Intracellular pH Regulation in Isolated Rat Bile Duct Epithelial Cells

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Abstract

To evaluate ion transport mechanisms in bile duct epithelium (BDE), BDE cells were isolated from bile duct–ligated rats. After short-term culture pHi was measured with a single cell microfluorimetric set-up using the fluorescent pHi indicator BCECF, and calibrated with nigericin in high K^+ concentration buffer. Major contaminants were identified using vital markers.

In HCO_3^--free media, baseline pHi (7.03±0.12) decreased by 0.45±0.18 pH units after Na^+ removal and by 0.12±.04 after amiloride administration (1 mM). After acid loading (20 mM NH_4Cl) pHi recovery was inhibited by both Na^+ removal and amiloride (JH^+ = 0.74±1.1, and JH^+ = 2.28±0.8, respectively, vs. 5.47±1.97 and 5.97±1.76 mM/min, in controls, respectively). In HCO_3^- containing media baseline pHi was higher (7.16±0.1, n = 36, $P < 0.05$) and was decreased by Na^+ substitution but not by amiloride. Na^+ removal inhibited pHi recovery after an intracellular acid load (0.27±0.26, vs. 7.7±4.1 mM/min, in controls), whereas amiloride reduced JH^+ only by 27%. pH recovery was inhibited by DIDS (0.5–1 mM), but not by Cl^- depletion. Finally, acute Cl^- removal increased pHi by 0.18 pH units in the absence but not presence of DIDS. These data indicate that BDE cells possess mechanisms for Na^+/H^+ exchange, Na^+:HCO_3^- symport and Cl^-/HCO_3^- exchange. Therefore BDE may be capable of transepithelial H^+/HCO_3^- transport. (*J. Clin. Invest.* 1991. 87:1503–1512.) Key words: bile duct epithelial cells • cell isolation and culture • centrifugal elutriation • intracellular pH regulation • Na^+/H^+ exchange • Na^+:HCO_3^- symport • Cl^-/HCO_3^- exchange • bile secretion • bicarbonate secretion

Introduction

Beginning with small cuboidal cells at the canal of Hering which adjoin the bile canaliculi, the bile duct epithelium (BDE)[1] branches progressively in columnar fashion ultimately forming the extrahepatic and common bile ducts. Although comprising only 3–5% of the overall population of liver cells (1), this epithelium provides a large surface area for transport between blood and bile and plays a vital anatomical and physiologic role in the elaboration of bile (2). In fact, notwithstanding considerable species variability, studies in the intact animal or perfused liver suggest that bile duct cells have both absorptive and secretory (3–6) capabilities consistent with their intimate relationship to the hepatic artery which forms its vascular supply.

Despite the importance of this epithelium in both the bile secretory process and in acute and chronic cholestatic disorders that comprise the "vanishing bile duct syndrome" (7), little is known regarding the biology and physiology of BDE cells. For example, largely because of the difficulties connected with their isolation, conventional transport techniques have not been used to explore specific transport systems in BDE cells.

To define the mechanisms mediating fluid and electrolyte transport by BDE in greater detail, we have modified recently described procedures for the isolation and short-term culture of BDE cells (1, 8, 9) and have utilized this preparation to begin characterization of the specific transport mechanisms possibly involved in H^+/HCO_3^- transport, using the pH-sensitive fluorescent dye BCECF and microfluorimetry of single cells.

Methods

Materials

EGTA, penicillin/streptomycin, calf serum, bovine serum albumin, heparin, Hepes, D(+)glucose, insulin, soybean trypsin inhibitor (type (I-S), amiloride, DMSO, deoxyribonuclease (DN-25), nigericin, DIDS, Na-gluconate, K-gluconate, hemicalcium gluconate, and Fast Blue BB salt were purchased from Sigma Chemical Co. (St. Louis, MO). 2,7,bis(carboxyethyl)-5(6)-carboxyfluorescein-acetomethylester (BCECF-AM) was obtained from Molecular Probes, Inc. (Eugene, OR). Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), Matrigel TM from Collaborative Research, Inc. (Bedford, MA), Collagenase A from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Pronase from Calbiochem-Behring Corp. (La Jolla, CA). Liebowitz 15 (L 15), MEM, Joklik modified MEM, α-MEM, L-glutamine, gentamicin, and FCS were from Gibco Laboratories (Grand Island, NY). Acetylated LDL labeled with 1,1-dioctadecyl-1-1,3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI) were from BioTechnology Inc. (Stoughton, MA). *N*(gamma-1-glutamyl)-4-methoxy-2-napthylamide was obtained from Polyscience, Inc. (Warrington, PA). Monoclonal anticytokeratin 7 and 19 antibodies (RPN 1162 and RPN 1165, respectively) were purchased from Amersham Corp. (Arlington Heights, IL), whereas monoclonal antibody anti-rat-leukocyte-common-antigen, clone MCR OX1, was from Serotec, Inc. (Oxford, UK), and Cell-Tak from BioPolymers, Inc. (Farmington, CT).

Bile ductular cell isolation

Bile duct epithelial cells were isolated from 230–250-g male Sprague-Dawley rats (Camm Research Laboratories) 3–5 wk after bile duct ligation, by a modification of the method of Mathis et al. (8). Modifications involved further purification by centrifugal elutriation and selective adherence of Kupffer cells and inflammatory cells to plastic. In

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1. *Abbreviations used in this paper:* BDE, bile duct epithelium; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein; DiI-Ac-LDL, DiI-conjugated acetyl derivative of low density lipoproteins; GGT, gamma-glutamyl transferase; L-15, Leibowitz 15 cell culture medium.

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addition, animals received alternate day s.c. injections of 3 mg phytonadione (AquaMephyton, MSD, West Point, PA) to prevent hemorrhage. Briefly, the portal tissue obtained after collagenase digestion of the liver and containing the hyperplastic bile duct epithelium was separated mechanically from parenchymal tissue, finely minced and further digested with collagenase, hyaluronidase, and DNase. The obtained crude nonparenchymal cell suspension was then subjected to a double Percoll density gradient centrifugation and viable cells banding at densities 1.060–1.075 were collected.

The pellet was resuspended in 50 ml Joklik modified MEM and then subjected to centrifugal elutriation with a model JE-6B elutriator (Beckman Instruments, Inc., Palo Alto, CA) Ice cold MEM supplemented with 0.004% DNase, 10% calf serum, penicillin/streptomycine, and L-glutamine was used as elutriation medium and the rotor speed was set as 2,500 rpm. Cells were loaded at a flow rate of 9 ml/min. Thereafter 150-ml aliquots were collected while sequentially increasing the flow rate to 16, 24, 30, 38 and 46 ml/min. All fractions were then pelleted and resuspended in L-15 medium containing insulin (0.1 μM), FCS (3%), L-glutamine (2 mM), Hepes (25 mM), gentamicin (50 μg/ml), penicillin/streptomycin (100,000 U–100 mg/liter) (plating medium).

