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 (pH_i) regulation in the presence ($+HCO_3^-$) and absence ($-HCO_3^-$) of bicarbonate. Baseline pH_i was higher (7.28±09) in $+HCO_3^-$ than in $-HCO_3^-$ (7.16±0.14). Blocking Na^+/H^+ exchange with amiloride had no effect on pH_i 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'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS). Acute Na^+ replacement in $+HCO_3^-$ also caused acidification which was amiloride independent but DIDS inhibitible.

The recovery of pH_i from an intracellular acid load (maximum H^+ efflux rate) was 50% higher in $+HCO_3^-$ than in $-HCO_3^-$. Amiloride inhibited H^+ $efflux_{max}$ by 75% in $-HCO_3^-$ but by only 27% in $+HCO_3^-$. The amiloride-independent pH_i 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 inhibitible, 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 pH_i in rat hepatocytes.

Introduction

Most cells can maintain a constant cytosolic pH (pH_i) in the face of continuous metabolic acid production and can rapidly recover from acute intracellular acid and alkaline loads. The best-characterized pH_i regulatory mechanism in mammals is sodium/hydrogen (Na^+/H^+) exchange (1–5). In some cells pH_i 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^+-independent Cl^-/HCO_3^- exchange (15–19), and (*c*) Na^+-HCO_3^- cotransport (20–25).

The mechanisms by which hepatocytes regulate pH_i 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)[1] (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, pH_i is regulated by Na^+/H^+ exchange. However, in HCO_3^--containing media, the inhibition of pH_i 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 pH_i and pH_i 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 pH_i 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'-diisothiocyano-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'-diisothiocyano-2,2'-disulfonic acid stilbene.

Table I. Composition of Buffer Solutions Used

Ion	A Hepes +Na	B Hepes −Na	C Krebs-HCO_3 +Na	D Krebs-HCO_3 −Na	E Krebs-HCO_3 −Cl	F Standards
			mM			
Na	136	0	139	0	139	0
K	5.7	5.7	5.7	5.7	5.7	121.2
Mg	1.0	1.0	1.0	1.0	1.0	1.0
Ca	2.5	2.5	2.5	2.5	2.5	0
Choline	0	130	0	0	0	0
TMA	0	6*	0	0	0	0
Cl	137.2	137.2	122.2	122.2	0	140
SO_4	1.0	1.0	1.0	1.0	1.0	1.0
PO_4	1.2	1.2	1.2	1.2	1.2	1.2
HCO_3	0	0	24	24	24	0
Hepes	20	20	0	0	0	0
Gluconate	0	0	0	0	122.2	0
CO_2 (%)	0	0	5	5	5	0
O_2 (%)	100	100	95	95	95	0
pH	7.40	7.40	7.40	7.40	7.40	6.4–7.6

In the NH_4^+-containing solutions, 20–25 mM NH_4^+ 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.

~ 10^3 cells/mm^2. 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 (F_i) at emission wavelength 530 nm were obtained during excitation at wavelengths 500 nm (F_i500) and 450 nm (F_i450), after 3–5 min perifusion (10 ml/min) with the experimental buffer (see Table I). Then the perifusion was stopped and BCECF-AM (33) was added to the cuvette (final concentration 10 μM) for 2–5 min. F_i measurements on dye-loaded cells were begun 8–10 min after restarting perifusion, when F_i450 values were 7.89±SD 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 F_i450, was 29.7±9% (7–51%). Leakage rates were not affected by DIDS preincubation or by Na^+, HCO_3^-, or Cl^- removal, but were greater by 28.95±10.0% in studies with amiloride. Photobleaching contributed little to the fall in F_i since in preliminary experiments, F_i values fell at similar rates when F_i measurements were made (*a*) every minute and (*b*) after 0 and 20 min only. The leaked dye was effectively washed away by perifusion: the contribution of extracellular dye to the total signal was always < 5%. pH_i was measured as the ratio of the F_i500 and F_i450 values, after correction by subtracting the corresponding values. F_i500 is directly proportional to intracellular dye concentration and also to pH_i. F_i450 is proportional to intracellular dye concentration but is independent of pH_i (33). Therefore, the F_i 500/450 ratio is directly proportional to pH_i but is independent of changes in intracellular dye concentration.

After each experiment, a calibration curve was generated by perifusing 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 F_i 500/450 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 perifusion with amiloride.

Protocol for intracellular acid loading. Cells were acid loaded by pulse exposure to ammonia (NH_4Cl, or NH_4 gluconate in the Cl^--free experiments [8]). Recovery of pH_i was assessed after ion substitution for Na^+, Cl^-, and HCO_3^- 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_i recovery was quantitated as: (*a*) maximum pH_i recovery rate dpH/dt_{max} over a 2-min period; pH_i at which this rate was measured was taken as pH_i after the first minute, (*b*) maximum H^+ efflux rate, calculated by multiplying dpH/dt_{max} by intracellular buffering capacity (see below), (*c*) percent pH_i recovery from nadir toward baseline value over an 8-min period after NH_4^+ withdrawal. Statistical comparisons were made using paired and Student's *t* tests as indicated in the figure legends and text.

