

## Effect of secretion on intracellular pH regulation in isolated rat bile duct epithelial cells.

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### Research Article

The effects of secretin on ion transport mechanisms involved in regulation of intracellular pH (pHi) and HCO<sub>3</sub><sup>-</sup> excretion were characterized in bile duct epithelial (BDE) cells isolated from normal rat liver. pHi was measured with 2,7-bis(carboxy-ethyl)-5(6)-carboxy-fluorescein-acetomethylester (BCECF-AM) using a microfluorimetric method. Basal pHi of BDE was 7.04 ± 0.06 in Hepes and 7.16 ± 0.10 in KRB and was unaffected by secretin (50-200 nM). Recovery rates from an acid load in Hepes or in KRB media (with and without amiloride) were also not altered by secretin, indicating that Na<sup>+</sup>/H<sup>+</sup> exchange and Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransport were not affected by this hormone. After acute Cl<sup>-</sup> removal, pHi rose 0.24 ± 0.08 pHU at a maximal rate of 0.125 ± 0.06 pHU/min (H<sup>+</sup> flux rates = 6.02 ± 3.27 mM/min) and recovered after Cl<sup>-</sup> readmission (0.188 ± 0.08 pHU/min; H<sup>+</sup> flux rates = 11.82 ± 5.34 mM/min). Pretreatment with 1 mM DIDS inhibited the effects of Cl<sup>-</sup> removal, while valinomycin, which induces cell depolarization, enhanced these effects, probably by stimulating electrogenic HCO<sub>3</sub><sup>-</sup> influx. Secretin significantly increased both the maximal rate of alkalinization after Cl<sup>-</sup> removal ( $P < 0.012$ ) and of pHi recovery after Cl<sup>-</sup> readmission ( $P < 0.025$ ), indicating stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange activity. These findings were reproduced with N6,2'-O-Dibutyryl adenosine-3',5'-cyclic monophosphate (DBcAMP). The Cl<sup>-</sup> channel blocker 5-nitro-2'-(3-phenylpropylamino)-benzoate (NPPB, 10 μM) significantly decreased the effects [...]

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# Effect of Secretin on Intracellular pH Regulation in Isolated Rat Bile Duct Epithelial Cells

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## Abstract

The effects of secretin on ion transport mechanisms involved in regulation of intracellular pH ( $pH_i$ ) and  $HCO_3^-$  excretion were characterized in bile duct epithelial (BDE) cells isolated from normal rat liver.  $pH_i$  was measured with 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein-acetomethylester (BCECF-AM) using a microfluorimetric method. Basal  $pH_i$  of BDE was  $7.04 \pm 0.06$  in Hepes and  $7.16 \pm 0.10$  in KRB and was unaffected by secretin (50–200 nM). Recovery rates from an acid load in Hepes or in KRB media (with and without amiloride) were also not altered by secretin, indicating that  $Na^+/H^+$  exchange and  $Na^+/HCO_3^-$  cotransport were not affected by this hormone. After acute  $Cl^-$  removal,  $pH_i$  rose  $0.24 \pm 0.08$  pHU at a maximal rate of  $0.125 \pm 0.06$  pHU/min ( $H^+$  flux rates =  $6.02 \pm 3.27$  mM/min) and recovered after  $Cl^-$  readmission ( $0.188 \pm 0.08$  pHU/min;  $H^+$  flux rates =  $11.82 \pm 5.34$  mM/min). Pretreatment with 1 mM DIDS inhibited the effects of  $Cl^-$  removal, while valinomycin, which induces cell depolarization, enhanced these effects, probably by stimulating electrogenic  $HCO_3^-$  influx. Secretin significantly increased both the maximal rate of alkalinization after  $Cl^-$  removal ( $P < 0.012$ ) and of  $pH_i$  recovery after  $Cl^-$  readmission ( $P < 0.025$ ), indicating stimulation of  $Cl^-/HCO_3^-$  exchange activity. These findings were reproduced with  $N^6,2'-O$ -Dibutyryl adenosine-3'-5'-cyclic monophosphate (DBcAMP). The  $Cl^-$  channel blocker 5-nitro-2'-(3-phenylpropylamino)-benzoate (NPPB, 10  $\mu$ M) significantly decreased the effects of secretin and DBcAMP on the  $pH_i$  changes promoted by acute  $Cl^-$  removal/readmission. These findings establish that secretin stimulates the activity of the  $Cl^-/HCO_3^-$  exchanger in BDE cells, probably by activating  $Cl^-$  channels via the intracellular messenger cAMP. This in turn depolarizes the cell, stimulating electrogenic  $Na^+/HCO_3^-$  symport. The cell depolarization induced by  $Cl^-$  channel activation should enhance  $HCO_3^-$  entrance through electrogenic  $Na^+/HCO_3^-$  symport, which in turn stimulates the  $Cl^-/HCO_3^-$  exchange. These mechanisms could account for secretin stimulated bicarbonate secretion in bile. (*J. Clin. Invest.* 1993. 92:1314–1325.) Key words: bile duct epithelium · intra-

cellular pH regulation · secretin ·  $Cl^-/HCO_3^-$  exchanger ·  $Cl^-$  channel

## Introduction

Bile duct epithelial (BDE)<sup>1</sup> cells secrete bicarbonate, both spontaneously and following hormonal stimulation with secretin (1–5). Together with pancreatic secretions, this process neutralizes gastric acid, thereby facilitating digestion. However in the normal adult rat, little spontaneous BDE secretion can be detected and responsiveness to secretin is minimal (4), unless BDE cells proliferate following bile duct ligation (1, 2, 5) or  $\alpha$ -naphthylisothiocyanate administration (5). A correlation between enhanced bile duct mass and secretin induced choleresis has also been observed in a rat model of cirrhosis (3). The mechanism by which secretin induces secretion is not known although BDE containing specific binding sites for secretin have been documented in rat BDE cells and not in hepatocytes (6). In the pig, secretin induces exocytosis of tubulo-vesicle structures from BDE cells to the lateral cell membrane (7).