Fraction 30 and 38 were combined into plastic tissue culture flasks (Falcon Plastics, Cockeysville, MD) and cells were allowed to settle and adhere to the plastic for 1 h at 37°C. Nonadherent cells were resuspended by swirling the flasks gently, pelleted, and again resuspended in plating medium. Aliquots were taken for determination of cell viability, yield, and cyto- or immunocytochemistry. The remaining cells were plated at a density of 4×10^3 cell/mm^2 in L-15 medium into tissue culture plastic wells (Corning Glass Works, Corning, NY) containing glass coverslip fragments coated with a thin layer of EHS matrix (Matrigel TM, Collaborative Research, Inc.) at 37°C in an air equilibrated incubator. Medium was changed after 4 h. Preparations used to study pHi regulation were cultured in these conditions for 6–24 h. In experiments performed in the presence of bicarbonate, medium was changed after 4 h to α-MEM containing the same additives, but 25 mM $NaHCO_3$ replaced 25 mM Hepes.

Cell identification

Viability, counting and sizing. Viability was determined by Trypan blue exclusion. Cells were counted and sized with a Channalizer 256 counter Coulter Electronics, Inc., Hialeah, FL.

Gammaglutamyl-transpeptidase (GGT). Freshly isolated cells (plated on Cell-Tak coated coverslips for 30 min at 37°C) and cells cultured for 15–24 h on Matrigel coated coverslip fragments were then fixed in cold acetone for 10 min. GGT cytochemistry was performed as described (10, 11).

Endogenous peroxidase activity. Cells were plated on Cell-Tak, fixed in 4% paraformaldehyde, preincubated in diaminobenzadine (DAB) (0.1%) and then reacted with 0.01% H_2O_2 for 15 min.

Cytokeratin immunocytochemistry. Cells were plated and fixed as described for GGT. After preincubation for 30 min in 10% normal goat serum, primary monoclonal antibodies (anti-cytokeratin 19 and 7) were then added (1:10 and 1:30, respectively) with 0.1 M PBS and 0.05% saponin. After incubation for 1 h at room temperature, secondary antibody (anti-mouse IgG FITC conjugated) was added (1:200) for 1 h at room temperature in the same buffer. When an immunoperoxidase technique was employed, 1:100 Fab anti–mouse peroxidase-conjugated antibody was used as a secondary antibody. Thereafter, the specimens were treated as described for peroxidase cytochemistry.

Leukocyte common antigen. Freshly isolated cells were incubated for 1 h with 1:100 monoclonal mouse anti–rat common antigen (12) in ice-cold PBS. Slides were then reacted with FITC-conjugated secondary goat anti–mouse IgG (1:200) for 30 min, washed, and mounted.

Vital markers. Vital markers for endothelial cells and Kupffer cells were also used to identify these contaminants directly on the stage of the microscope before selecting the cells to be studied. Acetylated LDL labeled with the fluorescent dye DiI was used to label endothelial cells and macrophages (13, 14) by incubating the plated cells for 3–4 h in L-15 medium containing 10 μg/ml Ac-DiI-LDL. Colloidal ink phagocytosis was demonstrated by administering Higgins India ink to the rat via the inferior vena cava 10 min before liver perfusion, at the dose of 9 mg (13, 15).

Electron microscopy. In selected experiments, freshly isolated cells and cells maintained in culture for 6 h were processed for electron microscopy as previously described for hepatocytes (16).

pHi determination

Intracellular pHi. This was measured using the fluorescent pHi indicator, BCECF given as the acetoxymethyl-ester. Because the yield of cells was low and the preparation contained contaminants, a microfluorimetric, single-cell method was employed (17).

Optical system. Bile duct cells on glass coverslips were loaded with BCECF (12 μM) for 30–40 min, washed for 10 min in BCECF-free medium, and transferred onto a thermostated perfusion chamber placed on the stage of a Zeiss IM 35 inverted microscope. Proper gassing of the various perfusion media (see Table I) was facilitated by an artificial lung. pHi was measured as the ratio between the pHi-sensitive and the isosbestic excitation wavelengths (495/440 nm). In preliminary experiments wavelengths were changed by rapid manual displacements. Subsequently the microscope was connected to a Spex-AR-CM-micro system (Spex Industries, Edison, NJ) equipped with a rotating chopper mirror able to rapidly alternate the light generated by a 150-W xenon lamp between two excitation beams. A pinhole device (Nikon) was inserted in the emission light pathway to restrict the measuring spot of the photometer to a selected area of the field. The emitted light was read at 530 nm by a photometer with a photon-counting attachment. Background fluorescence was then determined, measuring a cell-free area of the same size (18) and substracted from fluorescent intensity readings. Average signal to background ratio was 77:1.

Cell identification and selection. Clusters of at least 5–10 small (9–13 μm diameter) mononucleated cells were selected for study (9). Cells were avoided if they contained colloidal carbon particles (Kupffer cells) (13, 15), showed a fibroblastlike aspect, or contained cytoplasmic translucent vacuoles (Ito cells) (19). Endothelial cells and macrophages which take up DiI-Ac-LDL were also excluded (13–15).

pHi calibration. 495/490 F1 ratio data were converted to pHi values by generating a calibration curve at the end of each experiment. Cells were exposed to nigericin (24 μM) in a Na^+-free medium containing 120 mM KCl, buffered at three different pH values (pH 6.8, 7.2, 7.6), as previously described (20, 21).

Determination of total (β_{tot}) and intrinsic (β_i) intracellular buffering power. The intrinsic buffering power (β_i) (in the absence of the open buffering system HCO_3-CO_2) was determined at different pHi as recently described ($n = 7$) (18, 22). The total intracellular buffering power (in the presence of bicarbonate) β_{tot}, was then calculated from β_i as: $\beta_{tot} = \beta_i + 2.302 \times [HCO_3^-]_i$ (where $[HCO_3^-]_i$ is derived from the Henderson-Hasselbach equation). Transmembrane H^+ fluxes (JH^+) were calculated from $\delta pHi/\delta t$ as $JH^+ = \beta_i \times \delta pHi/\delta t$, where the $\delta pHi/\delta t$ was measured by hand-drawn tangent from the experimental plots.