Maximum pH_i recovery rates were converted to maximum acid efflux rates by multiplying by the calculated intracellular buffering capacity (B). For measurement of non bicarbonate or intrinsic buffering capacity B_{int}, hepatocytes were perifused with Na-free Hepes (solution B, Fig. 1, except Na^+ replaced by TMA) to prevent pH_i regulation by Na^+/H^+ exchange and were then exposed to a 4 minute 10 mM NH_4Cl pulse (replacing 10 mM TMACl) as illustrated in Fig. 1. B_{int} was calculated from the fall in pH_i resulting from pulse withdrawal, using the formula: $B = ([NH_4Cl] \times 10^{(pK-pHc)})/(1 + 10^{(pK-pHext)}) \times (pHc - pHd)$, where pK of NH_4Cl = 9.30, pHext = external pH (7.40), and pH*c* and pH*d* = pH_i values at points *c* and *d* of Fig. 1 (8). In six experiments from separate cell preparations, pH*c* was 7.30±0.08, pH*d* was 6.72±0.13, and B_{int} was 23.3±3.4 mM. This value was utilized as total buffering capacity (B_{tot}) for all experiments performed in the absence of bicarbonate (Table II, series A–D). B_{tot} in the presence of bicarbonate was calculated for each individual experiment as previously described (8) from the formula: $B_{tot} = B_{int} + 2.302 \times [HCO_3^-]$, where $[HCO_3^-]$ = intracellular $[HCO_3^-]$, calculated from the Henderson-Hasselbach equation during maximum pHi_i recovery. We assumed a constant value for B_{int} although it could in theory vary with pH_i. However, H^+ fluxes were usually quantitated over a narrow pH_i range of 6.70–6.90 in most paired experiments (Table II) and when this

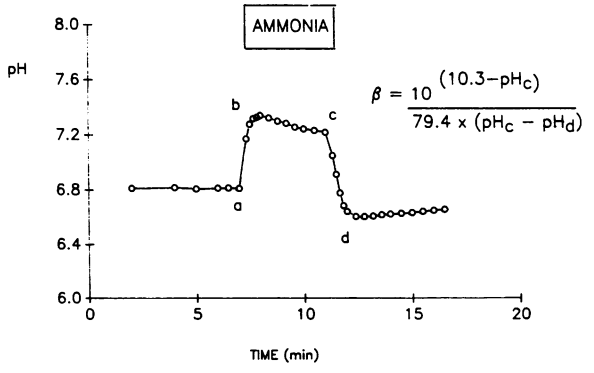

Figure 1. Example illustrating measurement of intrinsic (non-HCO_3^-) buffering capacity B_{int}. Cells perifused with Na^+-free Hepes (replaced by TMA) exposed to 10 mM NH_4^+ (replacing TMA) for 4 min and B_{int} was calculated as illustrated. Results of six estimates of B_{int} in six different preparations = 23.3±3.4 mM. For details, see text.

Table II. Parameters of pH_i Recovery from an Acute Acid Load under Various Experimental Conditions

Intervention	*n*	Baseline pH_i	Nadir pH_i	dpH/dt_{max}	pH_i where dpH_i/dt_{max} measured	Total buffer capacity	H^+ efflux max	Percent recovery (8 min)
							mM/min	
A. Hepes alone	10	7.18±0.15	6.72±0.19	0.16±0.05	6.85±0.12	23.3	3.6±1.17	90±10
B. Hepes, amiloride	4	7.13±0.12	6.40±0.17	0.05±0.02	6.44±0.13	23.3	1.22±0.42	33±12
C. Hepes, Na^+ removal	3	7.16±0.09	6.28±0.05	0.02±0.02	6.33±0.07	23.3	0.43±0.36	8±7
D. Hepes, 2 mM DIDS	5	7.19±0.19	6.82±0.19	0.16±0.04	6.89±0.16	23.3	3.73±1.0	97±5
E. KRB alone	9	7.30±0.07	6.79±0.05	0.12±0.04	6.91±0.04	42.2±1.7	5.43±1.8	89±6
F. KRB, amiloride	18	7.26±0.09	6.64±0.16	0.11±0.04	6.78±0.13	37.8±4.2	4.26±1.2	74±15
G. KRB, amiloride, 0.25 mM DIDS	4	7.23±0.14	6.62±0.21	0.04±0.01	6.70±0.19	35.9±5.7	1.92±0.73	43±19
H. KRB, amiloride, 2 mM DIDS	5	7.25±0.07	6.70±0.07	0.04±0.02	6.77±0	37±1.6	1.64±0.72	46±9
I. KRB, Na^+ removal	3	7.30±0.07	6.64±0.07	0.01±0.01	6.66±0.07	33.8±17	0.33±0.26	7±3.7
J. KRB, Na^+ removal, amiloride	2	7.21±0.04	6.31±0.04	0.04±0.005	6.39±0.04	29±0.5	1.16±0.2	15±2
K. KRB, Cl depletion	5	7.34±0.05	6.92±0.06	0.16±0.04	7.06±0.09	50±5.2	8.2±1.9	99±2
L. KRB, Cl depletion, amiloride (4-min NH_4)	3	7.43±0.04	7.06±0.09	0.02±0.02	7.09±0.10	52.6±7.5	1.34±1.06	31±15
M. KRB, Cl depletion, amiloride (6-min NH_4)	5	7.35±0.08	6.70±0.08	0.10±0.03	6.82±0.07	38.5±2.6	4.02±0.85	59±9

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 B_{int} 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 NH_4, DIDS, and amiloride but not nigericin, viability always exceeded 85%.