Studies of these secretory mechanisms have been limited due to difficulty in isolating BDE cells from liver tissue, where they are vastly outnumbered by hepatocytes and where endothelial, Kupffer, and inflammatory cells of similar size and density frequently contaminate the preparations. Recently, techniques have been developed in bile duct-obstructed rats to isolate BDE with reasonable purity and to identify both BDE cells and contaminating cells in vital preparations in cell culture (8–10). Using these techniques, we have been able to identify several ion transport mechanisms that regulate intracellular pH in BDE and that are likely to be utilized for absorptive and secretory functions in this epithelium (10). These transport systems include two acid extruding mechanisms ( $Na^+/H^+$  exchange and a  $Na^+/HCO_3^-$  symport) and an acid loading mechanism ( $Cl^-/HCO_3^-$  exchange) that would be capable of secreting  $HCO_3^-$ . Using similar preparations, other investigators have demonstrated an increase in intracellular cAMP levels in response to secretin stimulation, and an increase in exocytosis and stimulation of intracellular vesicle traffic (11, 12). However, the transport processes involved in  $H^+$  and  $HCO_3^-$  transport have been characterized in proliferated (“abnormal”) BDE cells isolated from bile duct-obstructed animals (10). We now have devised methods to isolate BDE in reasonable purity from normal rat liver and in this report examine the effects of secretin on the transport mechanisms involved in intracellular pH regulation.

1. Abbreviations used in this paper: BDE, bile duct epithelium; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein; DIDS, 4,4'-diisothiocyanato-2,2'-disulphonic acid stilbene; DiI, 1,1-dioctadecyl-1,3,3,3'-tetramethyl-indo-carbocyanine perchlorate; GGT, gamma-glutamyl transpeptidase; L-15, Leibowitz 15 cell culture medium; DBcAMP,  $N^6,2'-O$ -dibutyryl adenosine-3'-5'-cyclic monophosphate; NPPB, 5-nitro-2'-(3-phenylpropylamino)-benzoate.

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## Methods

**Materials.** Secretin (3,000 U/mg) was purchased from Bachem Bioscience Inc. (Philadelphia, PA). BSA (essentially fatty acid free), EDTA, penicillin/streptomycin, trypsin, heparin, Hepes, D(+)-glucose, insulin, soybean trypsin inhibitor (type I-s), amiloride, DMSO, deoxyribonuclease (DN-25), nigericin, calf serum, 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS), Na<sup>+</sup>-gluconate, K<sup>+</sup>-gluconate, hemicalcium gluconate and Fast Blue BB salt were purchased from Sigma Chemical Co. (St. Louis, MO). 2,7-bis(carboxyethyl)-5(6)-carboxy-fluorescein-acetomethylester (BCECF-AM), calcein AM and ethidium homodimer were obtained from Molecular Probes Inc. (Eugene, OR). Percoll was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ), Matrigel™ from Collaborative Research, Inc. (Bedford, MA), Collagenase A from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Pronase from Calbiochem (La Jolla, CA). Liebowitz-15 (L-15), MEM, Joklik modified MEM,  $\alpha$ -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'-tetramethyl-indo-carbocyanine perchlorate (DiI) were from BioTechnology Inc. (Stoughton, MA). N(gamma-1-glutamyl)-4-methoxy-2-naphthylamide was obtained from Polysciences, Inc. (Warrington, PA). Monoclonal anticytokeratin 7 and 19 antibodies (RPN 1162 and RPN 1165, respectively) were purchased from Amersham Corp. (Arlington Heights, IL). 5-nitro-2'-(3-phenylpropylamino)-benzoate (NPPB) was a kind gift of Prof. R. Greger (Freiburg, Germany).

**Isolation of bile duct epithelial cells.** Male Sprague-Dawley rats (Camm Research Lab Animals, Wayne, NJ) weighing 300–350 g, were housed in temperature- and light-controlled rooms and allowed free access to water and laboratory chow. BDE cells were isolated from normal rats as previously described from bile duct-ligated rats (10) with minor modifications consisting of: (a) an additional digestion step of the portal tissue residue by trypsin; (b) collection of the fractions elutriating at 24 ml/min in addition to 30 and 38 ml/min; and (c) adherence to plastic flasks was eliminated, because cell purity was not improved (In the bile duct-ligated model, contaminant cells were more prevalent (10), and this step increased the purity of the preparation by favoring their adherence.)