Solutions

The composition of solutions used for studies on pHi regulation are given in Table I. A 1 mM stock solution of BCECF was solubilized in DMSO, whereas amiloride was dissolved by gently warming in deionized water.

Statistical analysis

Data are presented as arithmetical means±SD, unless otherwise stated. Statistical analysis were conducted using the paired or unpaired t test, as appropriate.

Results

Bile duct epithelial cell isolation and culture

An average of $5.7 \pm 2 \times 10^6$ nonadherent cells with a median diameter of 10.5 μm and a viability of 89±3% were recovered

Table I. Composition of Buffer Solutions Used

	Hepes	Hepes 0 Na^+	Hepes NH_4	KRB	KRB 0 Na^+	KRB 0 Cl^-	KRB NH_4
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Na^+	141	0	121	140	0	140	110
K^+	5.9	5.9	5.9	5.9	5:9	5.9	5.9
Mg^+	1	1	1	1	1	1	1
Ca^{2+}	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Choline	0	135	0	0	140	0	0
TMA	0	6	0	0	0	0	0
Cl^-	142.2	142.2	142.2	122.2	122.2	0	122.2
SO_4^-	1	1	1	1	1	1	1
PO_4^-	1.2	1.2	1.2	1.2	1.2	1.2	1.2
HCO_3^-	0	0	0	25	25	25	25
Hepes	10	10	10	0	0	0	0
Gluconate	0	0	0	0	0	123.5	0
NH_4^+	0	0	20	0	0	0	30
Glucose	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Pyruvate	1	0	0	1	0	1	0
O_2 (%)	100	100	100	95	95	95	95
CO_2 (%)	0	0	0	5	5	5	5
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4

from the combined fractions 30 and 38 (n = 25). The majority of the cells stained positively for GGT (72±7.2%, range 60–85%) and were therefore identified as BDE cells (Fig. 1 *C*) (1, 2, 8, 9, 23). BDE cells were also positive for CK 19 + 7 (62±8%) Fig. 1, *A* and *B* (1, 2, 9). DiI and peroxidase positive cells (endothelial cells, macrophages, and Kupffer cells) were 13.4±3.7 and 12±6.5%, respectively. LCA positive cells were 3±0.4%. Contamination with hepatocytes was minimal.

BDE cells easily attached to Matrigel, when plated at relatively high density in L-15 medium supplemented with 3% FCS (10). Moreover, the percentage of GGT-positive cells increased by 8–10% after 15–24 h culture suggesting that the biomatrix favored attachment of BDE cells compared to contaminants. Cultured BDE cells appeared as small (8–13 μm) mononucleated rounded cells mostly organized in three-dimensional clusters rather than in monolayers (8). Fig. 1 *D* illustrates a cluster of BDE cells, 6 h after plating demonstrating preservation of cell ultrastructure and reestablishment of tight contacts between the cells. These cells were clearly distinguishable from occasional contaminants like hepatocytes, cells with fibroblastoid appearance and from very flattened cells with cytoplasmic refractile vacuoles, that were identified as Ito cells (19). Because clusters were mostly GGT-positive, cluster formation was also used as a criteria when selecting cells for functional studies.

Cells that phagocytosed colloidal ink (3% of the cell population) were easily distinguishable because of the presence of black cytoplasmic inclusions. However, in BDL rats, identification of Kupffer cells by colloidal ink was less effective than in normal rats, because the percent of ink positive cells was significantly lower than that of peroxidase positive cells presumably reflecting impairment of the reticuloendothelial system in the liver during cholestasis. DiI-Ac-LDL–positive cells (endothelial cells and macrophages) could also be easily identified on the stage of the microscope because of their bright fluorescence under rhodamine excitation.

pHi regulation in BDE cells

BCECF loading. BDE cells required a 30–40-min incubation in BCECF to load in contrast to hepatocytes where 3–5 min would be sufficient to give strong fluorescent signals. This finding is consistent with a deficiency of phase I reaction enzymes in BDE (23). On the other hand, dye leakage was not a significant problem in BDE cells, and fluorescent signals could be obtained from the same cells for up to 40 min.

Intrinsic buffering power. βi measured 27.6 mM/pH unit at pHi 6.85, in agreement with values reported for many cells, including rat hepatocytes in subconfluent monolayers (21). When measured at different pHi, βi changed from 17.88 mM/pH unit at pHi 7 to 65.64 mM/pH unit at pHi 6.55. A similar strong dependency of βi on pHi has also been demonstrated in mesangial (18), hepatoma (22), parietal cells (24), and hepatocytes (25).

pHi regulation in bicarbonate-free media: basal pHi. Basal pHi in a nominally bicarbonate-free Hepes-buffered medium, measured 7.03±0.12 (n = 14). As shown in Fig. 2 when external Na^+ was removed by substitution with choline (maneuver that reverses the mode of operation of the Na^+/H^+ exchanger), pHi decreased by 0.45±0.18 (n = 4) pHi units at a rate of −11.6±2.54 mM/min. After Na^+ readmission, pHi rapidly recovered to baseline. Amiloride (1 mM), a Na^+/H^+ exchange inhibitor also produced a reversible fall in pHi (0.12±0.04 pH units, n = 4) at a rate of −2.14 mM/min. This data indicates that the steady-state pHi of BDE cells, in the absence of HCO_3^- is maintained by a Na^+-dependent, amiloride-inhibitable mechanism (likely a Na^+/H^+ exchange).

Recovery of pHi from an acute acid load. To study transport mechanisms that mediate H^+ extrusion in BDE cells, we evaluated the recovery of pHi after an acute acid load with 20 mM NH_4Cl as described (21, 26). As shown in Fig. 3 *A*, BDE cells recover from the acid load (nadir pHi 6.70±0.13, n = 14) by extruding protons at a maximal rate of 7.45±2.66 mM/min (n = 14) (or 5.76±1.78 mM/min (n = 14) at pHi = 6.78±0.09).

