Bicarbonate-dependent and -independent Intracellular pH Regulatory Mechanisms in Rat Hepatocytes

Evidence for Na\(^{+}\)-HCO\(_3\)\(^{-}\) Cotransport

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Abstract

Using the pH-sensitive dye 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein and a continuously perfused subconfluent hepatocyte monolayer cell culture system, we studied rat hepatocyte intracellular pH (pHi) regulation in the presence (+HCO\(_3\)) and absence (−HCO\(_3\)) of bicarbonate. Baseline pHi was higher (7.28±0.09) in +HCO\(_3\) than in −HCO\(_3\) (7.16±0.14). Blocking Na\(^{+}/H^{+}\) exchange with amiloride had no effect on pHi in +HCO\(_3\) but caused reversible 0.1–0.2-U acidification in −HCO\(_3\) or in +HCO\(_3\) after preincubation in the anion transport inhibitor 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene (DIDS). Acute Na\(^{+}\) replacement in +HCO\(_3\) also caused acidification which was amiloride independent but DIDS inhibitable.

The recovery of pHi from an intracellular acid load (maximum H\(^{+}\) efflux rate) was 50% higher in +HCO\(_3\) than in −HCO\(_3\). Amiloride inhibited H\(^{+}\) efflux\(_{\text{max}}\) by 75% in −HCO\(_3\) but by only 27% in +HCO\(_3\). The amiloride-independent pHi recovery in +HCO\(_3\) was inhibited 50–63% by DIDS and 79% by Na\(^{+}\) replacement but was unaffected by depletion of intracellular Cl\(^{-}\), suggesting that Cl\(^{-}\)/HCO\(_3\) exchange is not involved. Depolarization of hepatocytes (raising external K\(^{+}\) from 5 to 25 mM) caused reversible 0.05–0.1-U alkalinization, which, however, was neither Na\(^{+}\) nor HCO\(_3\) dependent, nor DIDS inhibitable, findings consistent with electroneutral HCO\(_3\) transport.

We conclude that Na\(^{+}\)-HCO\(_3\) cotransport, in addition to Na\(^{+}/H^{+}\) exchange, is an important regulator of pHi in rat hepatocytes.

Introduction

Most cells can maintain a constant cytosolic pH (pHi) in the face of continuous metabolic acid production and can rapidly recover from acute intracellular acid and alkaline loads. The best-characterized pHi regulatory mechanism in mammals is sodium/hydrogen (Na\(^{+}/H^{+}\)) exchange (1–5). In some cells pHi is also regulated by bicarbonate (HCO\(_3\)) transport, including (a) Na\(^{+}\)-dependent Cl\(^{-}\)/HCO\(_3\) exchange in invertebrate (6–9) and also in mammalian cells (10–14), (b) Na\(^{+}\)-dependent Cl\(^{-}/HCO_{3}^{-}\) exchange (15–19), and (c) Na\(^{+}\)-HCO\(_3\) cotransport (20–25).

The mechanisms by which hepatocytes regulate pH have been only partly characterized. Studies using purified plasma membrane vesicles from rat liver have localized a Na\(^{+}/H^{+}\) exchanger to the basolateral (26, 27) and a Na\(^{+}\)-independent Cl\(^{-}/HCO_{3}^{-}\) exchanger to the canalicular membrane (28). Further work with pH-sensitive microelectrodes (29) and the pH-sensitive dye, 2,7-bis(carboxyethyl)-5-6-carboxy-fluorescein (BCECF)\(^{+}\) (30), in hepatocytes maintained in HCO\(_3\)-free media, has demonstrated that pH recovery from an acute intracellular acid load is inhibited by amiloride and is dependent on the presence of extracellular Na\(^{+}\), suggesting that under these conditions, pHi is regulated by Na\(^{+}/H^{+}\) exchange. However, in HCO\(_3\)-containing media, the inhibition of pHi recovery by amiloride was less complete (29), suggesting that alkalization of the hepatocyte may occur by additional mechanisms that involve the transport of HCO\(_3\)\(^{-}\).

To characterize these mechanisms further, we have measured baseline pHi and pHi recovery after an acid load, utilizing BCECF in subconfluent hepatocyte monolayers maintained in both the presence and absence of HCO\(_3\)\(^{-}\). In addition to confirming a role for Na\(^{+}/H^{+}\) exchange, these studies suggest that pHi in rat hepatocytes is also regulated by a mechanism characteristic of Na\(^{+}\)-HCO\(_3\) cotransport.

Methods

Materials. Hepatocytes were isolated from 150–200-g male Sprague-Dawley rats (Camm Research Laboratories, Wayne, NJ). Collagenase (type 1), dimethylsulfoxide (DMSO), nigericin, 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene (DIDS), and amiloride were obtained from Sigma Chemical Co. (St. Louis, MO). The fluorescent dye 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein-acetomethoxymethyl ester (BCECF-AM) was obtained from Molecular Probes Inc. (Eugene, OR) and made up as a 1.0 mM stock in DMSO. Matrigel was obtained from Collaborative Research Inc. (Lexington, MA) and Liebovitz-15 culture medium from Gibco Laboratories, (Grand Island, NY). All other chemicals were reagent grade. See Table I for composition of perfusion solutions.