Results

Baseline pH_i

Baseline pH in HCO_3^--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 HCO_3^- (mean of series E, F, and I, Table II) ($P < 0.01$). As shown in Fig. 2 *A*, amiloride has no effect on pH_i in the pres-

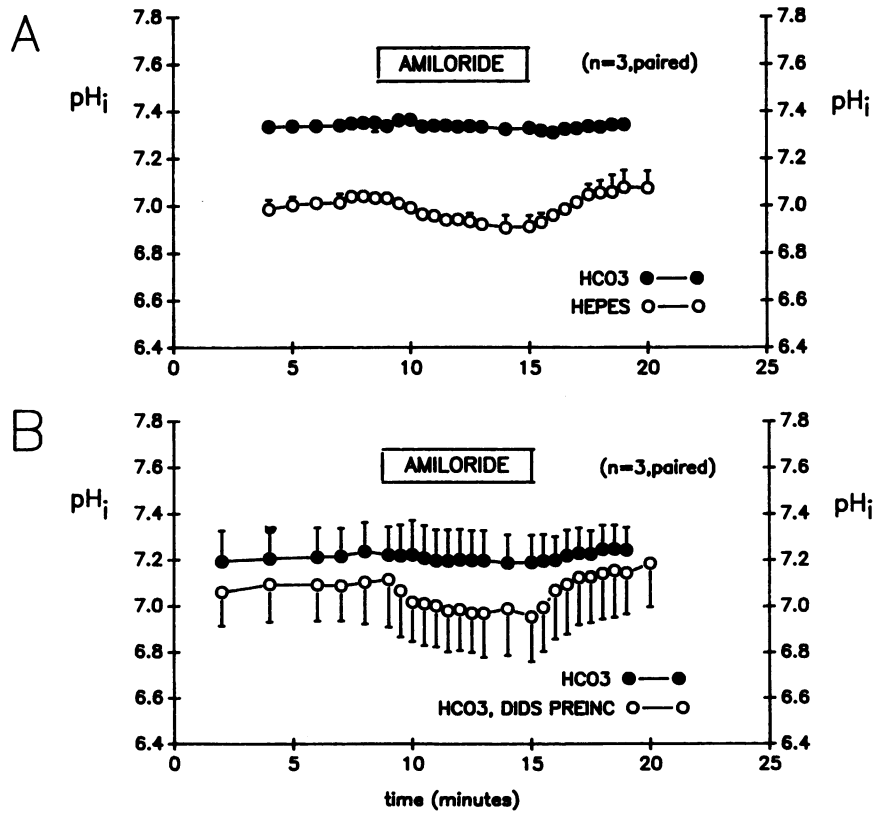

Figure 2. (*A*) Effect of perifusion with 1 mM amiloride on baseline pH_i in the presence (•) and absence (○) of HCO_3^-. (*B*) Effect of 1 mM amiloride on baseline pH_i in the presence of HCO_3^- with (○) and without (•) preincubation of cells in 1.0 mM DIDS.

ence of HCO_3^- but in the absence of HCO_3^-, resulted in a reversible fall in pH_i of 0.1–0.2 U. Amiloride also acidified cells preincubated with 1.0 mM DIDS despite the presence of HCO_3^- (Fig. 2 *B*). However, preincubation of cells in 0.25–2.0 mM DIDS had no significant effect on pH_i in the presence of HCO_3^- when amiloride was absent (series E–H, Table II). These experiments suggest that hepatocytes possess at least two mechanisms for maintaining basal pH_i: one is HCO_3^- independent and amiloride inhibitable and the other is HCO_3^- dependent and DIDS inhibitable. To characterize these mechanisms further, we proceeded to study the pH_i recovery after an acute intracellular acid load.

Recovery of pH_i from an acute acid load

HCO_3^--free media. As illustrated in Fig. 3 *A*, perifusion with 1 mM amiloride for 8 min greatly slowed pH_i recovery after cells were acid-loaded by withdrawal of NH_4^+. In four experiments (Table II, series B, Fig. 3 *B*) amiloride (1 mM) inhibited maximum pH_i recovery rate and H^+ $efflux_{max}$ by 71±8% and 75±9% relative to paired controls (from series A, Table II). After 8-min amiloride exposure, pH_i had recovered to only 6.65±0.08 and percent recovery was inhibited by 64±11% relative to controls. After withdrawal of amiloride, pH_i approached baseline values within 3–4 min.

As shown in Fig. 4 *A*, pH_i recovery in HCO_3^--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. 4 *B*), Na^+ removal inhibited dpH/dt_{max} and H^+ $efflux_{max}$ 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 HCO_3^--free media, pH_i recovery after an acute acid load in rat hepatocytes is mediated by Na^+/H^+ exchange.