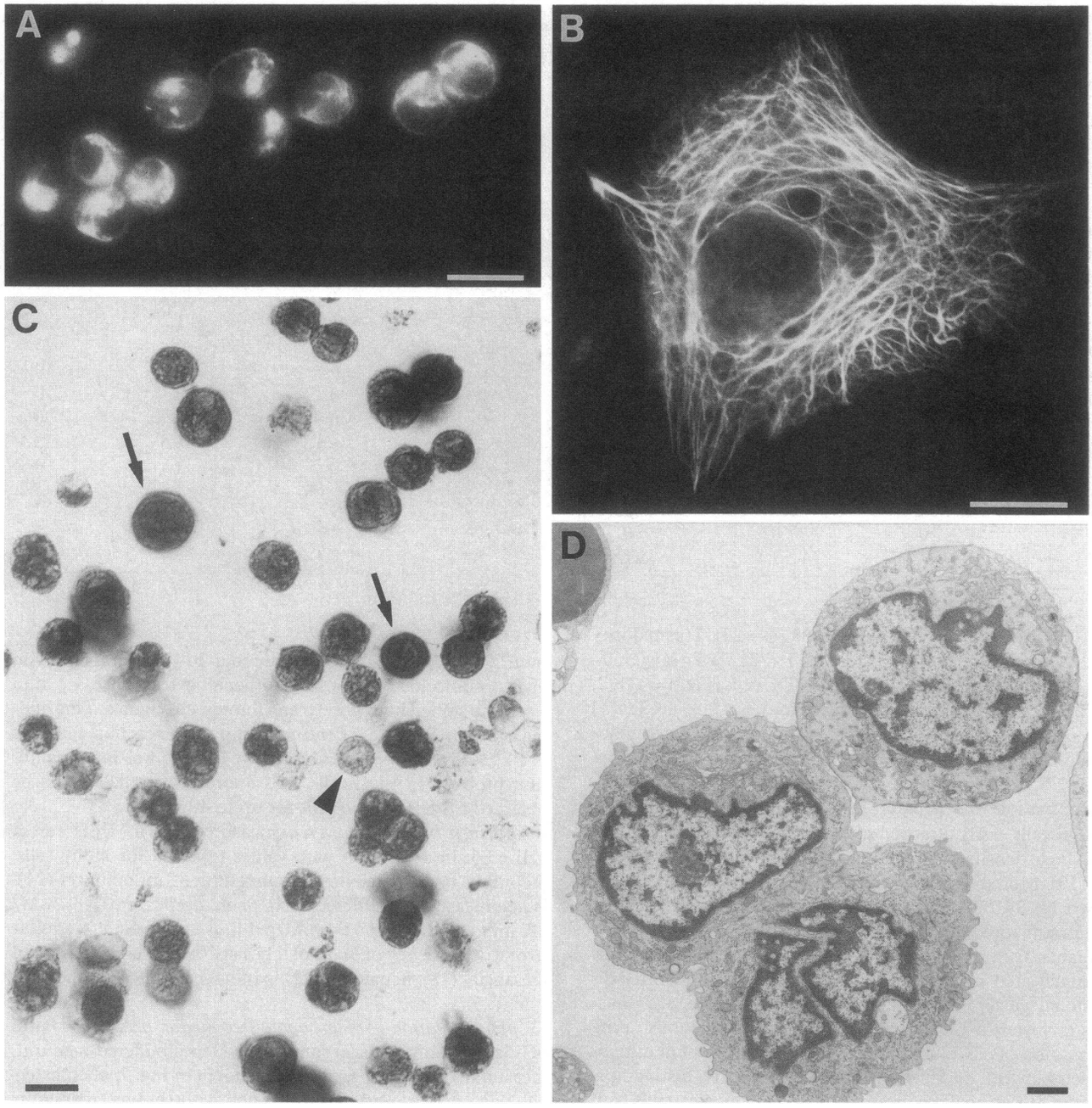

Figure 1. (*A*) Group of freshly isolated BDE cells from fractions 30 and 38 combined showing positive immunocytochemical staining for cytokeratin 19 + 7. Magnification, 1,213. Bar, 10 μm. (*B*) Cytokeratin 19 + 7 immunocytochemistry of a BDE cell cultured for 6 d on matrigel. Magnification, 1,535. Bar, 10 μm. (*C*) Freshly isolated BDE preparation from fractions 30 and 38 combined. GGT cytochemistry. Original magnification, 848. Bar, 10 μm. (*D*) Electron microscopy of BDE cells after 6 h culture on matrigel. Horizontal section through a cluster of cells. Note interdigitating intercellular spaces and the presence of tight contacts between the cells with desmosomes. Magnification, 6,125. Bar, 1.0 μm.

In six experiments, a second NH_4Cl pulse was performed and, at the moment of NH_4Cl withdrawal, external Na^+ was completely substituted with choline (Fig. 3 *A*). After Na^+ removal, intracellular acidification was much greater (nadir pHi reaching 6.5±0.2) and pHi recovery was completely blocked (JH^+ = 0.74±1.12 mM/min). pHi rapidly returned to basal values after Na^+ readmission.

Moreover, when BDE cells were superfused with 1 mM amiloride after withdrawal of NH_4Cl, (n = 8, Fig. 3 *B*), pHi recovery was greatly slowed (JH^+ = 2.28±0.8 mM/min and 0.03±0.01 δpHi/min during amiloride vs. 5.97±1.76 mM/min and 0.16±0.06 δpHi/min during control pulses). Inhibition was reversible and pHi recovered to baseline after withdrawal of amiloride.

Although 38% of H^+ efflux was insensitive to amiloride inhibition, these data are most consistent with the experiments on basal pHi and suggest that in nominally HCO_3^--free medium, BDE cells recover from an acute acid load primarily by

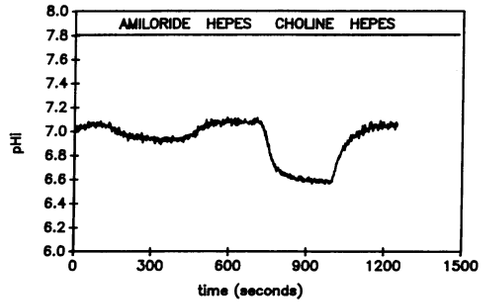

Figure 2. Effects of amiloride and sodium removal on baseline pH in isolated BDE cells. BDE cells were loaded with BCECF for 30 min and washed for 10 min. The tracing shows the fluorescence intensity ratio, calibrated as pHi, recorded from a cluster of 5–10 BDE cells. Solutions were changed as indicated above the trace. Cells, bathed in HCO_3^--free Hepes-buffered Ringers, were first perfused with the Na^+/H^+ inhibitor, amiloride (1 mM). pHi decreased and then returned to baseline after amiloride washout. pHi rapidly acidified also after external Na^+ substitution (choline) and rapidly returned to baseline after Na^+ readmission. The tracing is a representative example of four similar experiments.

