured chick heart cells by showing that (a) removing (HCO~ + CO2) buffer results in an increase in pH and a~ (SITS accentuates the alkalinization and attenuates the increase of a~), (b) reintroducing (HCO~ + CO2) buffer results in a SITS-sensitive pH recovery from acidification and a decrease in a~ and (c) removing Clo causes a
countergradient increase in HCO₃⁻.¹ The SITS-sensitive changes in pHᵢ and a_a that occur when the HCO₃⁻ gradient is manipulated suggest that Cl⁻/HCO₃⁻ exchange can regulate pHᵢ after cell acidification and can cause cell alkalization. In contrast, the suggestion proposed for sheep Purkinje fibers is that Cl⁻/HCO₃⁻ exchange appears to regulate pHᵢ only after alkalization (Vaughan-Jones, 1982). Interestingly, a_a decreased when sheep Purkinje fibers were exposed to HCO₃⁻-free solution (Vaughan-Jones, 1979), a finding contrary to what would be expected based on the results of the Cl⁻/HCO₃⁻ exchanger in cultured chick heart cells.

We showed that under physiological conditions application of SITS or DIDS increases a_a, reduces the rate constant of steady-state Cl⁻ efflux, and decreases pHᵢ, suggesting that Cl⁻/HCO₃⁻ exchange provides a steady-state Cl⁻ efflux and HCO₃⁻ influx. Heinemeyer and Bay (1987) similarly reported that 0.5 mM SITS acidified pHᵢ in mammalian ventricle. If measurements obtained from our data are used to calculate the thermodynamic driving force for a putative Cl⁻/HCO₃⁻ exchange that is solely dependent on the Cl⁻ and HCO₃⁻ gradient, one would expect a net Cl⁻ influx and HCO₃⁻ efflux. This being contrary to our experimental observations, we thus propose that the stilbene derivative-sensitive Cl⁻/HCO₃⁻ exchange in cultured chick heart cells must be coupled to an additional ion gradient to reverse the net direction.

Removal of (HCO₃⁻ + CO₂) causes a transient alkalinization (due to rapid CO₂ efflux) that is accompanied by a countergradient increase of a_a, and a countergradient decrease of a_a. As shown in Table II, inhibition of Cl⁻/HCO₃⁻ exchange in the presence of SITS or DIDS enhances the rate and magnitude of alkalization, attenuates the increase in a_a, and inhibits the transient decrease of a_a. These findings demonstrate that upon removing (HCO₃⁻ + CO₂) buffer HCO₃⁻ efflux via Cl⁻/HCO₃⁻ exchange attenuates cell alkalization induced by a rapid CO₂ efflux. SITS has no effect on the rate of pHᵢ recovery from alkalization because this pHᵢ is rather due to dissipation of CO₂ and HCO₃⁻ gradients. Conversely, the introduction of (HCO₃⁻ + CO₂) results in a transient acidification (due to rapid CO₂ influx) followed by a SITS-sensitive pHᵢ recovery that is accompanied by a transient increase in a_a and a decrease in a_a. Similar changes in ion content occurred on exposure to NH₄Cl (alkalinization) and on its removal (acidification). Consequently, Na⁺ transport must be coupled with HCO₃⁻ movement in exchange for Cl⁻, supporting the hypothesis that manipulating the HCO₃⁻ gradient can change a_Na, a_K, and pHᵢ via a Na⁺-dependent Cl⁻/HCO₃⁻ exchange mechanism.

Using the NH₄⁺ prepulse method, we presented evidence in Fig. 10 showing that the induced acid load cannot be regulated in Na⁺-free solution, thus rendering unlikely the possibility that Na⁺-independent Cl⁻/HCO₃⁻ exchange is involved in pHᵢ regulation. Therefore, under acid conditions pHᵢ regulation only involves a Na⁺-dependent process. We also confirmed that the maintained acidification in Na⁺-free (with or without Ca²⁺-free) amiloride-containing solution cannot be attributed to the combined effect of Na⁺ removal on Na⁺/Ca²⁺ exchange and Ca²⁺-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 Clᵢ⁻ in our preparations (Liu et al., 1987).
cotransport (0.1 mM bumetanide) throughout the NH4Cl prepulse experiment does not affect the inhibition of pH recovery caused by removing Na+. Furthermore, the decrease in aH by <5 mM during a 5-min exposure to Na+-free MEBSS causes an insignificant change to the calculated driving force of Cl-/HCO3 exchange. Therefore, inhibition of pH recovery upon removal of Na+ cannot be secondary to a decrease in Cl- via (Na+ + K+ + 2Cl-) cotransport.