A HCO_3^--dependent component of pH_i recovery. After NH_4Cl withdrawal in the presence of HCO_3^- (series E, Table II) dpH/dt_{max} was comparable to that seen in the absence of HCO_3^- (series A) (0.13±0.04 vs. 0.15±0.05) and H^+ $efflux_{max}$ 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^+ $efflux_{max}$ only slightly: by 16±8% and

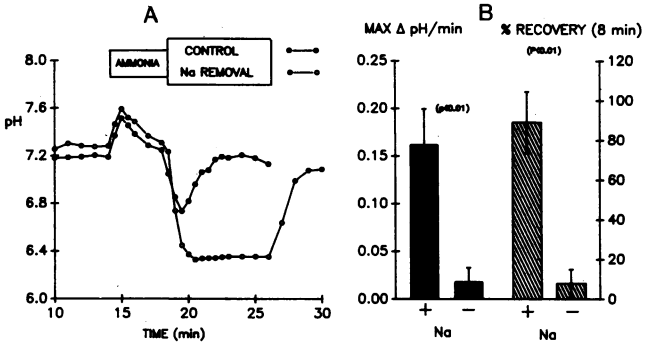

Figure 4. (*A*) NH_4^+ pulse in the nominal absence of HCO_3^-; effect of acute Na^+ removal. (○) Na^+ replaced isoosmotically by choline (Table I, solution B) for 8 min after NH_4 withdrawal. (●) Paired control experiment. (*B*) Maximum change in pH_i/min and percent recovery in three experiments, compared by paired *t* test.

27±7%, respectively, relative to paired controls (from series E and F, Table II). After 8-min amiloride exposure, pH_i had recovered to 7.15±0.13 and percent recovery was inhibited by only 15±5% relative to control. Thus in the presence but not in the absence of HCO_3^-, the pH_i recovery is largely amiloride independent.

Effect of DIDS. Recovery of pH_i from an NH_4^+ pulse in the presence of HCO_3^- and amiloride was substantially inhibited by preincubation of cells in 0.25 mM DIDS for 20 min (Fig. 6A). In four experiments (Table II, series G, Fig. 6 *B*) dpH/dt_{max}, H^+ $efflux_{max}$ 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 pH_i, the rise in pH_i after NH_4^+ exposure nor its subsequent fall after NH_4^+ 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 HCO_3^- (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^+ $efflux_{max}$ (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-

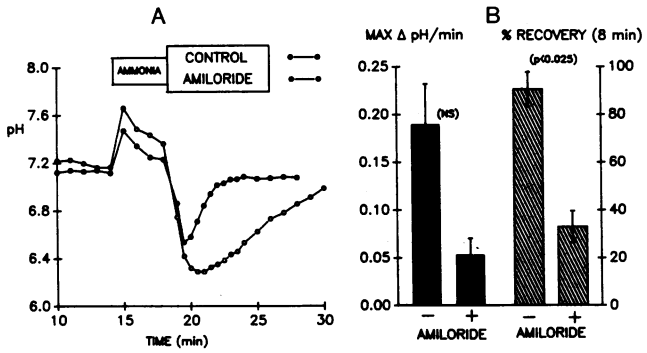

Figure 3. (*A*) NH_4^+ pulse in the nominal absence of HCO_3^- (Na^+-Hepes: Table I, solution A). Effect of amiloride. Hepatocytes were exposed to 20 mM NH_4Cl (replacing NaCl) between 14 and 18 min. (○) Amiloride 1 mM in Na^+-Hepes perfused for 8 min after NH_4Cl withdrawal. (●) Paired control experiment. (*B*) Maximum change in pH_i/min and percent recovery over 8 min, compared by paired *t* test.

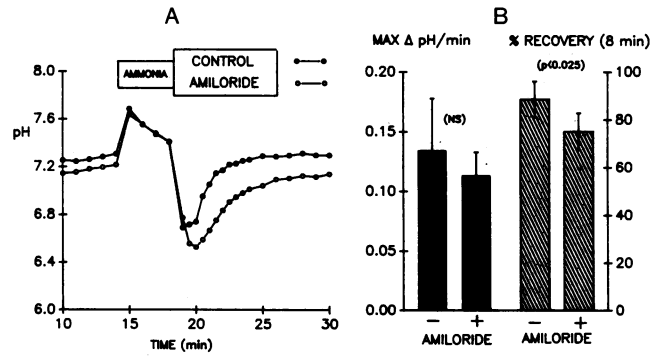

Figure 5. (*A*) NH_4^+ pulse in Krebs-HCO_3^- (Table I, solution C); effect of amiloride. (○) Amiloride 1 mM in Krebs-HCO_3 perfused for 8 min after NH_4Cl withdrawal. (●) Paired control. (*B*) Results of this and four similar experiments, compared by paired *t* test.

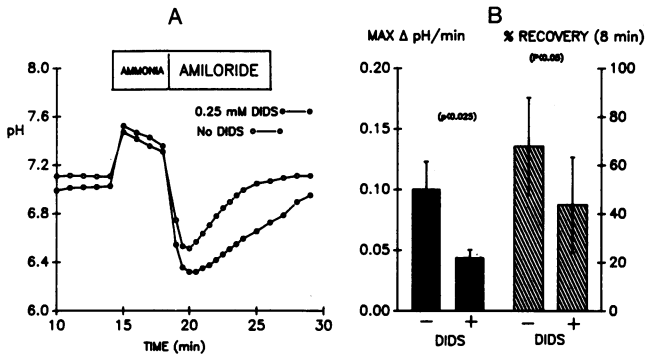

Figure 6. (*A*) NH_4^+ pulse in Krebs-HCO_3^-; effect of amiloride plus 0.25 mM DIDS. In both experiments, cells were perfused with 1 mM amiloride (in KRB-HCO_3^-) for 8 min after NH_4Cl withdrawal. (•) Cells incubated in 0.25 mM DIDS (in Liebovitz-15 medium) for 20 min before loading with BCECF. (○) Paired control experiment. (*B*) Results of this and three similar experiments, compared by paired *t* test.