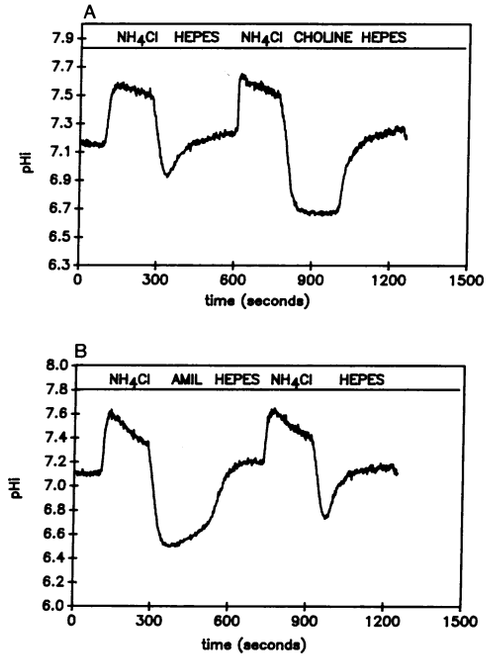

Figure 3. (*A*) Effects of external Na^+ removal on pHi recovery from an acid load induced by pulse withdrawal of 20 mM NH_4Cl in the nominal absence of HCO_3^-. pHi changes during two different pulses are shown. pHi rises upon exposure to NH_4Cl due to the entry of the permeant weak base NH_3 and its protonation to the impermeant NH_4^+. After withdrawal of external NH_4Cl, NH_4^+ releases a proton and leaves the cell as NH_3, thereby acutely acidifying the cell. BDE cells rapidly recover to baseline in normal Hepes-buffered Ringer media. When the acid load is performed in Na^+-free Ringer's (second pulse), nadir pHi is more acid and pHi recovery is completely blocked until Na^+ is readmitted. The tracing is representative of six similar experiments. (*B*) Effects of the Na^+/H^+ exchange inhibitor amiloride on pHi recovery from an acid load in the absence of HCO_3^-. BDE cells were acidified as described above. When amiloride was present (first pulse) pHi recovery was greatly inhibited compared to control pulses (second part of the tracing). Data are representative of eight similar experiments.

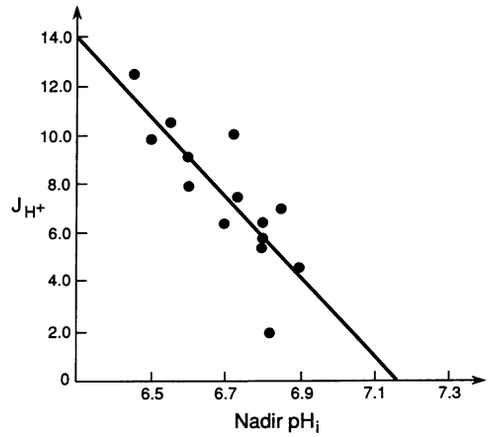

Figure 4. Relationship between maximal H^+ efflux rates (JH^+) and pHi after NH_4Cl pulse. In HCO_3^--free medium, JH^+ is presumed to reflect Na^+/H^+ exchange activity. JH^+_{max} was calculated as described in the methods section, from βi and maximal recovery rates.

Na^+/H^+ exchange. Moreover, when maximal acid extrusion rates were plotted versus nadir pHi (Fig. 4) an inverse relationship between these two parameters was found ($r = 0.83$, $y = 1.17 - 1.64x$) consistent with activation of BDE cell Na^+/H^+ exchange by intracellular acidification, as described for a number of other cell types (27).

pHi regulation in the presence of bicarbonate: basal pHi. In the presence of 25 mM HCO_3^-/5% CO_2, basal pHi of BDE cells was significantly higher than in the absence of bicarbonate (7.16±0.11 vs. 7.03±0.12, $P < 0.05$, $n = 36$). In four experiments acute Na^+ removal with choline decreased pHi by 0.52±0.2 pHU, whereas amiloride had no effect on baseline pHi, when HCO_3^- was present (Fig. 5). However, after incubation with 0.5 mM DIDS, an inhibitor of HCO_3^--dependent transport processes (Table II), pHi was significantly reduced to 6.95±0.09, a value not significantly different from baseline pH in Hepes buffer. These data suggest that a Na^+-dependent amiloride-insensitive, DIDS-inhibitable HCO_3^- transport mechanism maintains pHi above the one measured in bicarbonate-free media.

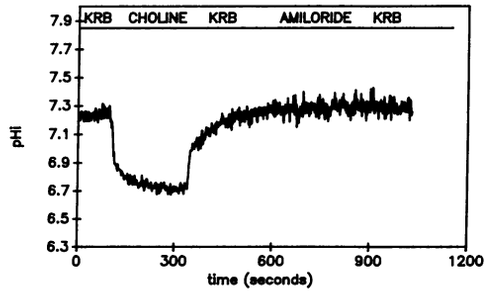

Figure 5. Effect of external Na^+ removal and amiloride on baseline pHi in the presence of HCO_3^-. BDE cells, bathed in KRB, gassed with 95% O_2/5% CO_2, rapidly acidified when exposed to a Na^+-free KRB (equimolar substitution with choline). pHi rapidly recovered to baseline when Na^+ was readmitted. On the other hand, amiloride (1 mM) had no effect on baseline pHi. Data are representative of four similar experiments.

Table II. pHi Recovery from Acute Intracellular Acid Load

Condition	Basal pHi	Recovery rates	JH^+
	pHi units	*δpHi/min*	*mM/min*
Hepes controls (n = 6)	7.05±0.12	0.14±0.04	5.47±1.97
Hepes Na-free (n = 6)	7.04±0.15	0.01±0.016	0.74±1.12
Hepes controls (n = 8)	7.01±0.12	0.16±0.06	5.97±1.76
Hepes amiloride (n = 8)	7.00±0.13	0.03±0.01	2.28±0.8
KRB controls (n = 6)	7.08±0.01	0.14±0.05	7.77±4.1
KRB Na-free (n = 6)	7.07±0.11	0.001±0.002	0.27±0.26
KRB controls (n = 20)	7.15±0.11	0.16±0.05	8.47±3.12
KRB amiloride (n = 20)	7.14±0.1	0.12±0.06	6.16±2.75
KRB DIDS 0.5 mM (n = 4)	6.95±0.08	0.072±0.008	3.6±0.66
KRB DIDS 1 mM (n = 8)	6.91±0.09	0.07±0.02	3.54±0.98
KRB Cl-depleted amiloride (n = 4)	7.38±0.07	0.12±0.003	5.86±1.22

For media composition, see Table I. DBE cells were acid loaded by pulse withdrawal of NH_4Cl (20 mM in Hepes-buffered media or 30 mM in media containing bicarbonate; NH_4-gluconate substituted NH_4Cl in Cl-free experiments). Recovery rates were calculated as δpHi/min during the recovery phase after acidification. H^+ efflux rates (JH^+) were calcualted from recovery rates and β_i as described in the text. (Values are expressed as mean±SD.)