From a thermodynamic standpoint, a Na+-dependent Cl-/HCO3 exchange should provide an inward transport of HCO3 in exchange for Cl-. This would allow a complete recovery of pH to occur after removal of NH4Cl in the presence of amiloride (Figs. 6 and 11). Under these conditions, the driving force for a putative (Na+-independent) Cl-/HCO3 exchange would allow pH to return only to ~6.8 (based on calculations from the values of aCl and [HCO3-]). In the absence of Na+, the lack of pH recovery indicates that this stilbene-sensitive, HCO3-dependent Cl- transport and acid extrusion (amiloride-insensitive) mechanism must be a Na+-dependent Cl-/HCO3 exchange.

Amiloride-insensitive Increase of aNa

When NH4Cl 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+ recovery so that the net effect is a gain of aNa (Fig. 9). The relatively slow aNa 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 NH4Cl prepulse experiment (see Methods), and (b) aCl and aNa measurements are compatible with Cl- and Na+ 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+-dependent Cl-/HCO3 exchange.

However, in one experiment 1 mM DIDS did not block, but rather augmented, the amiloride-insensitive gain of aNa. This unexpected net aNa gain may result from inhibition of the Na+/K+ pump by an increase in aCa (>1 μM; Turi and Somogyi, 1988) and by intracellular acidification (Breitwieser et al., 1987), as well as stimulation of Na+/Ca2+ exchange by an increase of aCa caused by an intracellular acidification and a rapid depolarization. In the absence of amiloride or DIDS, removal of NH4Cl caused increases both in aCa in sheep Purkinje fibers (Bers and Ellis, 1982) and in free Ca2+ (<1 μM) as recently measured with fura-2 in cultured chick heart cells (Freudennich, C. C., personal communication). The augmented and sustained acidification (pH 6.5) that was accompanied by Em depolarization in the presence of amiloride and DIDS would be expected to increase Ca2+ 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+/Ca2+ exchange.
Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} Exchange vs. Na\textsuperscript{+}/H\textsuperscript{+} Exchange

We demonstrated that pH\textsubscript{i} recovery from acidification involves both Na\textsuperscript{+}/H\textsuperscript{+} exchange and Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange. Inhibition of both mechanisms leads to a complete block of pH\textsubscript{i} recovery after an acid load. Although caution must be used in applying pharmacological agents to quantitatively define a transport mechanism, Na\textsuperscript{+}/H\textsuperscript{+} exchange seems slightly more sensitive to acidification than Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange. Upon removal of NH\textsubscript{4}Cl, our findings show a greater acidification in 1 mM amiloride than in 1 mM DIDS. However, the rate of pH\textsubscript{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\textsubscript{i} recovery by 27%. In addition, the amiloride-sensitive H\textsuperscript{+} efflux is 16.9 ± 4.0 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} as compared with the DIDS-sensitive H\textsuperscript{+} efflux of 28.2 ± 4.5 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1}. Under these conditions, Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange is more efficient than Na\textsuperscript{+}/H\textsuperscript{+} exchange in regulating pH\textsubscript{i} from an acid load.