Briefly, liver was digested by perfusion with MEM supplemented with collagenase A (360 U/liter). The portal tissue residue was then mechanically separated from parenchymal tissue and finely minced and digested by sequential incubation in trypsin (0.25%, 10 min) and hyaluronidase (35,000 U, 60 min) in MEM. The cell suspensions obtained from trypsin and hyaluronidase digestion were combined and purified by isopycnic centrifugation (Percoll) and counterflow elutriation (JE-6B elutriator; Beckman Instruments, Inc., Palo Alto, CA) as previously described (10). Fractions elutriating at flow rates 24, 30, and 38 ml/min were combined and used for the functional studies. Small aliquots were used for determination of cell viability (trypan blue exclusion or Calcein AM/ethidium homodimer (13), number and size (counter Channalizer 256; Coulter Electronics, Inc. Hialeah, FL), and purity (GGT cytochemistry [14] and cytokeratin 19 + 7 [10]). The remaining cells were plated on small cover slip fragments (4 × 2 mm), layered in 22- or 12-mm diameter tissue culture plastic wells (Corning Glass Works, Corning, NY), covered with a thin layer of Engelbreth-Holm-Swarm (EHS) mouse tumor matrix (Matrigel™; Collaborative Research Inc.) and incubated at 37°C in an air-equilibrated incubator. The medium (L-15 or  $\alpha$ -MEM) was changed after 4 h, and all experiments were performed 8–18 h after plating.

Colloidal ink (Higgins india ink, administered via inferior vena cava) was used as vital marker of Kupffer cells (15, 16) and acetylated LDL labeled with the fluorescent DiI (added to the cell plate 3–4 h before starting the experiments) as a vital marker of endothelial cells and macrophages as previously described (16, 17).

**Intracellular pH determination.** Intracellular pH (pH<sub>i</sub>) of cultured BDE cells was measured by employing a microfluorimetric single-cell

method using a SPEX-AR-CM-micro system (Spex Industries, Inc., Edison, NJ) and BCECF-AM as a fluorescent pH<sub>i</sub> indicator as described from this laboratory (10). BDE cells on glass coverslips were loaded with BCECF-AM (12  $\mu$ M) for 40 min, washed 10 min in a BCECF free medium, and transferred into a thermostated perfusion chamber placed on the stage of an inverted microscope (IM 35; Carl Zeiss, Inc., Thornwood, NY). Clusters of at least five small mononucleated cells were selected under DIC optics. Cells were avoided when they contained ink particles (Kupffer cells), showed a fibroblast-like aspect, contained cytoplasmic translucent vacuoles (Ito cells) or were positive for DiI-Ac-LDL (endothelial cells and macrophages). Hepatocytes, found rarely, were distinguished by their large size (15–20  $\mu$ m) and morphology. The 490/440 fluorescent intensity (Fi) ratio data were converted to pH<sub>i</sub> values by using the nigericin (12  $\mu$ M) calibration curve technique as described (18, 19). Over the pH range 6.4–7.6 pH<sub>i</sub>, fluorescence ratio varied in a linear fashion with pH<sub>o</sub>. Fluorescence intensity was found to exceed background autofluorescence by at least 40-fold.

**Total and intrinsic intracellular buffering power.** The intrinsic buffering power ( $\beta$ <sub>i</sub>) was determined as described (20–25) by evaluating the pH<sub>i</sub> change induced by the administration and withdrawal of a known amount of base. Giving that the  $\beta$ <sub>i</sub> is related to pH<sub>i</sub> (10, 20–25), we estimated the  $\beta$ <sub>i</sub> values at different pH<sub>i</sub>, by exposing the cells to 30 or 20 mM NH<sub>4</sub>Cl in Hepes, Na<sup>+</sup>-free buffered solutions and then decreased the NH<sub>4</sub>Cl concentration by 5 or 10 mM for each step to 0 mM. The  $\beta$ <sub>i</sub> was then calculated from the midpoint change in pH<sub>i</sub> at each step.  $\beta$ <sub>i</sub> values were then plotted vs. pH<sub>i</sub> using a best fit Enzfitter program (Elsevier-Biosoft, Cambridge, UK). To exclude the possibility that the findings of our study could be influenced by the addition of secretin or DBcAMP on  $\beta$ <sub>i</sub>, we also evaluated the  $\beta$ <sub>i</sub>/pH<sub>i</sub> curves from experiments performed with cells preincubated (10 min) and perfused with 200 nM secretin ( $n = 8$ ) or 100  $\mu$ M DBcAMP ( $n = 5$ ). As in other studies (10, 23) a single exponential function better describes the known  $\beta$ <sub>i</sub>/pH<sub>i</sub> relationships.  $\beta$ <sub>i</sub> changed from 6.8 mM/pH unit at pH<sub>i</sub> 7.5 to 22.1 mM/pH unit at pH<sub>i</sub> 7 and to 72.1 mM/pH unit at pH<sub>i</sub> 6.5 (controls,  $n = 9$ ) and was not significantly influenced by secretin (6.5, 22.4, and 77.3 mM/pH unit at pH<sub>i</sub> 7.5, 7, and 6.5 respectively) or by DBcAMP (7, 21.3, and 70.3 mM/pH unit at pH<sub>i</sub> 7.5, 7, and 6.5 respectively) at every pH<sub>i</sub> value. The values of  $\beta$ <sub>i</sub> measured in this study are very close to those measured in BDE cells isolated from bile duct ligated rat liver (10) and are also in agreement with those measured in many other cell types including hepatocytes (23).  $\beta$ <sub>tot</sub> was calculated from the formula:  $\beta$ <sub>tot</sub> =  $\beta$ <sub>i</sub> + 2.303 × (HCO<sub>3</sub><sup>-</sup>)<sub>i</sub> where intracellular (HCO<sub>3</sub><sup>-</sup>)<sub>i</sub> is derived from the Henderson-Hasselbach equation.