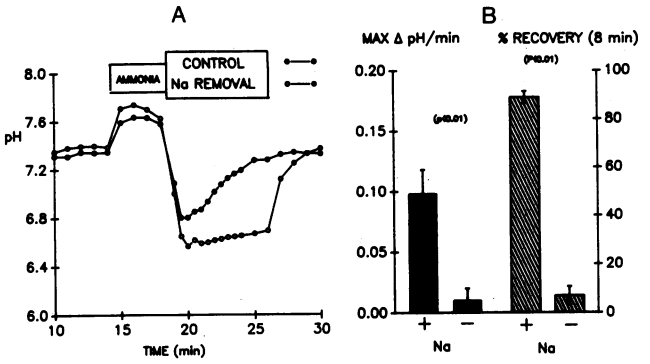

Figure 8. (*A*) NH_4^+ pulse in Krebs-HCO_3^-; effect of Na^+ removal. (○) Na^+ replaced isosmotically with choline (Table 1, solution D) for 8 min after NH_4Cl withdrawal. (•) Paired control experiment. (*B*) Results of this and two similar experiments, compared by paired *t* test.

trols (from series A, Table II). Thus, DIDS specifically inhibited the HCO_3^--dependent, amiloride-independent component of the pH_i recovery, suggesting that it is mediated in part by a HCO_3^- transport system.

Na^+ dependence. As was seen in HCO_3^--free media (Fig. 4), Na^+ replacement (Table 1, solution D) reversibly inhibited the pH_i recovery after an acid load in the presence of HCO_3^- (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/dt_{max} and H^+ $efflux_{max}$ by 72–79% and by 76–83%, respectively (Fig. 9 and Table II, series J). Thus, the amiloride-independent, HCO_3^--dependent component of pH_i recovery also seems to be Na^+ dependent.

Cl^- independence. Possible carriers mediating the Na^+- and HCO_3^--dependent pH_i recovery include Na^+-dependent Cl^-/HCO_3^- exchange and Na^+-HCO_3^- cotransport. To distinguish between these, the effects of intracellular Cl^- depletion on pH_i recovery in HCO_3^- 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 pH_i 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 pH_i fell only to 7.06±0.09 after exposure to 20 mM NH_4 for 4 min and recovered very slowly in the presence of 1 mM amiloride (series L). However, after 6-min exposure to 25 mM NH_4, pH_i fell to 6.70±0.08 in the presence of amiloride (series M), a value similar to the nadir pH_i values seen after 4-min exposure to 20 mM NH_4 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 presence and in the absence of Cl^- (Fig. 10, Table II, series M; paired controls from series F). Furthermore, pH_i 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 pH_i values. Neither was pH_i recovery in the absence of amiloride inhibited by Cl^- depletion. Indeed, dpH/dt_{max}, H^+ $efflux_{max}$ and percent recovery over 8 min seemed to be greater in the absence (Table II, series K) than in the pres-

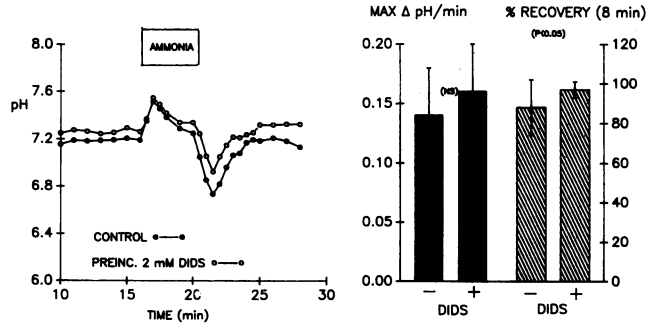

Figure 7. (*A*) NH_4^+ pulse in the nominal absence of HCO_3^-; effect of DIDS. (○) Cells incubated in 2.0 mM DIDS (in L-15) for 20 min before loading with BCECF. (•) Paired control experiment. (*B*) results of this and four similar experiments compared by paired *t* test.

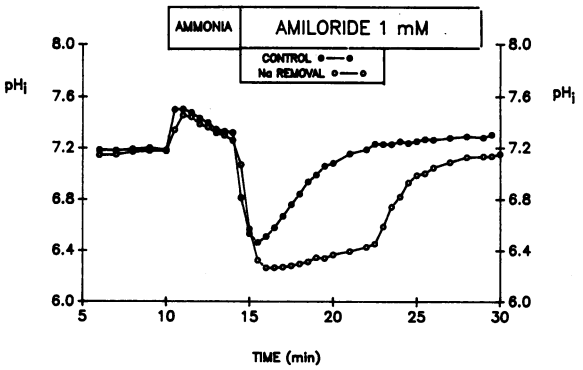

Figure 9. NH_4^+ pulse in Krebs-HCO_3^-; effect of Na^+ removal in the presence of 1.0 mM amiloride. (○) Na^+ replaced by choline for 8 min after NH_4^+ withdrawal. (•) Paired control. Results were similar in two experiments.