Our results also show that on NH\textsubscript{4}Cl removal, inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange augments the initial rate of decrease in a\textsubscript{CO\textsubscript{2}} by 23.6 ± 10.4% (n = 3, P < 0.05), indicating an enhanced rate of Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange. The other difference between Na\textsuperscript{+}/H\textsuperscript{+} exchange and an amiloride-insensitive, Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange is that Na\textsuperscript{+}/H\textsuperscript{+} exchange is activated only after pH\textsubscript{i} decreases beyond a threshold (Roos and Boron, 1981), whereas Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange remains active under physiological conditions and is stimulated after acidification. Even though different modes of Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange exist in a variety of cell preparations, kinetic studies consistently imply that Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange is more sensitive to the HCO\textsubscript{3}\textsuperscript{-} gradient, namely, pH gradient, than to the Cl gradient (Boron and Russell, 1983; Vanghan-Jones, 1986; Ahearn et al., 1987). This may account for the response of Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange to pH\textsubscript{i} change in cultured chick heart cells.

Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} Exchange vs. Other pH\textsubscript{i}-regulating Systems

When NH\textsubscript{4}Cl is removed, total acid extrusion rate (60.5 ± 4.9 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1}, n = 16) induces a Na\textsuperscript{+} influx (33.6 ± 5.7 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1}, n = 6) and a Cl\textsuperscript{-} efflux (35.7 ± 6.9 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1}, n = 9) that is calculated from the change in a\textsubscript{CO\textsubscript{2}} and agrees with Cl\textsuperscript{-} efflux measurements. After acidification, we observed an amiloride-insensitive Na\textsuperscript{+} influx of 20.8 ± 7.0 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} (n = 6), a Cl\textsuperscript{-} efflux of 27.9 ± 4.1 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} (n = 9), and an acid extrusion of 38.6 ± 5.2 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} (n = 12). The ratio of acid extrusion, Na\textsuperscript{+} influx, and Cl\textsuperscript{-} efflux is close to 2:1:1, in agreement with an electroneutral (Na\textsuperscript{+} + HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} + H\textsuperscript{+}) 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\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange system may be consistent with an ion pair model, i.e., NaCO\textsubscript{3}/Cl\textsuperscript{-} exchange (Becker and Duhm, 1978); however, this NaCO\textsubscript{3}/Cl\textsuperscript{-} exchange has not been shown to be reversible, i.e., inducing a Cl\textsuperscript{-} influx (Boron, 1986). This system may also be similar to an electrogenic Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransport (Fitz et al., 1989), which should cause change.
in \( E_m \) by 10–20 mV in response to the \( \text{HCO}_3^- \) 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 \( \text{NH}_4\text{Cl} \) removal activates \( \text{Na}^+ \)-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 \( \text{Na}^+ \)-dependent \( \text{Cl}^-/\text{HCO}_3^- \) exchange is an electroneutral process and not an electrogenic \( \text{Na}^+/-\text{HCO}_3^- \) cotransport.

In cultured chick heart cells, in addition to \( (\text{Na}^+ + \text{HCO}_3^-)/\text{Cl}^- + \text{H}^+ \) exchange, our findings reveal an acidification-induced, amiloride-sensitive \( \text{Na}^+ \) uptake and acid extrusion. The similar dual \( \text{pH} \)-regulating system, i.e., \( \text{Na}^+/-\text{H}^+ \) exchange and \( (\text{Na}^+ + \text{HCO}_3^-)/\text{Cl}^- + \text{H}^+ \) exchange, is also found in crayfish neurones (Moody, 1981) and leech neurones (Schlue and Thomas, 1985; Deitmer and Schlue, 1987).