**Solutions.** The composition of solutions used in the study has been previously detailed (10). The Hepes-buffered solution contained (in mM) Na<sup>+</sup> 140, K<sup>+</sup> 5.9, Mg<sup>++</sup> 1, Ca<sup>++</sup> 1.25, Cl<sup>-</sup> 142.2, SO<sub>4</sub> = 1, PO<sub>4</sub> = 1.2, Hepes 10, glucose 5, pH 7.4; the HCO<sub>3</sub><sup>-</sup>-CO<sub>2</sub> buffered solution (KRB) contained (in mM) Na<sup>+</sup> 140, K<sup>+</sup> 5.9, Mg<sup>++</sup> 1, Ca<sup>++</sup> 1.25, Cl<sup>-</sup> 122.2, SO<sub>4</sub> = 1, PO<sub>4</sub> = 1.2, HCO<sub>3</sub><sup>-</sup> 25, glucose 5, pH 7.4. Na<sup>+</sup> free and Cl<sup>-</sup> free solutions were prepared by equimolar replacement with choline and gluconate respectively. Tetramethylammonium (TMA-OH) was used for pH titration in the nigericin containing solutions (pH 6.8–7.6) as well as in the Na<sup>+</sup>-free Hepes-buffered solution (pH 7.4). Secretin was made up as a concentrated solution in the appropriate perfusion buffer containing 1% (wt/vol) BSA. The secretin containing solution was then infused (1:60 vol/vol dilution) into the perfusion fluid, at a rate calculated to produce the required final concentration, via a Y-connection sited close to the chamber where the coverslips were placed for pH<sub>i</sub> recording, and where temperature and pH were periodically checked. NPPB was dissolved in DMSO and then diluted (1:1,000, vol/vol) in the appropriate perfusion solution to a final concentration of 10  $\mu$ M (0.1% DMSO in the perfusion solutions). Valinomycin was dissolved in DMSO to a final concentration of 5 × 10<sup>-8</sup> M.

**Statistical analysis.** Data are presented as the arithmetic means ± standard deviations. Statistical analysis was conducted using the paired or unpaired Student's *t* test as appropriate or the analysis of the variance (Anova one way) when three groups were compared.