Hepatocyte isolation and culture. Isolated hepatocytes (viability 86–94%) were prepared as previously described by this laboratory (31, 32), washed twice in Liebovitz-15 medium, and sedimented, and 1.5 ml of the cell pellet was resuspended in 30 ml of L-15 medium supplemented with 10% fetal calf serum and 10 mM Hepes, pH 7.40. The suspension was then poured into plastic petri dishes (10 ml per dish), containing glass coverslips (3 × 0.5 cm) precoated with the extracellular biomatrix, Matrigel, diluted 1:1 with Na-Hepes buffer (solution A, Table I). Incubation at 37°C for 2–6 h resulted in a subconfluent monolayer of hepatocytes plated on the glass slides with a density of

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1. Abbreviations used in this paper: BCECF, 2,7-bis(carboxyethyl)-5,6-carboxy-fluorescein; DIDS, 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene.
Table I. Composition of Buffer Solutions Used

<table>
<thead>
<tr>
<th>Ion</th>
<th>A Heps</th>
<th>B</th>
<th>C HCO3</th>
<th>D Krebs-HCO3</th>
<th>E</th>
<th>F</th>
<th>Standards</th>
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<td>Na+</td>
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<td>139</td>
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<td>139</td>
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<tr>
<td>K+</td>
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<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
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</tr>
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<td>1.0</td>
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<tr>
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<td>2.5</td>
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</tr>
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<td>TMA</td>
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<td>Cl-</td>
<td>137.2</td>
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<td>122.2</td>
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<td>SO4</td>
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<td>HCO3</td>
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<td>24</td>
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<td>Hepes</td>
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<td>0</td>
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<td>Glucose</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>CO2 (%)</td>
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<td>O2 (%)</td>
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<td>7.40</td>
<td>7.40</td>
<td>6.4-7.6</td>
<td></td>
</tr>
</tbody>
</table>

In the NH4+-containing solutions, 20–25 mM NH4+ replaced 20–25 mM Na+. In the depolarization experiments, “baseline” solutions contained 116 mM Na+ and 20 mM TMA+. [K+] was increased from 5.7 to 25.7 mM by replacing 20 mM TMA+ by 20 mM K+. *pH was adjusted to 7.40 with TMA-OH.

~ 10^5 cells/mm². In preliminary studies, Matrigel precoating resulted in prolonged cell attachment to the glass coverslips, preserved cuboidal cell shape, and prolonged cell viability as compared with cells cultured on uncoated glass slides (88% vs. 50% trypan blue exclusion after 20 h).

Measurement of intracellular pH. After 2–6 h in culture, the glass coverslips were fixed vertically at 20° to the excitation light path in a cuvette mounted in a spectrofluorometer (model LS5, Perkin-Elmer Corp., Norwalk, CT). Background fluorescence intensity values (F0) at emission wavelength 530 nm were obtained during excitation at wavelength 500 nm (F500) and 450 nm (F450), after 3–5 min perfusion (10 ml/min) with the experimental buffer (see Table I). Then the perfusion was stopped and BCECF-AM (33) was added to the cuvette (final concentration 10 μM) for 2–5 min. F1 measurements on dye-loaded cells were begun 8–10 min after restarting perfusion, when F450 values were 7.89±2SD 4.06 (range 3.0–21.8)-fold higher than background values. Dye leakage over the subsequent 20-min experimental period, measured as the percentage decline in F450, was 29.7±4% (7–51%). Leakage rates were not affected by DIDS preincubation or by Na+, HCO3, or Cl- removal, but were greater by 28.95±10.0% in studies with amiloride. Photobleaching contributed little to the fall in F1 since in preliminary experiments, F1 values fell at similar rates when F1 measurements were made (a) every minute and (b) after 0 and 20 min only. The leaked dye was effectively washed away by perfusion: the contribution of extracellular dye to the total signal was always < 5%. pH was measured as the ratio of the F250 and F450 values, after correction by subtracting the corresponding values. F500 is directly proportional to intracellular dye concentration and also to pH. F450 is proportional to intracellular dye concentration but is independent of pH (33). Therefore, the F 500/450 ratio is directly proportional to pH, but is independent of changes in intracellular dye concentration. After each experiment, a calibration curve was generated by perfusing the cells with pH standards (6.4, 6.8, 7.2, 7.6; see Table I) (34). These standards contained the K+/H+ ionophore nigericin (12.5 μM) and 120 mM K+, corresponding to cytosolic K+ in hepatocytes as measured by microelectrodes (35). After 4 min of exposure, corrected F500/450 ratios were measured until stable values were obtained (after a further 1–2 min). Correlation coefficients for these standard curves invariably exceeded 0.99 by linear regression analysis. The slopes and y-axis intercepts of the regression lines were 2.82±0.33 and 15.87±2.26, respectively, and were not affected by preincubation of cells in DIDS or by continuous perfusion with amiloride.