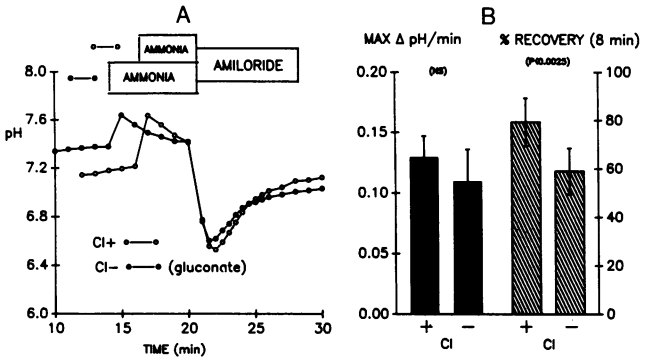

Figure 10. (*A*) NH_4^+ pulse in Krebs-HCO_3; effect of Cl^- removal. In both experiments, hepatocytes were exposed to 1 mM amiloride for 8 min after NH_4Cl withdrawal. (●) Cells exposed to Cl^--free media (Table I, solution E) as of time zero. (○) Cl^- present throughout. Note that in Cl^--free medium, baseline pH_i is higher than when Cl^- is present and that a 6-min NH_4Cl pulse is required to reduce pH_i 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^+-HCO_3^- cotransport rather than Na^+-dependent Cl^-/HCO_3^- exchange in hepatocyte pH_i recovery after intracellular acidification.

Regulation of Na^+/H^+ exchange and Na^+-HCO_3^- cotransport by pH_i. Analysis of data from all experiments performed in HCO_3^--free medium (Table I, series A, $n = 10$), revealed an inverse correlation between H $efflux_{max}$ and nadir pH_i ($r = 0.63$, $P < 0.05$, $y = 1.32 - 1.17x$). In contrast, when data were pooled from all experiments performed in the presence of HCO_3^- and amiloride ($n = 18$), no correlation was found between nadir pH_i 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-HCO_3^- cotransport.

Electrogenicity of Na-HCO_3^- cotransport in hepatocytes. Na^+-HCO_3^- cotransport is electrogenic in renal proximal tubular and corneal epithelial cells (20–23, 38); in rabbit proximal tubule, the HCO_3^-/Na^+ stochiometry is 3:1 (38). To determine whether the cotransporter is electrogenic in hepatocytes, we assessed the effect on pH_i of acutely increasing external K^+ ($[K^+_{ext}]$) from 5 to 25 mM, which this laboratory has previously shown to depolarize hepatocytes by about 15 mV (35). If the HCO_3^-/Na^+ stochiometry is 3:1, such a depolarization should result in a HCO_3^-- and Na^+-dependent and DIDS-inhibitable increase in pH_i of about 0.1 U.

In the experiment shown in Fig. 11 *A*, baseline pH_i was 7.20 in the presence of Na^+, HCO_3^-, and 1 mM amiloride. Increasing $[K^+_{ext}]$ did indeed result in a reversible alkalinization of 0.06 U. Acute Na^+ removal then caused a rapid fall in pH_i to 6.78. However, a subsequent increase in $[K^+_{ext}]$ 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 pH_i (in the presence of Na^+, HCO_3^-, and amiloride) was only 6.85 and the fall after acute Na^+ removal was almost completely inhibited. However, increasing $[K^+_{ext}]$ again resulted in alkalinization in both the presence

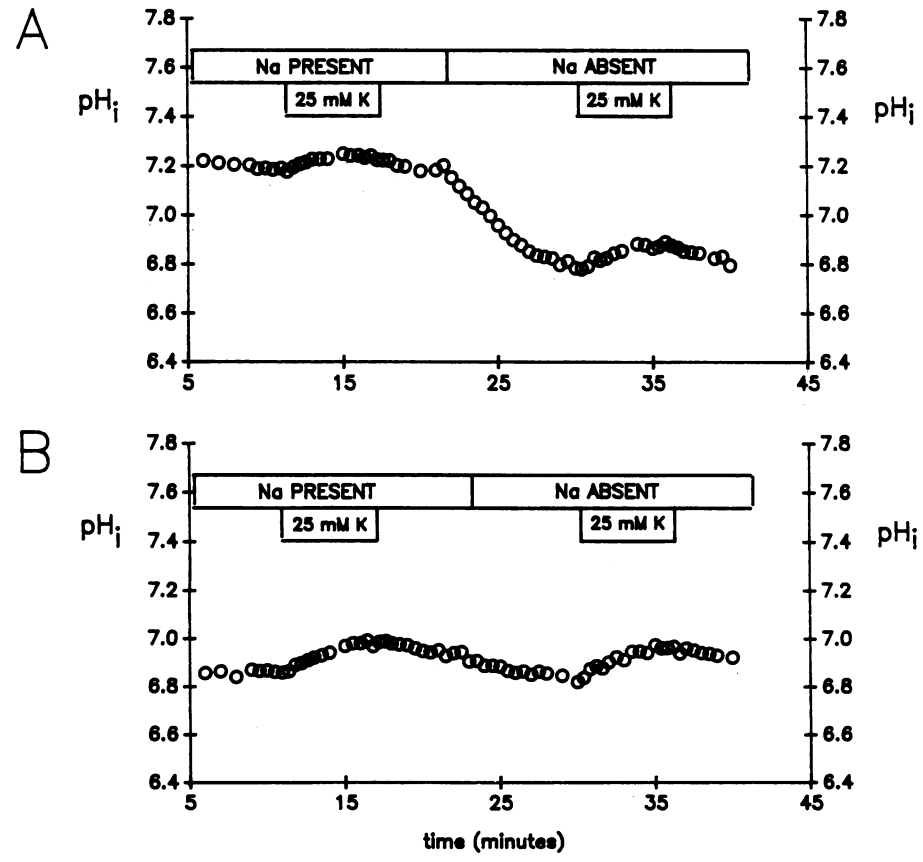

Figure 11. (*A*) Effect of acutely increasing external $[K^+]$ from 5 to 25 mM on pH_i first in the presence and then in the absence of Na^+. (*B*) Same experiment performed in cells preincubated in 1.0 mM DIDS. HCO_3^- and 1.0 mM amiloride were present throughout these experiments.