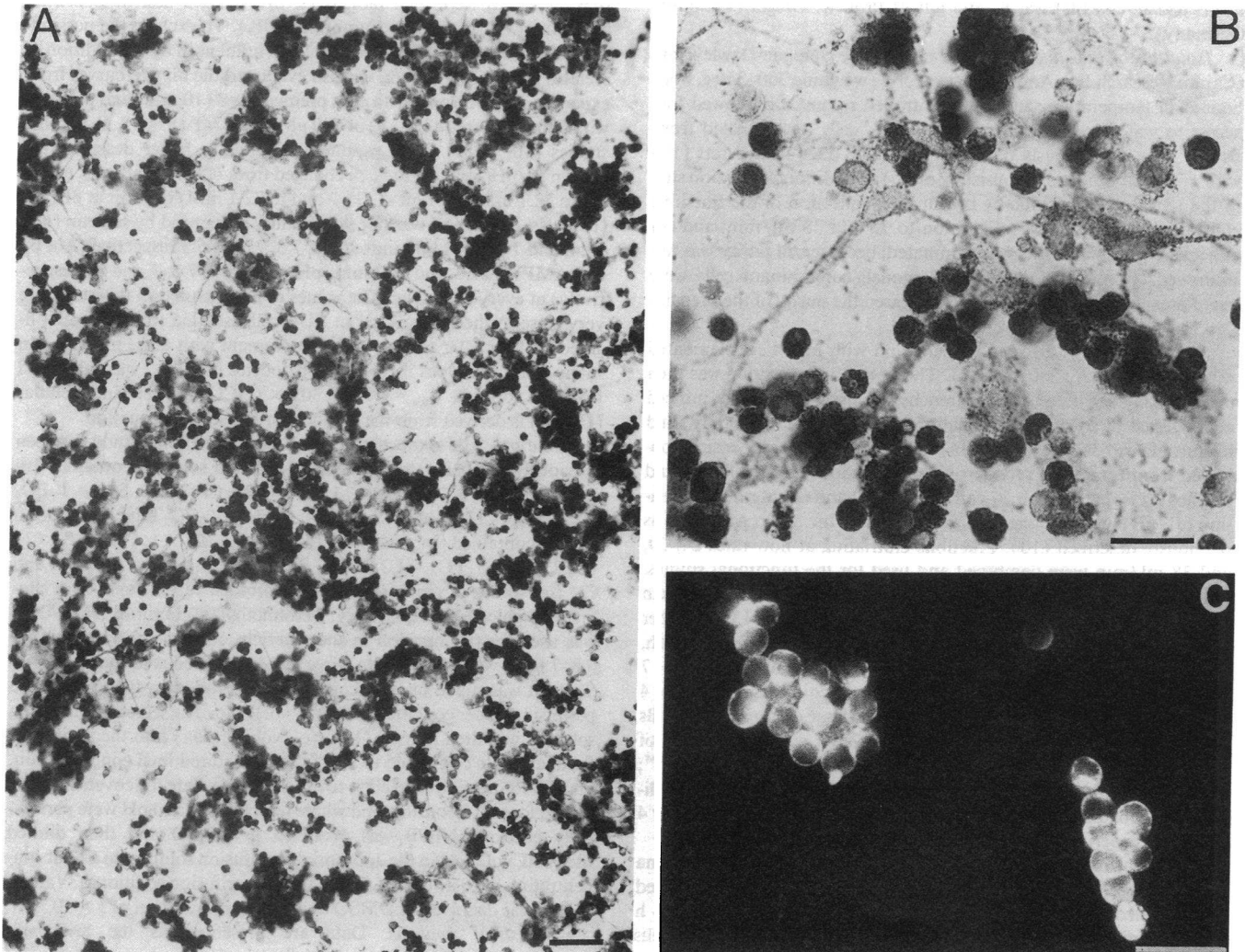
## Results

**Properties of BDE cell preparation.** An average of  $870 \pm 291 \times 10^3$  cells with a viability  $> 90\%$  (trypan blue exclusion) were recovered from the combined fractions elutriating at flow rates of 24, 30, and 38 ml/min ( $n = 59$  preparations). BDE cells with average diameters of  $7.2 \pm 0.3 \mu\text{m}$  and  $8.3 \pm 0.3 \mu\text{m}$ , respectively, were usually present in two main peaks in the cell counter tracing. Before plating, GGT positive staining, indicative of BDE cells (8–10), was observed in  $63 \pm 7\%$  (range 50–78%) of the cells. 10 preparations were also tested for cytokeratin 19 and 7 (Fig. 1 C), specific for BDE cells (8–10) and  $69.2 \pm 15.7\%$  were positive (range 46–92%). When BDE cells were cultured (L-15 or  $\alpha$ -MEM media) on Matrigel-coated coverslips, they organized in three-dimensional clusters. After overnight culture (Fig. 1 B), the percentage of GGT positive cells (tested in cells attached to coverslips) increased to  $80.7 \pm 7.6\%$  (range 63–93%) indicating that matrigel favored the attachment of BDE compared with contaminating cells. In clusters of more than five cells,  $> 90\%$  were GGT positive, indicating that cluster formation could also be used as a criteria for cell selection for the functional studies. Endothelial cells ( $10 \pm 4\%$ , range 8–15%) as evaluated by DiI-Ac-LDL positive

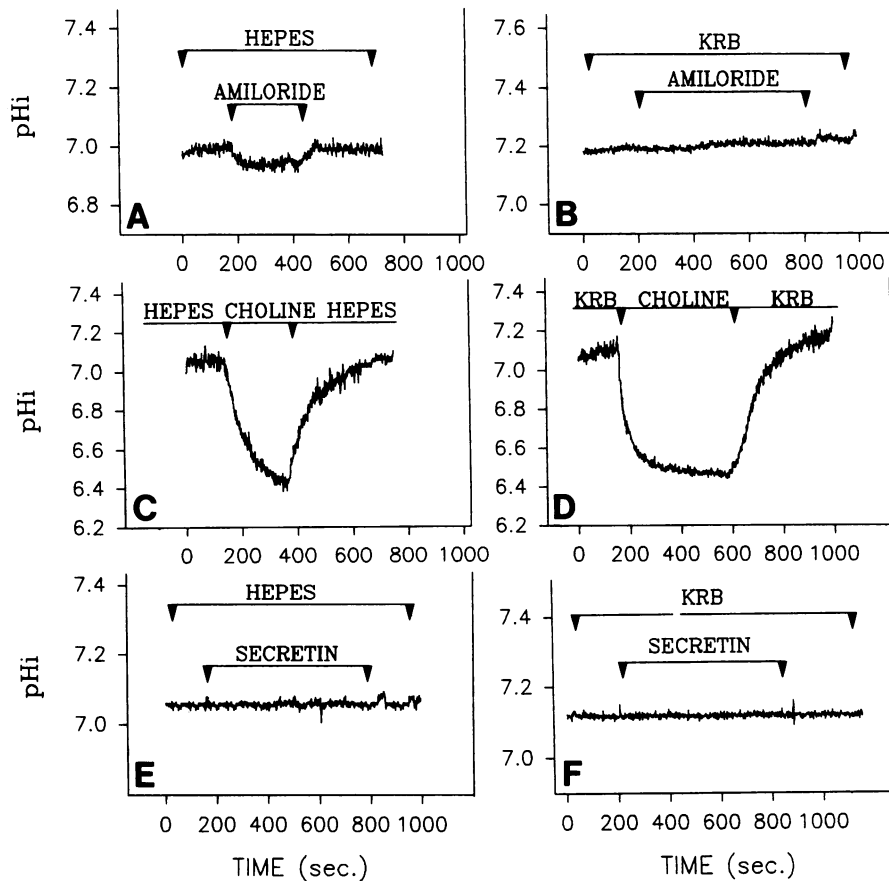
fluorescence (rhodamine excitation) were the major contaminants, while Kupffer cells (India ink positive) was  $< 1\%$ . Cell viability was  $> 83\%$  within 18 h after plating as judged by trypan blue exclusion. None of the ion substitutions or inhibitors diminished cell viability below 80% ( $\text{Na}^+$ , or  $\text{Cl}^-$  free solutions, DIDS [1 mM], Amiloride [1 mM] or NPPB [10  $\mu\text{M}$ ] when also assessed by the calcein AM/ethidium homodimer method) (13).