Protocol for intracellular acid loading. Cells were acid loaded by pulse exposure to ammonia (NH4Cl, or NH4 gluconate in the CT-free experiments [8]). Recovery of pH, was assessed after ion substitution for Na+, Cl-, and HCO3 as well as during perfusion with amiloride and after preincubation in DIDS, inhibitors of Na+/H+ exchange (4), and anion transport (28, 36, 37), respectively. Each intervention was assessed relative to a paired control experiment, performed in varying order in three to five separate cell preparations on different days. pH recovery was quantitated as: (a) maximum pH, recovery rate dpH/dtmax over a 2-min period; pH, at which this rate was measured was taken as pH1 after the first minute, (b) maximum H+ efflux rate, calculated by multiplying dpH/dtmax by intracellular buffering capacity (see below), (c) percent pH, recovery from nadir toward baseline value over an 8-min period after NH4+ withdrawal. Statistical comparisons were made using paired and Student’s t tests as indicated in the figure legends and text.

Maximum pH, recovery rates were converted to maximum acid efflux rates by multiplying by the calculated intracellular buffering capacity (B). For measurement of non bicarbonate or intracellular buffering capacity Bint, hepatocytes were perfused with Na-free HEPES (solution B, Fig. 1, except Na+ replaced by TMA) to prevent pH, regulation by Na+/H+ exchange and were then exposed to a 4 minute 10 mM NH4Cl pulse (replacing 10 mM TMACl) as illustrated in Fig. 1. Bint was calculated from the fall in pH, resulting from pulse withdrawal, using the formula: B = ([NH4Cl] × 10^(i=0) [pHd]) / ([1 + 10^(i=0) [pHd]) × (pHc - pHd)), where pK of NH4Cl = 9.30, pHex = external pH (7.40), and pHc and pHd = pH, values at points c and d of Fig. 1 (8). In six experiments from separate cell preparations, pHc was 7.30±0.08, pHd was 6.72±0.13, and Bint was 23.3±3.4 mM. This value was utilized as total buffering capacity (Btot) for all experiments performed in the absence of bicarbonate (Table II, series A-D). Bint in the presence of bicarbonate was calculated for each individual experiment as previously described (8) from the formula: Bint = Bint + 2.302 × [HCO3], where [HCO3] = intracellular [HCO3]; calculated from the Henderson-Hasselbach equation during maximum pH, recovery. We assumed a constant value for Btot although it could in theory vary with pH, however, H+ fluxes were usually quantitated over a narrow pH, range of 6.70–6.90 in most paired experiments (Table II) and when this value was used (8).

Figure 1. Example illustrating measurement of intracellular (non-HCO3) buffering capacity Bint. Cells perfused with Na+-free Hepes (replaced by TMA) exposed to 10 mM NH4 (replacing TMA) for 4 min and Bint was calculated as illustrated. Results of six estimates of Bint in six different preparations = 23.3±3.4 mM. For details, see text.
Table II. Parameters of pHr Recovery from an Acute Acid Load under Various Experimental Conditions