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 alkalinization were not inhibited by DIDS, amiloride, Na^+ removal, or HCO_3^- removal. Indeed, they appeared accentuated by Na^+ removal and by DIDS in the presence of amiloride and by HCO_3^- 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 HCO_3^- removal; we feel this is a result of diminished buffering capacity and does not imply HCO_3^- dependence of depolarization-induced alkalinization. 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_i is maintained by a Na^+-dependent, DIDS-inhibitable mechanism consistent with Na^+-HCO_3^- cotransport. However, together with the data in Table III, they suggest that the depolarization-induced alkalinization is neither Na^+ dependent, HCO_3^- dependent, nor DIDS inhibitable and, consequently, is unlikely to result from electrogenic Na^+-HCO_3^- cotransport. Rather, these data suggest that depolarization may alkalinize the cell by a mechanism dependent on proton conductance. These results therefore provide no evidence that Na^+-HCO_3^- cotransport in hepatocytes is electrogenic and suggest that it may be electroneutral.

Discussion

In the present study, baseline values ($\sim$ 7.20) were comparable to values of $\sim$ 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 couplets 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 NH_3-induced pH_i 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 HCO_3^-, pH_i regulation is mediated by Na^+/H^+ exchange. The inhibition of pH_i 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_i 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 NH_4^+ 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^+-independent pH_i 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 HCO_3^-, H^+ $efflux_{max}$ after an acute acid load was $\sim$ 50% greater than in the absence of HCO_3^-. Secondly, amiloride markedly inhibited pH_i recovery in the absence of HCO_3^- but had little effect in the presence of HCO_3^-. Finally, the amiloride-independent pH_i recovery was substantially inhibited by the stilbene DIDS. Higher concentrations of DIDS were used than have been used to block HCO_3^- 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_i 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_i, degree of alkalinization, and subsequent acidification on NH_4 exposure (making an effect on intracellular buffering capacity unlikely), or the pH_i recovery when HCO_3^- was absent.

These results indicate that hepatocyte pH_i recovers in part via a HCO_3^- transport mechanism. Na^+-independent Cl^-/HCO_3^- exchange appears an unlikely mediator, first because this exchanger normally functions in the direction of HCO_3^- extrusion (15–19) and secondly because most of the HCO_3^--dependent recovery is also Na^+ dependent (Figs. 8 and 9). To distinguish between the other two possibilities, Na^+-dependent

Table III. Effect on pH_i of Hepatocyte Depolarization by Acutely Increasing External K^+ from 5 to 25 mM under Various Experimental Conditions

Experimental conditions	n	Baseline pH_i	Rise in pH_i	Total acid efflux	dpH/dt_{max}	Max H^+ efflux
				mM		*mM/min*
Without amiloride, with Na^+, HCO_3^- present	5	7.26±0.11	0.06±0.03	4.32±2.6	0.028±0.02	1.83±0.40
Without amiloride, with Na^+, HCO_3^- absent	3	7.09±0.02	0.14±0.02	3.30±0.44	0.063±0.02	1.49±0.48
Without amiloride, without Na^+, HCO_3^- present	2	6.78±0.07	0.065±0.02	3.02±0.33	0.05±0.00	1.97±0.11
With amiloride, with Na^+, HCO_3^- present	6	7.20±0.06	0.053±0.02	4.26±0.97	0.032±0.01	1.97±0.11
With amiloride, with Na^+, HCO_3^- absent	3	6.92±0.03	0.065±0.01	1.53±0.35*	0.033±0.11	0.77±0.28*
With amiloride, without NA^+, HCO_3^- present	3	6.74±0.03	0.12±0.014	5.20±0.17	0.07±0.015	2.66±0.61
With amiloride, with Na^+, HCO_3^- present, plus 1 mM DIDS preincubation	3	6.98±0.08	0.12±0.022	6.81±1.74	0.065±0.017	3.36±0.61

* $P < 0.005$ in absence of HCO_3^- compared to its presence.