### *pH<sub>i</sub> regulation in BDE cells*

**Basal pH<sub>i</sub>.** BDE cells, isolated from normal rat liver, maintained a basal pH<sub>i</sub> of  $7.04 \pm 0.06$  ( $n = 30$ ) in a nominally bicarbonate-free, Hepes-buffered media. However basal pH<sub>i</sub> was significantly higher ( $7.16 \pm 0.10$ ,  $n = 44$ ;  $P < 0.001$ ) when BDE cells were cultured and perfused with media supplemented with 25 mM  $\text{HCO}_3^-$  and gassed with 95%  $\text{O}_2/5\% \text{CO}_2$ , suggesting that a  $\text{HCO}_3^-$  loading mechanism is present. Indeed, when BDE cells in bicarbonate were preincubated with 1 mM DIDS (60–80 min), an inhibitor of  $\text{HCO}_3^-$  transport, basal pH<sub>i</sub> significantly decreased ( $6.95 \pm 0.08$ ,  $P < 0.001$ ,  $n = 10$ ). As shown in Fig. 2, acute exposure of cells to 1 mM amiloride (inhibitor of  $\text{Na}^+/\text{H}^+$  exchange) produced a  $0.11 \pm 0.04$  pHU decrease in



**Figure 1.** A. GGT cytochemistry of BDE cells cultured overnight (L-15 medium) on Matrigel covered coverslips. Bar, 50  $\mu\text{m}$ . B. Clusters of overnight cultured BDE cells, positive for GGT. Bar, 20  $\mu\text{m}$ . C. Clusters of freshly isolated BDE cells positive for cytokeratin 19 + 7. Bar, 20  $\mu\text{m}$ .



**Figure 2.** Effect of amiloride, sodium removal, and secretin on basal  $pH_i$ . **A.** Cells perfused with  $HCO_3^-$ -free, HEPES buffered solution were acutely exposed to the  $Na^+ / H^+$  inhibitor, amiloride (1 mM;  $n = 4$ ). Basal  $pH_i$  decreased and then returned to baseline after amiloride washout. (The tracing is a representative example.) **B.** Cells perfused with KRB, gassed with 95%  $O_2 / 5\% CO_2$ , were acutely exposed to 1 mM amiloride. No measurable change of  $pH_i$  was observed ( $n = 4$ ). **C and D.** BDE cells were exposed to  $Na^+$ -free medium (substitution with choline) either in absence (**C**,  $n = 5$ ) or in presence (**D**,  $n = 4$ ) of  $HCO_3^-$ .  $pH_i$  rapidly decreased and recovered only after  $Na^+$  readmission. **E and F.** BDE cells were exposed to Secretin (200 nM) either in absence (**E**,  $n = 8$ ) or in presence of  $HCO_3^-$  (**F**,  $n = 12$ ). No measurable effect on basal  $pH_i$  was observed.

the absence of bicarbonate (Fig. 2 *A*,  $n = 4$ ) but was almost without effect in its presence (Fig. 2 *B*,  $n = 4$ ). In contrast the acute removal of  $Na^+$ , which stimulates intracellular  $Na^+$  exit in exchange with  $H^+$ , decreased basal  $pH_i$  by  $0.49 \pm 0.08$  pHU in the absence of bicarbonate (Fig. 2 *C*,  $n = 5$ ) and by  $0.71 \pm 0.06$  pHU in its presence (Fig. 2 *D*,  $n = 4$ ).  $pH_i$  rapidly recovered to the basal values only after  $Na^+$  readmission.

**Effect of secretin on basal  $pH_i$ .** In bicarbonate-free media, secretin had no measurable effect on BDE cell basal  $pH_i$ , either when cells were acutely exposed to the hormone (50 nM,  $n = 4$ ; 200 nM,  $n = 4$ ; Fig. 2 *E*) or were pretreated (10 min) and perfused with 200 nM secretin ( $7.02 \pm 0.06$ ,  $n = 10$ ) compared with carrier (i.e., albumin) controls ( $7.05 \pm 0.14$ ,  $n = 9$ ). Similar results were obtained where BDE cells were cultured and perfused with bicarbonate-enriched media (Fig. 2 *F*). Acute exposure to 200 nM secretin ( $n = 12$ ) or preincubation (10 min) and perfusion with 200 nM secretin ( $n = 45$ ) showed no measurable effect on basal  $pH_i$  ( $7.14 \pm 0.09$ ) compared with the carrier controls ( $7.16 \pm 0.07$ ;  $n = 32$ ). These findings demonstrate that secretin has no effect on the basal  $pH_i$  of BDE cells. To test the hypothesis that a fall in  $pH_i$  might be counteracted by acid extruders at  $pH_i$  values close to or slightly higher than the basal values, if secretin stimulated the activity of the  $Cl^- / HCO_3^-$  exchanger, BDE cells were preincubated and perfused with  $\alpha$ -MEM, superfused with 1 mM amiloride, and then exposed to secretin (200 nM). However amiloride had no effect on basal  $pH_i$ , and even under these conditions BDE cells showed no significant changes in basal  $pH_i$  when acutely exposed to secretin ( $n = 6$ ). These experiments suggest that  $Na^+ / H^+$  activity is not stimulated as a compensatory mechanism to maintain basal  $pH_i$  during secretin exposure.