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>Baseline pHr</th>
<th>Nadir pHr</th>
<th>dpHr/dt measured</th>
<th>pHr where buffer capacity was measured</th>
<th>Total buffer capacity</th>
<th>H+ efflux max</th>
<th>Percent recovery (8 min)</th>
<th>mM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Hepes alone</td>
<td>10</td>
<td>7.18±0.15</td>
<td>6.72±0.19</td>
<td>0.16±0.05</td>
<td>6.85±0.12</td>
<td>23.3</td>
<td>3.6±1.17</td>
<td>90±10</td>
<td>96±10</td>
</tr>
<tr>
<td>B. Hepes, amiloride</td>
<td>4</td>
<td>7.13±0.12</td>
<td>6.40±0.17</td>
<td>0.05±0.02</td>
<td>6.44±0.13</td>
<td>23.3</td>
<td>1.22±0.42</td>
<td>33±12</td>
<td>8±7</td>
</tr>
<tr>
<td>C. Hepes, Na+ removal</td>
<td>3</td>
<td>7.16±0.09</td>
<td>6.28±0.05</td>
<td>0.02±0.02</td>
<td>6.33±0.07</td>
<td>23.3</td>
<td>0.43±0.36</td>
<td>8±7</td>
<td>8±7</td>
</tr>
<tr>
<td>D. Hepes, 2 mM DIDS</td>
<td>5</td>
<td>7.19±0.19</td>
<td>6.82±0.19</td>
<td>0.16±0.04</td>
<td>6.89±0.16</td>
<td>23.3</td>
<td>3.73±1.0</td>
<td>97±5</td>
<td>97±5</td>
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<tr>
<td>E. KRB alone</td>
<td>9</td>
<td>7.30±0.07</td>
<td>6.79±0.05</td>
<td>0.12±0.04</td>
<td>6.91±0.04</td>
<td>42.2±1.7</td>
<td>5.43±1.8</td>
<td>89±6</td>
<td>89±6</td>
</tr>
<tr>
<td>F. KRB, amiloride</td>
<td>18</td>
<td>7.26±0.09</td>
<td>6.64±0.16</td>
<td>0.11±0.04</td>
<td>6.78±0.13</td>
<td>37.8±4.2</td>
<td>4.26±1.2</td>
<td>74±15</td>
<td>74±15</td>
</tr>
<tr>
<td>G. KRB, amiloride, 0.25 mM DIDS</td>
<td>4</td>
<td>7.23±0.14</td>
<td>6.62±0.21</td>
<td>0.04±0.01</td>
<td>6.70±0.19</td>
<td>35.9±5.7</td>
<td>1.92±0.73</td>
<td>43±19</td>
<td>43±19</td>
</tr>
<tr>
<td>H. KRB, amiloride, 2 mM DIDS</td>
<td>5</td>
<td>7.25±0.07</td>
<td>6.70±0.07</td>
<td>0.04±0.02</td>
<td>6.77±0.02</td>
<td>37±1.6</td>
<td>1.64±0.72</td>
<td>46±9</td>
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<tr>
<td>I. KRB, Na+ removal</td>
<td>3</td>
<td>7.30±0.07</td>
<td>6.64±0.07</td>
<td>0.01±0.01</td>
<td>6.66±0.07</td>
<td>33.8±17</td>
<td>0.33±0.26</td>
<td>7±2</td>
<td>7±2</td>
</tr>
<tr>
<td>J. KRB, Na+ removal, amiloride</td>
<td>2</td>
<td>7.21±0.04</td>
<td>6.31±0.04</td>
<td>0.04±0.005</td>
<td>6.39±0.04</td>
<td>29±0.5</td>
<td>1.16±0.2</td>
<td>15±2</td>
<td>15±2</td>
</tr>
<tr>
<td>K. KRB, Cl depletion</td>
<td>5</td>
<td>7.34±0.05</td>
<td>6.92±0.06</td>
<td>0.16±0.04</td>
<td>7.06±0.09</td>
<td>50±5.2</td>
<td>8.2±1.9</td>
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<tr>
<td>L. KRB, Cl depletion, amiloride (4-min NH4)</td>
<td>3</td>
<td>7.43±0.04</td>
<td>7.06±0.09</td>
<td>0.02±0.02</td>
<td>7.09±0.10</td>
<td>52.6±7.5</td>
<td>1.34±1.06</td>
<td>31±15</td>
<td>31±15</td>
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<tr>
<td>M. KRB, Cl depletion, amiloride (6-min NH4)</td>
<td>5</td>
<td>7.35±0.08</td>
<td>6.70±0.08</td>
<td>0.10±0.03</td>
<td>6.82±0.07</td>
<td>38.5±2.6</td>
<td>4.02±0.85</td>
<td>59±9</td>
<td>59±9</td>
</tr>
</tbody>
</table>

KRB, Krebs-Ringer buffer.

was not the case as with the Na+ removal experiments, the inhibition of recovery was profound and should remain so even if Bint were to vary considerably.

After each set of experiments, the viability of the preparation was assessed. Trypan blue exclusion always exceeded 85% except following exposure to nigericin, which reduced cell viability to 30-80%. However, when cells were exposed to NH4, DIDS, and amiloride but not nigericin, viability always exceeded 85%.

Results

Baseline pHr

Baseline pH in HCO3-free medium was 7.16±0.14 (mean±SD of series A-C, Table II) and was 7.28±0.09 in the presence of HCO3 (mean of series E, F, and I, Table II) (P < 0.01). As shown in Fig. 2A, amiloride has no effect on pHr in the pres-
ence of HCO₃⁻ but in the absence of HCO₃⁻, resulted in a reversible fall in pH of 0.1–0.2 U. Amiloride also acidified cells preincubated with 1.0 mM DIDS despite the presence of HCO₃⁻ (Fig. 2B). However, preincubation of cells in 0.25–2.0 mM DIDS had no significant effect on pHₕ in the presence of HCO₃⁻ when amiloride was absent (series E–H, Table II). These experiments suggest that hepatocytes possess at least two mechanisms for maintaining basal pHₕ; one is HCO₃⁻ independent and amiloride inhabitable and the other is HCO₃⁻ dependent and DIDS inhabitable. To characterize these mechanisms further, we proceeded to study the pHₕ recovery after an acute intracellular acid load.

**Recovery of pHₕ from an acute acid load**

**HCO₃⁻-free media.** As illustrated in Fig. 3A, perfusion with 1 mM amiloride for 8 min greatly slowed pHₕ recovery after cells were acid-loaded by withdrawal of NH₄Cl. In four experiments (Table II, series B, Fig. 3B) amiloride (1 mM) inhibited maximum pHₕ recovery rate and H⁺ effluxₚₚₚ_max by 71±8% and 75±9% relative to paired controls (from series A, Table II). After 8-min amiloride exposure, pHₕ had recovered to only 6.65±0.08 and percent recovery was inhibited by 64±11% relative to controls. After withdrawal of amiloride, pHₕ approached baseline values within 3–4 min.