Recovery of pHi from an acute acid load. Because intracellular total buffering power increased in HCO_3^--containing medium (26), it was necessary to administer 30 mM NH_4Cl to induce comparable degrees of acidification as observed in HCO_3^--free medium (28). As shown in Fig. 6 *A*, pHi decreased to 6.7±0.14 and then recovered to baseline at a significantly higher rate than in the absence of bicarbonate (8.14±3.5 mM/min at pH 6.8±0.06, n = 25 vs. 5.76±1.78 mM/min at pHi 6.78±0.09, n = 14, $P < 0.01$).

As shown in Fig. 6 *A*, external Na^+ removal produced a greater degree of acidification (6.41±0.19, n = 6) and, as in the absence of HCO_3^-, completely blocked pHi recovery (JH^+ = 0.27±0.26 mM/min, N = 6).

In contrast, as shown in Fig. 6 *B*, in the presence of amiloride (1 mM), nadir acidification was lower (6.6±0.14, n = 20), and pHi recovery was only slightly impaired, JH^+ = 6.16±2.75 mM/min at pHi 6.77±0.08, n = 20. Therefore, in the presence of bicarbonate, the amiloride-inhibitable component of pHi recovery amounted to only 27% of the total recovery rate, versus 62% in Hepes.

Recovery from an acid load in the presence of amiloride and HCO_3^- was substantially inhibited by preincubation of BDE cells for 20 min to 1 h with 0.5 mM or 1 mm DIDS (Fig. 7). Nadir pHi was 6.5±0.11 (n = 4) and 6.53±0.12 (n = 8),

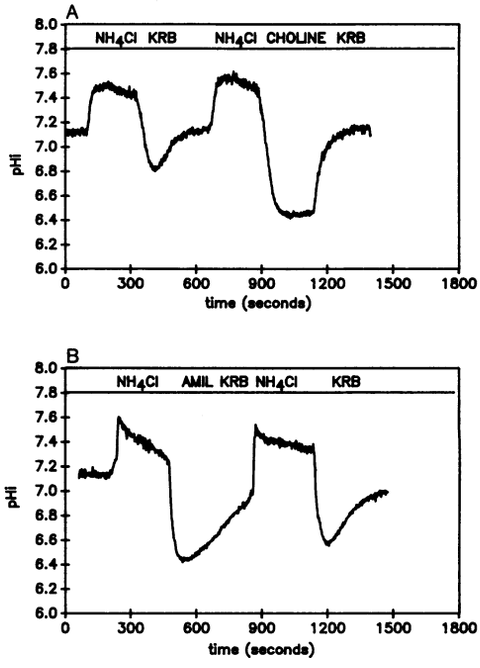

Figure 6. (*A*) Effect of external Na^+ removal (equimolar substitution with choline) on pHi recovery from an intracellular acid load, in the presence of HCO_3^-. Cells were acidified by pulse withdrawal of 30 mM NH_4Cl. pHi recovery was completely inhibited in the absence of Na^+ (second pulse) and quickly recovered after Na^+ readmission. (*B*) Effect of amiloride on pHi recovery from an acid load in the presence of HCO_3^-. Amiloride (1 mM) (first pulse), only slightly inhibited pHi recovery, compared to the control experiment (second pulse). The recording is representative of 20 similar experiments.

respectively, and JH^+ was reduced to 3.66±0.66 mM/min and 3.54±0.98, respectively. Acid extrusion rates in the presence of DIDS and amiloride are thus similar to those measured in the absence of HCO_3^- during amiloride administration, indicating that DIDS specifically inhibited the amiloride-insensitive component of pHi recovery. In addition, the effect of DIDS suggests that this phenomenon is, in part, dependent on HCO_3^- transport.

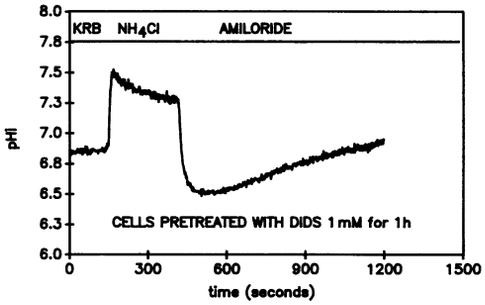

Figure 7. Effect of DIDS on pHi recovery from an acid load in the presence of HCO_3^-. Cells were preincubated with 1 mM DIDS for 1 h before acid load. Amiloride (1 mM) was added during the recovery phase to inhibit Na^+/H^+-dependent recovery. DIDS greatly inhibited pHi recovery. The tracing is representative of eight similar experiments. Similar results were obtained when cells were preincubated with 0.5 mM DIDS for 20 min.

Acid extrusion rates were also measured in cells depleted of Cl^- by incubating them for 30–40 min in a Cl^--free medium (equimolar substitution with gluconate). After Cl^- removal, basal pHi was significantly higher (7.38±0.07, n = 4 vs. 7.14±0.11) (Table II). After administration and withdrawal of 25 mM NH_4^+, cells were perfused with Cl^--free KRB containing 1 mM amiloride. Acid extrusion rates were not significantly different from those measured in the presence of amiloride in Cl^--containing KRB (5.84±1.22 at pHi 6.94±0.1, n = 4, vs. 6.16±2.75, n = 20) (Fig. 8). The lack of effect of Cl^- depletion on pHi recovery suggests the involvement of a Na^+:HCO_3^- symport rather than a Na^+-dependent Cl^-/HCO_3^- exchange.

Effects of acute Cl^- removal. To detect the presence of a Cl^-/HCO_3^- exchanger, cells were grown in α-MEM medium, perfused with bicarbonate containing buffer, and then exposed to acute removal of Cl^-_{out} by substitution with equimolar amounts of gluconate. If an active Cl^-/HCO_3^- exchanger is present, this maneuver should increase pHi, as Cl^-_{in} is exchanged with $HCO_{3\,out}^-$. As shown in Fig. 9 *A*, Cl^- removal resulted in a rapid alkalinization of 0.18±0.008 pHi units, after which pHi recovered after Cl^- readmission (n = 7). In cells pretreated with DIDS (Fig. 9 *B*), the rise in pHi during Cl^- removal was completely inhibited and pHi decreased by 0.048±0.035 pHi units (n = 5).