**Recovery of  $pH_i$  from an acute acid load (Table I and Table II).** When BDE cells were exposed to 20 mM  $NH_4Cl^-$ ,  $pH_i$  promptly rose (Fig. 3), as  $NH_3$  rapidly diffused into the cell, due to the trapping of intracellular  $H^+$  as impermeant  $NH_4^+$ . After  $NH_4Cl^-$  was withdrawn,  $NH_3$  leaves the cell after releasing  $H^+$ , promoting a rapid intracellular acidification. In the absence of bicarbonate, the cells recovered spontaneously from this acid load (nadir  $pH_i = 6.55 \pm 0.08$ ), at a rate of  $0.29 \pm 0.09$  pHU/min at  $pH_i$   $6.60 \pm 0.04$  ( $jH^+ 16.95 \pm 6.08$  mM/min,  $n = 10$ , Table I, controls). This recovery was completely blocked when external  $Na^+$  was removed (substitution with choline) at the moment of  $NH_4Cl^-$  withdrawal.  $pH_i$  returned to baseline values only when external  $Na^+$  was readmitted (nadir  $pH_i$   $6.43 \pm 0.04$ ,  $n = 3$ ). When 1 mM amiloride was superfused at the moment of  $NH_4Cl^-$  withdrawal (Table I),

**Table I.** Recovery from  $NH_4Cl$  Acid Load in HEPES

Condition	Basal $pH_i$	Nadir $pH_i$	Recovery rates	
			pHU/min	$jH^+$ mM/min
HEPES controls ( $n = 10$ )	$7.03 \pm 0.05$	$6.55 \pm 0.08$	$0.29 \pm 0.09$ ( $pH_i$ 6.60)*	$16.95 \pm 6.08$
HEPES amiloride ( $n = 4$ )	$7.02 \pm 0.05$	$6.48 \pm 0.04$	$0.05 \pm 0.02$ ( $pH_i$ 6.51)*	$3.75 \pm 1.33$
HEPES secretin ( $n = 10$ )	$7.02 \pm 0.06$	$6.56 \pm 0.07$	$0.28 \pm 0.10$ ( $pH_i$ 6.61)*	$16.78 \pm 6.9$
HEPES albumin ( $n = 9$ )	$7.05 \pm 0.14$	$6.58 \pm 0.07$	$0.27 \pm 0.12$ ( $pH_i$ 6.63)*	$15.30 \pm 7.91$

Data are given as means  $\pm$  SD. \*  $pH_i$  common at all the experiments, where the recovery rate was calculated.

Table II. Recovery from  $\text{NH}_4\text{Cl}$  Acid Load in KRB

Condition	Basal $\text{pH}_i$	Nadir $\text{pH}_i$	Recovery rates	
			$\text{pHU}/\text{min}$	$\text{jH}^+$ $\text{mM}/\text{min}$
KRB controls ( $n = 11$ )	$7.14 \pm 0.05$	$6.64 \pm 0.07$	$0.20 \pm 0.07$ ( $\text{pH}_i$ 6.74)*	$11.60 \pm 4.05$
KRB amiloride ( $n = 6$ )	$7.15 \pm 0.07$	$6.55 \pm 0.08$	$0.13 \pm 0.05$ ( $\text{pH}_i$ 6.67)*	$7.79 \pm 3.01$
KRB amiloride + DIDS ( $n = 4$ )	$6.94 \pm 0.05$	$6.46 \pm 0.09$	$0.053 \pm 0.006$ ( $\text{pH}_i$ 6.55)*	$4.21 \pm 0.52$
KRB $\text{Cl}^-$ depleted + amiloride ( $n = 5$ )	$7.35 \pm 0.04$	$6.88 \pm 0.05$	$0.15 \pm 0.05$ ( $\text{pH}_i$ 6.91)*	$6.92 \pm 2.42$
KRB secretin ( $n = 10$ )	$7.15 \pm 0.10$	$6.64 \pm 0.08$	$0.22 \pm 0.08$ ( $\text{pH}_i$ 6.75)*	$12.04 \pm 4.76$
KRB albumin ( $n = 9$ )	$7.15 \pm 0.05$	$6.67 \pm 0.09$	$0.23 \pm 0.10$ ( $\text{pH}_i$ 6.75)*	$12.38 \pm 4.96$
KRB amiloride + secretin ( $n = 8$ )	$7.12 \pm 0.09$	$6.56 \pm 0.08$	$0.15 \pm 0.08$ ( $\text{pH}_i$ 6.66)*	$9.62 \pm 5.35$
KRB amiloride + albumin ( $n = 8$ )	$7.15 \pm 0.05$	$6.59 \pm 0.10$	$0.14 \pm 0.07$ ( $\text{pH}_i$ 6.67)*	$8.68 \pm 4.35$

Data are given as means  $\pm$  SD. \*  $\text{pH}_i$  common at all the experiments, where the recovery rate was calculated.