As shown in Fig. 4A, pHₕ recovery in HCO₃⁻-free media was almost totally and reversibly inhibited by acute extracellular Na⁺ withdrawal (Table I, solution B). In three experiments (Table II, series C and Fig. 4B), Na⁺ removal inhibited dpH/dt_max and H⁺ effluxₚₚₚ_max by 85±15% and 89±12%, respectively, and inhibited percent recovery over 8 min by 92±8% relative to paired controls (from series A, Table II). These data confirm previous studies using both microelectrodes (29) and BCECF (30) and suggest that, in nominally HCO₃⁻-free media, pHₕ recovery after an acute acid load in rat hepatocytes is mediated by Na⁺/H⁺ exchange.

**A HCO₃⁻-dependent component of pHₕ recovery.** After NH₄Cl withdrawal in the presence of HCO₃⁻ (series E, Table II) dpH/dt_max was comparable to that seen in the absence of HCO₃⁻ (series A) (0.13±0.04 vs. 0.15±0.05) and H⁺ effluxₚₚₚ_max was significantly greater (5.43±1.82 vs. 3.60±1.17, P < 0.05). Furthermore, in five experiments (Fig. 5), amiloride inhibited dpH/dt_max and H⁺ effluxₚₚₚ_max only slightly: by 16±8% and 15±5% relative to paired controls (Fig. 6A). In four experiments (Table II, series G, Fig. 6B) dpH/dt_max, H⁺ effluxₚₚₚ_max and percent recovery over 8 min were inhibited by 55±17%, 52±11%, and 39±19%, respectively, relative to paired controls (from series F, Table II). Similar effects were observed after 2.0 mM DIDS (Table II, series H). Neither baseline pHₕ, the rise in pHₕ after NH₄Cl exposure nor its subsequent fall after NH₄Cl withdrawal were significantly affected by either concentration of DIDS, suggesting that DIDS did not affect intracellular buffering capacity. Furthermore, in five paired experiments, performed in the absence of HCO₃⁻ (series D, Table II, Fig. 7), preincubation of cells in 2.0 mM DIDS did not inhibit dpH/dt_max (0.16±0.04 vs. 0.14±0.04 min⁻¹), H⁺ effluxₚₚₚ_max (3.33±1.01 vs. 3.24±0.94 mM) or percent recovery over 8 min (97±4% vs. 88±14%) with respect to paired con-
trols (from series A, Table II). Thus, DIDS specifically inhibited the HCO₃⁻-dependent, amiloride-independent component of the pHᵢ recovery, suggesting that it is mediated in part by a HCO₃⁻ transport system.

Na⁺ dependence. As was seen in HCO₃⁻-free media (Fig. 4), Na⁺ replacement (Table I, solution D) reversibly inhibited the pHᵢ recovery after an acid load in the presence of HCO₃⁻ (Fig. 8, Table II, series I). This inhibition could result in part from simultaneous cellular acidification due to acute Na⁺ withdrawal via reversal of Na⁺/H⁺ exchange. However, even when Na⁺/H⁺ was blocked by simultaneously perfusing with 1 mM amiloride, acute Na⁺ withdrawal in two paired experiments still reversibly inhibited dpH/dtmax and H⁺ efflux_max by 72–79% and by 76–83%, respectively (Fig. 9 and Table II, series J). Thus, the amiloride-independent, HCO₃⁻-dependent component of pHᵢ recovery also seems to be Na⁺ dependent.

Cl⁻ independence. Possible carriers mediating the Na⁺- and HCO₃⁻-dependent pHᵢ recovery include Na⁺-dependent Cl⁻/HCO₃⁻ exchange and Na⁺-HCO₃⁻ cotransport. To distinguish between these, the effects of intracellular Cl⁻ depletion on pHᵢ recovery in HCO₃⁻ were assessed, both in the presence (Fig. 10, Table II, series L, M) and in the absence (Table II, series K) of amiloride. Immediately after loading with BCECF (time zero), cells were perfused with Cl⁻-free media (Table I, solution E) for 20 min to deplete them of intracellular Cl⁻. Cl⁻ concentrations should be < 1 mM, according to previous measurements from this laboratory of Cl⁻ activity with microelectrodes in isolated hepatocytes (Henderson, R. M., unpublished observations).