In Purkinje fibers \( \text{Na}^+/\text{H}^+ \) exchange appears to be the main \( \text{pH} \) 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 \( \text{Cl}^-/\text{HCO}_3^- \) exchange because a controversy exists over the potency of anion exchanger inhibitors used to demonstrate \( \text{Cl}^-/\text{HCO}_3^- \) exchange. For example, Deitmer and Ellis (1980) showed that in the presence of 1 mM amiloride \( \text{pH}_i \) still recovers slowly in a \( \text{HCO}_3^- \)-free 1-mM SITS solution. Perhaps 1 mM DIDS, a more potent inhibitor of \( \text{Cl}^-/\text{HCO}_3^- \) exchange, would have been more effective for such a study in \( \text{HCO}_3^- \)-buffered solution. In addition, Vanheel et al. (1984) also demonstrated that 0.1 mM SITS and \( \text{Cl}^- \)-free solutions do not impair \( \text{pH}_i \) recovery from acidification. However, Vaughan-Jones (1982) showed that in \( \text{HCO}_3^- \)-buffered solution 0.18 mM DIDS inhibits the \( a_\Delta \) changes in response to \( \text{NH}_4^+ \) prepulse and slightly reduces \( \text{pH}_i \) recovery, and attenuates \( \text{pH}_i \) recovery after an acid load induced by \( \text{NH}_4\text{Cl} \) (Vaughan-Jones, 1982, Fig. 6). These findings are, in fact, consistent with \( \text{Cl}^-/\text{HCO}_3^- \) exchange in \( \text{pH}_i \) regulation after alkalization and acidification. If the experiments to examine the effect of DIDS were combined with the inhibition of \( \text{Na}^+/-\text{H}^+ \) exchange, then the results would have more clearly identified the transport mechanisms that participate in \( \text{pH}_i \) 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 \( \text{Cl}^-/\text{HCO}_3^- \) exchange in \( \text{pH}_i \) recovery. First, DIDS (0.1 mM)-sensitive \( \text{H}^+ \) extrusion did not occur upon removal of \( \text{NH}_4\text{Cl} \). However, the experiment was performed in \( \text{HCO}_3^- \)-free solution so that \( \text{Cl}^-/\text{HCO}_3^- \) exchange was inactivated. Second, in \( \text{HCO}_3^- \)-buffered solution, 0.1 mM DIDS only slightly decreased the acid-induced \( \text{Na}^+ \) uptake. We now show that \( \text{pH}_i \) recovery via \( \text{Na}^+/-\text{H}^+ \) 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 \( \text{pH}_i \) regulation, the effect of maximal inhibitory doses of DIDS on \( \text{Na}^+ \) uptake should be examined in the presence of amiloride.

**Steady-state \( \text{pH}_i \) Maintenance and Physiological Importance of \( \text{Na}^+/-\text{dependent} \text{Cl}^-/\text{HCO}_3^- \) Exchange**

Steady-state \( \text{pH}_i \) of cultured heart cells is ~1 pH unit more alkaline than expected for \( \text{H}^+ \) 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\(^+\)-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\(_{\text{Na}}\) by 0.05 ± 0.06 mM (n = 4) and decreases pH\(_{\text{i}}\) 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\(_{\text{i}}\). 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\(^+\)-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\(_{\text{i}}\) and a\(_{\text{Na}}\) and increases a\(_{\text{Cl}}\). The stilbene-sensitive change in a\(_{\text{Cl}}\) is equivalent to a Cl\(^-\) efflux of 7.1 ± 1.0 pmol·cm\(^{-2}\)·s\(^{-1}\) (n = 6), comparable with a SITS-sensitive \(^{36}\text{Cl}\) 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\(^-\) via (Na\(^+\) + HCO\(_3\)\(^-\)/Cl\(^-\) + H\(^+\)) exchange contributes to the observed low physiological H\(_{\text{i}}\). This Cl\(^-\) efflux is counterbalanced by (Na\(^+\) + K\(^+\) + 2Cl\(^-\)) cotransport, the mechanism maintaining high Cl\(^-\) in cultured chick heart cells (Liu et al., 1987). Mechanisms responsible for pH\(_{\text{i}}\) maintenance other than Na\(^+\)/H\(^+\) exchange and Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\)\(^-\) exchange will require further investigation.

**Conclusion**

When cultured chick heart cells are acidified in HCO\(_3\)\(^-\)-buffered solution, pH\(_{\text{i}}\) 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\(_{\text{Cl}}\) and a\(_{\text{Na}}\). Furthermore, under physiological conditions (Na\(^+\) + HCO\(_3\)\(^-\)/Cl\(^-\) + H\(^+\)) exchange can maintain pH\(_{\text{i}}\) and, when combined with Na\(^+\)/H\(^+\) exchange, may play an important role in regulating pH\(_{\text{i}}\) under pathophysiological conditions.

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