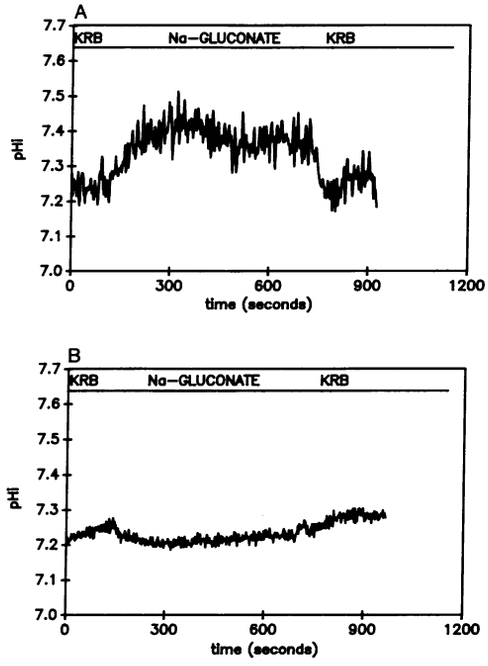

Figure 9. (*A*) Effects of acute removal of external Cl^- (substitution with equimolar gluconate) on baseline pHi in BDE cells (KRB medium). pHi rapidly alkalinized after Cl^- removal and returned to baseline after Cl^- readmission. The tracing is representative of seven similar experiments. (*B*) Baseline pHi did not alkalinize when the same maneuver was performed in BDE cells preincubated with 1 mM DIDS for 1 h. Data are representative of five similar experiments.

Discussion

In this study, we developed a technique for the isolation of a population of liver nonparenchymal cells enriched in bile ductular epithelial cells and utilized vital cell markers and microfluorometric procedures to characterize specific ion transport systems that regulate BDE cell intracellular pHi. Our data indicate that these cells possess a Na^+/H^+ exchange mechanism that is mainly responsible for pHi regulation in the absence of bicarbonate, mediates recovery from an intracellular acid load and is activated by intracellular acidification. When HCO_3^- is present, a Na^+-dependent, amiloride-insensitive, DIDS-inhibitable, Cl^--independent transport system is also activated (consistent with Na^+:HCO_3^- symport). This system maintains a higher steady-state pHi and is involved in the recovery from an intracellular acid load. In addition, the DIDS-inhibitable pHi alkalinization induced by acute Cl^- removal indicates the presence of a Cl^-/HCO_3^- exchange, which could act as the acid-loading component in the BDE cell pHi regulatory system. These studies indicate that BDE cells, like other epithelia, have the capability of transporting H^+ and HCO_3^- and thus might be involved in modifying the electrolyte composition of ductular bile. Future studies will be necessary to determine the membrane polarity of these transporters, their electrophysiological features, and their potential role in biliary electrolyte transport.

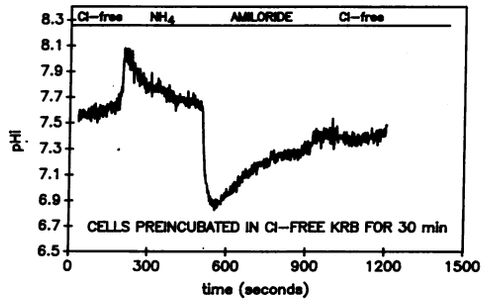

Figure 8. Effects of Cl^- depletion on pHi recovery from an acid load in the presence of HCO_3^-. Cells were preincubated for 30–40 min in Cl^--free KRB (equimolar substitution with gluconate) and then acid loaded by pulse withdrawal of 25 mM NH_4-gluconate. 1 mM amiloride was added during the recovery phase to eliminate the Na^+/H^+-dependent component of pHi recovery. JH^+ measured in these conditions were similar to the one recorded in the presence of normal Cl^- concentrations and amiloride.

Knowledge of the function of BDE cells has been limited because of difficulties in their isolation, related to their small number (3–5%) (2, 9), compared to other cell populations in the liver, and the similarity in their size and density to other nonparenchymal cell contaminants, including Kupffer, endothelial, and inflammatory cells (2, 29). Recently, several techniques have been reported that provided the background upon which our present procedure for isolation of BDE cells is based (8, 9, 29).

These procedures utilize the known proliferative properties of BDE cells after bile duct ligation, which leads to a selective increase in the number of bile duct cells up to 30–40% of the hepatic parenchyma mass (type I hyperplasia) (2, 7, 8, 30). Although it is uncertain whether BDE cells isolated from bile duct ligated rats originate from proliferated ductular cells or preexisting hepatocytes, these cells are well differentiated (9), lined by basement membrane–containing laminin (31), and retain phenotypic features of normal BDE cells, including their ultrastructural appearance, GGT staining, and immunoreactivity to cytokeratins 7 and 19 (2, 8, 9, 23). Thus, it seems likely

that these cells are phenotypically similar to normal BDE cells and that the transport systems identified in this report approximate normal BDE physiology. These assumptions will, however, need to be validated by developing techniques in the future to isolate BDE cells in sufficient yield from normal animals.

A cell population containing up to 85% (average, 72%) GGT-positive cells was isolated, by combined isopycnic centrifugation in Percoll, centrifugal elutriation, and negative selection by adherence to plastic. These cells were positive for cytokeratins 19 and 7 and showed ultrastructural features of normal BDE cells. The use of vital markers allowed more precise identification of BDE cells, whereas the use of a single-cell microfluorimetric approach enabled reliable recordings of BCECF fluorescence even if the cell yield was low.

Using this approach, a basal pHi of 7.03 pH units was measured in cells incubated in 3% FCS in the absence of bicarbonate. These values were comparable to most other cells studied (32) but slightly lower than basal pH's observed in hepatic parenchymal cells (21). Difference in concentration of FCS which may activate Na^+-proton exchange could account for these differences. Baseline pHi was dependent on extracellular Na^+ and was decreased by the Na^+/H^+ exchanger inhibitor amiloride (1 mM), suggesting that BDE cell's steady-state pHi is maintained by a Na^+/H^+ exchanger that counteracts a background acid loading process (Fig. 2). The presence of an active Na^+/proton exchange system in BDE cells was also confirmed by evaluating the effects of Na^+ removal and amiloride on pHi recovery from an acid load, as discussed in the result section (Fig. 3 *A* and *B*). At least 70% of acid extrusion during pH recovery from an acid load was mediated by this transporter, as assessed from the effect of amiloride inhibition. As also observed in hepatocytes, part of H^+ efflux seems to be independent from amiloride inhibition. While this could signal the presence of other mechanisms for H^+ efflux in addition to Na^+/H^+ exchange, BDE cells may also be capable of conducting H^+ across their plasma membranes as observed previously in isolated hepatocytes (21) and hepatocyte membrane vesicles (33). Intracellular acidification usually results in depolarization which would favor H^+ extrusion (34). However electrophysiologic approaches will be necessary to evaluate this possibility (35).