Baseline pHᵢ was significantly higher when Cl⁻ was removed, Fig. 10 A (7.35±0.08 vs. 7.22±0.05; P < 0.01, seven paired experiments from Table II, series E, F, K, L, and M). Furthermore, in Cl⁻-free media, hepatocyte pHᵢ fell only to 7.06±0.09 after exposure to 20 mM NH₄ for 4 min and recovered very slowly in the presence of 1 mM amiloride (series L). However, after 6-min exposure to 25 mM NH₄, pHᵢ fell to 6.70±0.08 in the presence of amiloride (series M), a value similar to the nadir pHᵢ values seen after 4-min exposure to 20 mM NH₄ in the presence of Cl⁻ (series F). When comparable acidification was achieved in the presence of amiloride, dpH/dt and H⁺ efflux_max were not significantly different in the present and in the absence of Cl⁻ (Fig. 10, Table II, series M; paired controls from series F). Furthermore, pHᵢ recovered to similar absolute values over 8 min (Table II) although percent recovery was less in the absence of Cl⁻ (Fig. 10 B) because of the higher baseline pHᵢ values. Neither was pHᵢ recovery in the absence of amiloride inhibited by Cl⁻ depletion. Indeed, dpH/dtmax, H⁺ efflux_max, and percent recovery over 8 min seemed to be greater in the absence (Table II, series K) than in the pres-
Figure 10. (A) NH₄⁺ pulse in Krebs-HCO₃⁻: effect of Cl⁻ removal. In both experiments, hepatocytes were exposed to 1 mM amiloride for 8 min after NH₄Cl withdrawal. (•) Cells exposed to Cl⁻-free media (Table I, solution E) as of time zero. (○) Cl⁻ present throughout. Note that in Cl⁻-free medium, baseline pHᵢ is higher than when Cl⁻ is present and that a 6-min NH₄Cl pulse is required to reduce pHᵢ to a comparable degree. See text for further details. (B) Results of this and four similar experiments, compared by paired t test.

ence (series E) of Cl⁻ (NS, P > 0.05 and P < 0.05 respectively by unpaired t test). These results favor a role for Na⁺-HCO₃⁻ cotransport rather than Na⁺-dependent Cl⁻/HCO₃⁻ exchange in hepatocyte pHᵢ recovery after intracellular acidification.

Regulation of Na⁺/H⁺ exchange and Na⁺-HCO₃⁻ cotransport by pHᵢ. Analysis of data from all experiments performed in HCO₃⁻-free medium (Table I, series A, n = 10), revealed an inverse correlation between H⁺ efflux max and nadir pHᵢ (r = 0.63, P < 0.05, y = 1.32 - 1.17x). In contrast, when data were pooled from all experiments performed in the presence of HCO₃⁻ and amiloride (n = 18), no correlation was found between nadir pHᵢ and H⁺ efflux max (r = 0.04). These data are consistent with activation of hepatocyte Na⁺/H⁺ exchange by intracellular acidification as is the case in several other cells (5) but provide no evidence that this is the case for Na⁺-HCO₃⁻ cotransport.

Electrogenicity of Na⁺-HCO₃⁻ cotransport in hepatocytes. Na⁺-HCO₃⁻ cotransport is electrogenic in renal proximal tubular and corneal epithelial cells (20–23, 38); in rabbit proximal tubule, the HCO₃⁻/Na⁺ stochiometry is 3:1 (38). To determine whether the cotransporter is electrogenic in hepatocytes, we assessed the effect on pHᵢ of acutely increasing external K⁺ ([K⁺ₑ]) from 5 to 25 mM, which this laboratory has previously shown to depolarize hepatocytes by about 15 mV (35). If the HCO₃⁻/Na⁺ stochiometry is 3:1, such a depolarization should result in a HCO₃⁻- and Na⁺-dependent and DIDS-inhibitable increase in pHᵢ of about 0.1 U.

In the experiment shown in Fig. 11 A, baseline pHᵢ was 7.20 in the presence of Na⁺, HCO₃⁻, and 1 mM amiloride. Increasing [K⁺ₑ] did indeed result in a reversible alkalinization of 0.06 U. Acute Na⁺ removal then caused a rapid fall in pHᵢ to 6.78. However, a subsequent increase in [K⁺ₑ] in the absence of Na⁺ again caused a reversible alkalinization. When cells were depolarized first in the absence and then in the presence of Na⁺, identical results were obtained (data not shown). When the same experiment was performed after preincubation in DIDS (Fig. 11 B), baseline pHᵢ (in the presence of Na⁺, HCO₃⁻, and amiloride) was only 6.85 and the fall after acute Na⁺ removal was almost completely inhibited. However, increasing [K⁺ₑ] again resulted in alkalinization in both the presence

![Figure 11.](image-url)
and in the absence of Na⁺. Table III summarizes the results of all experiments involving acute increases in external K⁺, including similar experiments performed in the absence of amiloride. The degree and rate of alkalization were not inhibited by DIDS, amiloride, Na⁺ removal, or HCO₃⁻ removal. Indeed, they appeared accentuated by Na⁺ removal and by DIDS in the presence of amiloride and by HCO₃⁻ removal in the absence of amiloride. Total H⁺ flux and maximum H⁺ efflux rate after exposure to high K⁺ in the presence of amiloride were significantly diminished by HCO₃⁻ removal; we find this is a result of diminished buffering capacity and does not imply HCO₃⁻ dependence of depolarization-induced alkalization. Furthermore, these parameters again appeared accentuated by Na⁺ removal and by DIDS.

The results in Fig. 11 complement data shown in Fig. 1 in suggesting that when Na⁺/H⁺ exchange is blocked by amiloride, baseline pH is maintained by a Na⁺-dependent, DIDS-inhibitable mechanism consistent with Na⁺-HCO₃ cotransport. However, together with the data in Table III, they suggest that the depolarization-induced alkalization is neither Na⁺ dependent, HCO₃⁻ dependent, nor DIDS inhibitable and, consequently, is unlikely to result from elective Na⁺-HCO₃ cotransport. Rather, these data suggest that depolarization may alkalize the cell by a mechanism dependent on proton conductance. These results therefore provide no evidence that Na⁺-HCO₃ cotransport in hepatocytes is electrogeneric and suggest that it may be electroneutral.