Cl^-/HCO_3^- exchange and Na^+-HCO_3^- cotransport, we studied the effects of intracellular Cl^- depletion. Cl^- depletion resulted in higher baseline pH_i values (Fig. 10 *A*, Table II). Possible reasons for this include: (*a*) HCO_3^- entry into the cell via Cl^-/HCO_3^- exchange. (*b*) Activation of Na^+/H^+ exchange by cell shrinkage after replacement of Cl^- by the nonpermeant anion gluconate; this may explain the faster pH_i 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 NH_4 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 pH_i. Therefore, to achieve comparable acidification, we exposed Cl^--depleted cells to higher [NH_4] (25 mM) and for longer time (6 min). Under these conditions, Cl^- depletion failed to inhibit the amiloride-independent pH_i recovery significantly. These findings suggest that the pH_i recovery is not mediated by Na^+-dependent Cl^-/HCO_3^- exchange unless this system is functional in hepatocytes with a very low K_m for intracellular Cl^-. In other cells where the presence of Na^+-dependent Cl^-/HCO_3^- exchange has been inferred, pH_i 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 HCO_3^- transporter mediating pH_i recovery in hepatocytes has properties most consistent with Na^+-HCO_3^- cotransport. In renal proximal tubular cells (20–22) and in corneal epithelial cells (23), Na^+-HCO_3^- cotransport is electrogenic; in rabbit renal proximal tubule the stochiometry is 3:1 (38). In the presence of electrogenic Na^+-HCO_3^- cotransport, depolarization of cells should lead to Na^+ and HCO_3^- 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 HCO_3^- nor Na^+ dependent, nor is it DIDS inhibitable, findings that argue against electrogenic Na^+-HCO_3^- cotransport. In contrast, others have provided evidence favoring electrogenic Na^+-HCO_3^- 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 HCO_3^- dependent and were inhibitable by the stilbene SITS. In these experiments, acidification may have resulted from HCO_3^- exit from the cells via "reverse" Na^+-HCO_3^- 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 pH_i per se by a decrease in plasma membrane K^+ conductance, as recently demonstrated even in the absence of HCO_3^- (48, 49). Therefore, evidence for electrogenic hepatocyte Na^+-HCO_3^- cotransport remains inconclusive.

In other cells (20–23), electrogenic Na^+-HCO_3^- cotransport normally mediates HCO_3^- exit from the cell across the basolateral membrane, driven by the negative intracellular potential (− 60 mV). The intracellular membrane potential in hepatocytes is considerably lower (35–45 mV) (31, 35, 50) so the driving force for HCO_3^- exit should be smaller, even if Na^+/HCO_3^- cotransport is electrogenic. Assuming a 3 HCO_3^- to 1 Na^+ stoichiometry, then with extracellular and intracellular [Na^+] of 140 and 20 mM respectively, external pH of 7.40 and a transmembrane potential gradient of −40 mV, the calculated equilibrium pH_i of Na^+-HCO_3^- cotransport is ~ 7.20 (38). Equilibrium pH_i would be higher still if the cotransporter were electroneutral, as is more consistent with our data and as also seems to be the case in a recently described Na^+-HCO_3^- cotransport system in smooth muscle (51). Therefore Na^+-HCO_3^- cotransport may normally function as an HCO_3^- extruder in other tissues but as a HCO_3^- loader (acid extruder) in the liver under conditions of acid loading. The data in Fig. 11 and two preliminary reports (47, 52) suggest that in hepatocytes also, Na^+-HCO_3^- cotransport may operate in the direction of HCO_3^- efflux under some circumstances such as after acute Na^+ withdrawal and acutely lowering extracellular [HCO_3^-] and pH (at constant P_{CO_2}).

Na^+-HCO_3^- cotransport contributed substantially to acid extrusion in the present study. Maximum H^+ efflux after an acid load was 50% higher in the presence (Table II, series E) than in the absence (series A) of HCO_3^-, suggesting that at least one-third of H^+ extrusion is due to Na^+-HCO_3^- cotransport. This is probably an underestimate since these series were unpaired and, furthermore, H^+ efflux was calculated at lower pH_i in the absence than in the presence of HCO_3^- (6.85 vs. 6.91). This should overestimate the role of Na^+/H^+ exchange, which appears activated at lower pH_i (see Results and Fig. 11). Indeed, the modest (~ 25%) inhibition of pH_i recovery in HCO_3^- by amiloride suggests that Na^+-HCO_3^- may be even more important than Na^+/H^+ exchange in mediating recovery from an acid load. Na^+-HCO_3^- cotransport may also be the dominant transporter maintaining baseline pH_i. Baseline pH_i was significantly higher in the presence than in the absence of HCO_3^- and was unaffected by blocking Na^+/H^+ exchange with amiloride. However, when in addition, Na^+-HCO_3^- cotransport was blocked or reversed by either DIDS peincubation (Figs. 1 *B* and 11 *A*), HCO_3^- removal (Fig. 1 *A*) or Na^+ removal (Fig. 11 A), baseline pH_i always fell.

The present studies do not permit definitive localization of hepatocyte Na^+-HCO_3^- cotransport to the basolateral or the canalicular membrane. However, since the H^+ extruding capacity of the transporter is comparable to that of Na^+/H^+ exchange, a basolateral localization seems likely, as suggested by preliminary studies in isolated membrane vesicles (54). Functional roles of Na^+-HCO_3^- cotransport in hepatocytes other than regulation of pH_i remain to be defined. Possibilities include: (*a*) systemic pH regulation, where the liver may play an important role by modifying the rate of urea synthesis (54); (*b*) hepatocyte volume regulation; we have recently reported (46) that regulatory volume increase in hepatocytes after hypertonic shrinkage is mediated by Na^+/H^+ exchange, together with a HCO_3^- transport process consistent with either Na^+-HCO_3^- cotransport or Cl^-/HCO_3^- exchange; (*c*) growth regulation: in several cells, growth promoting agents elevate pH_i via activation of Na^+/H^+ exchange (55) but in mesangial cells and in ascites tumor cells, these pH_i responses are modified by HCO_3^- transport processes (56, 57). Characterization of the role of Na^+-HCO_3^- cotransport in these important hepatocyte functions will require further study.

Acknowledgments

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