When compared to similar data from rat hepatocytes, the BDE cell's Na^+ proton exchange mechanism is more active. Maximal H^+ flux rates of 7.45 mM/min were observed in BDE cells, twice the rate of proton flux observed in this laboratory in subconfluent monolayers of rat hepatocytes (21). This greater activity was also reflected in higher rates of acidification after Na^+ removal, compared to findings in hepatocytes. As in the other cells, the rate of Na^+/proton exchange was inversely proportional to pHi, reflecting the well-known proton-modifying effects on this exchange mechanisms (27). Analysis of Fig. 4 suggests that BDE cell Na^+/H^+ exchange is inactive at pHi values > 7.15.

In physiological conditions the regulation of pHi of living animal cells, takes place in the presence of HCO_3^-. As observed in other cells (18, 21), basal pHi was significantly higher when BDE cells were incubated in media containing bicarbonate. Baseline pHi decreased after Na^+ removal, but was unaffected by amiloride (Fig. 5). On the other hand, when cells were pretreated with DIDS, an inhibitor of HCO_3^--dependent transport systems, pHi was lower, and did not differ significantly from values recorded in the absence of bicarbonate. These data suggest that in the presence of HCO_3^-, resting pHi is maintained by a Na^+- and HCO_3^--dependent but amiloride-insensitive mechanism.

When pHi recovery from an intracellular acid load was measured in the presence of HCO_3^-, acid extrusion rates were increased 41% compared to Hepes-buffered media (Fig. 10). In this condition, pHi recovery is mostly mediated by a HCO_3^--dependent mechanism that is completely Na^+-dependent, partly inhibited by DIDS and minimally affected by amiloride or by Cl^- removal (the amiloride-sensitive component of pHi recovery being only 27% of the total recovery rate).

The Na^+-dependence excludes mechanisms like Cl^-/HCO_3^- exchange and H^+ (or OH^-, HCO_3^-) conductances, whereas the inhibition by DIDS confirms that a HCO_3^- transport system is involved and effectively excludes proton or H^+-K^+ ATPases. By exclusion, this HCO_3^- transporter must represent Na^+-dependent Cl^-/HCO_3^- exchange or Na^+:HCO_3^- symport. However, recovery rates from an acid load in cells that had been depleted of intracellular Cl^- were not affected suggesting that the mechanism responsible for acid extrusion is a Na^+:HCO_3^- symport.

Na^+:HCO_3^- symports are usually (renal proximal tubular [36], corneal epithelial [37]) but not always (smooth muscle [38]) electrogenic, transporting (three or two) HCO_3^- to one Na^+. At present it is debated whether rat hepatocyte Na^+:HCO_3^- is electrogenic (39, 40) or not (21), and definitive determination of electrogenicity of the BDE HCO_3^- transporter will require electrophysiological measurements of the cell's membrane potential during variations in extracellular [HCO_3^-] (40). If electrogenic, this transporter could function as an acid extruder (HCO_3^- uptake) or loader (HCO_3^- extrusion) depending on the transmembrane electrical potential. Nevertheless, the higher pHi in the presence of HCO_3^- and the lower pHi in cells pretreated with DIDS suggest that the Na^+:HCO_3^- symport functions as a base loader (acid extruder) in resting conditions as well as following an acid load.

The acute removal of extracellular Cl^- resulted in a rise in pHi that was inhibited by pretreatment with DIDS. Although Cl^- removal could theoretically produce intracellular alkalinization by causing intracellular depolarization, and thus en-

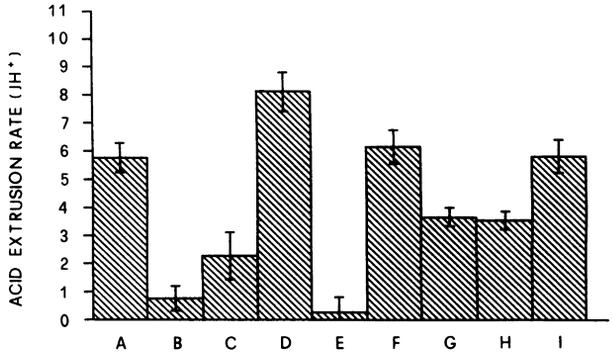

Figure 10. Comparison of measured H^+ extrusion rates (JH^+, millimolar/minute) during recovery from an acid load in the absence (*A–C*) and in the presence (*D–I*) of HCO_3^-. *A*, Hepes controls; *B*, Hepes Na^+ removal; *C*, Hepes 1 mM amiloride, *D*, KRB controls; *E*, KRB Na^+ removal; *F*, KRB 1 mM amiloride; *G*, KRB 0.5 mM DIDS pretreatment + 1 mM amiloride; *H*, KRB 1 mM DIDS + 1 mM amiloride; *I*, Cl^--free KRB + 1 mM amiloride.

hancing an electrogenic Na^+:HCO_3^- symport, these findings are most consistent with the presence in BDE cells of a Cl^-/HCO_3^- exchanger (41). A Cl^-/HCO_3^- exchange mechanism has also been identified in canalicular membranes of hepatocytes (42) where it functions as the primary acid loader (bicarbonate extruder) (22, 25), as it does in many other cells (24, 43, 44).

DIDS concentrations used in this study are somewhat higher than ones generally used with other cell types, but are similar to those employed in hepatocytes. Hepatocyte anion transporters are known to be relatively insensitive to DIDS (21, 39, 40). Moreover, in the isolated perfused rat liver preparation where the integrated function of hepatocytes and ductal system can be evaluated, the liver excretes DIDS into the bile at biliary concentrations up to 5 mM resulting in a decrease in biliary HCO_3^- concentration that is reversible after DIDS withdrawal (45). Thus, in the intact organ, high DIDS concentrations are tolerated without irreversible inhibition.

Although the present study does not provide information on the localization of the transporters in bile duct epithelial cells, and indeed their polarity might even be altered in BDE cells from bile duct–obstructed animals (46), the battery of ion transporters identified in BDE cells in this study is remarkably similar to the one responsible for transepithelial HCO_3^- movement in guinea pig gallbladder (47). Na^+:HCO_3^- symports have been described primarily in epithelial cells involved in electrolyte secretion (21, 28, 36, 37) and their presence in BDE cells, together with active Na^+/H^+ and Cl^-/HCO_3^- exchangers, supports the involvement of BDE cells in transepithelial H^+/HCO_3^- transport.

Further studies will be necessary in BDE cells isolated from normal animals and maintained in cultures with established epithelial polarity before definitive answers to these questions can be obtained. In the meantime, the approaches developed in the present study should facilitate the development of further information about hepatic bile duct epithelial cell transport systems.

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