**Discussion**

In the present study, baseline values (~ 7.20) were comparable to values of ~ 7.20 obtained in isolated hepatocytes using nuclear magnetic resonance (39) and in isolated perfused rat liver using microelectrodes (40) but were higher than values of 6.9-7.0 recorded in isolated hepatocytes using DMO (41, 42) and in isolated hepatocyte couples using microelectrodes (29). In hepatocytes, BCECF may be compartmentalized into nuclei or mitochondria which have pH values higher than in the cytosol, although in other cells such as the parietal cell (43), the dye appears confined to the cytosol. Also, hepatocytes were cultured in the presence of fetal calf serum which has been shown to stimulate Na⁺/H⁺ exchange (44). However, the NH₃-induced pH changes observed in the present study are similar to those obtained using microelectrodes in hepatocyte couplets (29), suggesting that they reflected cytosolic pH transients.

The present results confirm previous microelectrode work in isolated hepatocytes (29), suggesting that in the nominal absence of HCO₃⁻, pH regulation is mediated by Na⁺/H⁺ exchange. The inhibition of pH recovery by amiloride was less complete than that after Na⁺ removal, a finding consistent with studies in isolated plasma membrane vesicles (26), where only 50% of pH gradient-driven Na⁺ uptake was inhibited by 1 mM amiloride. The small Na⁺-independent pH recovery may represent a finite plasma membrane H⁺ conductance, as has also been observed in isolated membrane vesicles (26). Hepatocytes (29) as well as other cells (45) depolarize during acidification, therefore after NH₃ withdrawal at an extracellular pH of 7.40, a large in-to-out electrochemical gradient for H⁺ would exist. A further possible mediator of the Na⁺-indepen
dent pH recovery is H⁺ATPase, however, previous studies have failed to demonstrate such a system on hepatocyte plasma membranes (27).

The present study also suggests an additional mechanism for acid extrusion. First, in the presence of HCO₃⁻, H⁺ efflux is after an acute acid load was ~ 50% greater than in the absence of HCO₃⁻. Second, amiloride markedly inhibited pH recovery in the absence of HCO₃⁻ but had little effect in the presence of HCO₃⁻. Finally, the amiloride-independent pH recovery was substantially inhibited by the stilbene DIDS. Higher concentrations of DIDS were used than have been used to block HCO₃⁻ transport in other tissues because hepatocyte anion transporters are relatively resistant to DIDS (28). However, in the present study, inhibition of the amiloride-independent pH recovery was nearly complete at 0.25 mM DIDS since 2.0 mM DIDS produced comparable inhibition. The inhibition seems specific because 2.0 mM DIDS did not affect cell viability, baseline pH, degree of alkalization, and subsequent acidification on NH₃ exposure (making an effect on intracellular buffering capacity unlikely), or the pH recovery when HCO₃⁻ was absent.

These results indicate that hepatocyte pH recovering in part via a HCO₃⁻ transport mechanism. Na⁺-independent Cl⁻/HCO₃⁻ exchange appears an unlikely mediator, first because this exchanger normally functions in the direction of HCO₃⁻ extrusion (15-19) and secondly because most of the HCO₃⁻-dependent recovery is also Na⁺ dependent (Figs. 8 and 9). To distinguish between the other two possibilities, Na⁺-dependent

### Table III: Effect on pH of Hepatocyte Depolarization by Acutely Increasing External K⁺ from 5 to 25 mM under Various Experimental Conditions

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>n</th>
<th>Baseline pH</th>
<th>Rise in pH</th>
<th>Total acid efflux</th>
<th>dpH/dt max</th>
<th>Max H⁺ efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mM</td>
<td>mM</td>
<td>mM/min</td>
<td></td>
<td>mM/min</td>
</tr>
<tr>
<td>Without amiloride, with Na⁺, HCO₃⁻ present</td>
<td>5</td>
<td>7.26±0.11</td>
<td>0.06±0.03</td>
<td>4.32±2.6</td>
<td>0.028±0.02</td>
<td>1.83±0.40</td>
</tr>
<tr>
<td>Without amiloride, with Na⁺, HCO₃⁻ absent</td>
<td>3</td>
<td>7.09±0.02</td>
<td>0.14±0.02</td>
<td>3.30±0.44</td>
<td>0.063±0.02</td>
<td>1.49±0.48</td>
</tr>
<tr>
<td>Without amiloride, without Na⁺, HCO₃⁻ present</td>
<td>2</td>
<td>6.78±0.07</td>
<td>0.06±0.02</td>
<td>3.02±0.33</td>
<td>0.05±0.00</td>
<td>1.97±0.11</td>
</tr>
<tr>
<td>With amiloride, with Na⁺, HCO₃⁻ present</td>
<td>6</td>
<td>7.20±0.06</td>
<td>0.05±0.02</td>
<td>4.26±0.97</td>
<td>0.032±0.01</td>
<td>1.97±0.11</td>
</tr>
<tr>
<td>With amiloride, with Na⁺, HCO₃⁻ absent</td>
<td>3</td>
<td>6.92±0.03</td>
<td>0.06±0.01</td>
<td>1.53±0.35*</td>
<td>0.033±0.11</td>
<td>0.77±0.28*</td>
</tr>
<tr>
<td>With amiloride, without Na⁺, HCO₃⁻ present</td>
<td>3</td>
<td>6.74±0.03</td>
<td>0.12±0.014</td>
<td>5.20±0.17</td>
<td>0.07±0.015</td>
<td>2.66±0.61</td>
</tr>
<tr>
<td>With amiloride, with Na⁺, HCO₃⁻ present, plus 1 mM DIDS preincubation</td>
<td>3</td>
<td>6.98±0.08</td>
<td>0.12±0.022</td>
<td>6.81±1.74</td>
<td>0.065±0.017</td>
<td>3.36±0.61</td>
</tr>
</tbody>
</table>

*P < 0.005 in absence of HCO₃⁻ compared to its presence.
Cl⁻/HCO₃⁻ exchange and Na⁺-HCO₃ cotransport, we studied the effects of intracellular Cl⁻ depletion. Cl⁻ depletion resulted in higher baseline pH₁ values (Fig. 10 A, Table II). Possible reasons for this include: (a) HCO₃⁻ entry into the cell via Cl⁻/HCO₃⁻ exchange. (b) Activation of Na⁺/H⁺ exchange by cell shrinkage after replacement of Cl⁻ by the nonpermeant anion gluconate; this may explain the faster pH₁ recovery in Cl⁻-depleted cells when amiloride was absent (Table II, series E and K). We have recently reported (46) that shrinkage of hepatocytes by exposure to hypertonic media activates Na⁺/H⁺ exchange; and (c) cell alkalinization (Fig. 11) as a result of depolarization of hepatocytes (35) after Cl⁻ removal.

Exposure of Cl⁻-depleted hepatocytes to a “standard” 4 min 20 mM NH₄ pulse produced less alkalinization and subsequent acidification than when Cl⁻ was present in cells (Table II, series F and L), suggestive of a higher buffering capacity in Cl⁻-depleted cells, as expected from their higher baseline pH₁. Therefore, to achieve comparable acidification, we exposed Cl⁻-depleted cells to higher [NH₄] (25 mM) and for longer time (6 min). Under these conditions, Cl⁻ depletion failed to inhibit the amiloride-independent pH₁ recovery significantly. These findings suggest that the pH₁ recovery is not mediated by Na⁺-dependent Cl⁻/HCO₃⁻ exchange unless this system is functional in hepatocytes with a very low Kₘ for intracellular Cl⁻. In other cells where the presence of Na⁺-dependent Cl⁻/HCO₃⁻ exchange has been inferred, pH₁ recovery after an acid load in the presence of amiloride is substantially inhibited by Cl⁻ withdrawal (10–12, 14) and this did not occur in the present study.

Thus, the HCO₃⁻ transporter mediating pH₁ recovery in hepatocytes has properties most consistent with Na⁺-HCO₃ cotransport. In renal proximal tubular cells (20–22) and in corneal epithelial cells (23), Na⁺-HCO₃ cotransport is electrogenic; in rabbit renal proximal tubule the stoichiometry is 3:1 (38). In the presence of electrogenic Na⁺-HCO₃ cotransport, depolarization of cells should lead to Na⁺ and HCO₃⁻ and DIDS-inhibitable alkalinization. The present results (Fig. 11, Table III) indicate that depolarization of hepatocytes (35) by increasing external [K⁺] is indeed associated with reversible intracellular alkalinization. However, this phenomenon is neither HCO₃⁻ nor Na⁺ dependent, nor is it DIDS inhabitable, findings that argue against electrogenic Na⁺-HCO₃ cotransport. In contrast, others have provided evidence favoring electrogenic Na⁺-HCO₃ cotransport in hepatocytes (47, 48). In these studies, acidification and depolarization of hepatocytes was seen after either acutely lowering extracellular pH or removing external Na⁺. The effects of Na⁺ withdrawal were HCO₃⁻ dependent and were inhibitable by the stilbene SITS. In these experiments, acidification may have resulted from HCO₃⁻ exit from the cells via “reverse” Na⁺-HCO₃ cotransport and the depolarization is consistent with exit of an negatively charged species. However, the depolarization of hepatocytes could also result from the fall in pH₁ per se by a decrease in plasma membrane K⁺ conductance, as recently demonstrated even in the absence of HCO₃⁻ (48, 49). Therefore, evidence for electrogenic hepatocyte Na⁺-HCO₃ cotransport remains inconclusive.

In other cells (20–23), electrogenic Na⁺-HCO₃ cotransport normally mediates HCO₃⁻ exit from the cell across the basolateral membrane, driven by the negative intracellular potential (~60 mV). The intracellular membrane potential in hepato-

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