$\text{pH}_i$  recovery was inhibited by more than 75% ( $P < 0.002$ ). Recovery rate at  $\text{pH}_i$  6.51 was only  $0.05 \pm 0.02$   $\text{pHU}/\text{min}$  ( $\text{jH}^+$   $3.75 \pm 1.33$   $\text{mM}/\text{min}$ ,  $n = 4$ ), an effect that was reversible after withdrawal of amiloride. These findings ( $\text{Na}^+$  dependence and amiloride inhibition of  $\text{pH}_i$  recovery) indicate that in nominally bicarbonate-free media, the recovery from an acute acid load is driven by the  $\text{Na}^+/\text{H}^+$  exchanger. Since the  $\text{pH}_i$  recovery was not completely inhibited by amiloride, other mechanisms could also be involved.

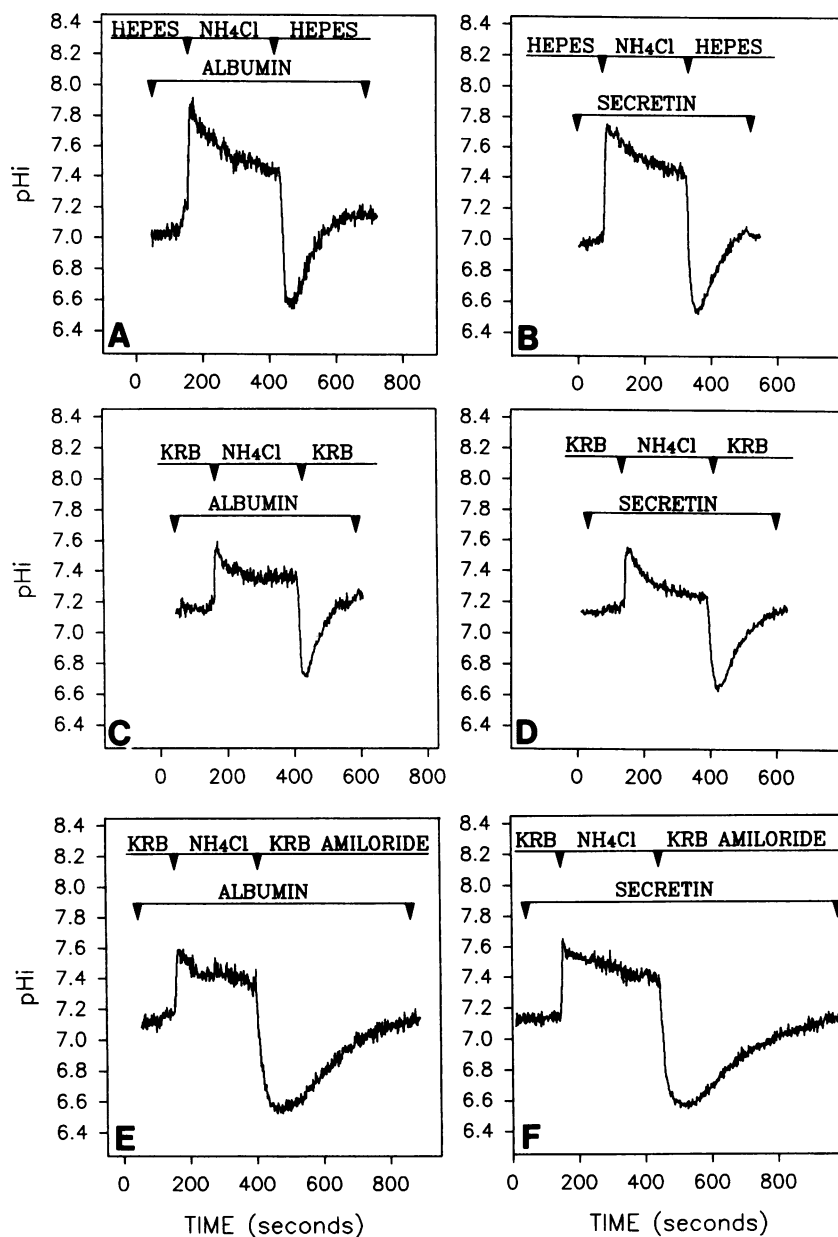
In BDE cells cultured in  $\text{HCO}_3^-$  enriched media ( $\alpha\text{MEM}$ ) and perfused with bicarbonate buffered solutions (KRB), the  $\text{pH}_i$  decreased to  $6.64 \pm 0.07$  ( $n = 11$ ) after  $\text{NH}_4\text{Cl}$  administration and withdrawal, and recovered at a rate of  $0.204 \pm 0.073$   $\text{pHU}/\text{min}$  at  $\text{pH}_i$  6.74 ( $\text{jH}^+$   $11.60 \pm 4.05$   $\text{mM}/\text{min}$ , Table II). Removal of external  $\text{Na}^+$  at the moment of  $\text{NH}_4\text{Cl}$  withdrawal produced a higher degree of acidification (nadir  $\text{pH}_i$   $6.42 \pm 0.04$ ,  $n = 4$ ) and the recovery to basal  $\text{pH}_i$  was completely inhibited, until external  $\text{Na}^+$  was readmitted. Amiloride (1 mM) administered at the time of  $\text{NH}_4\text{Cl}$  withdrawal decreased the nadir acidification ( $6.55 \pm 0.08$ , Table II), and significantly ( $P < 0.05$ ) decreased the rate of  $\text{pH}_i$  recovery to  $0.13 \pm 0.05$  (at  $\text{pH}_i$  6.67,  $\text{jH}^+$   $7.79 \pm 3.01$   $\text{mM}/\text{min}$ ) with respect to controls, indicating that  $\sim 35\%$  of the recovery is driven by the  $\text{Na}^+/\text{H}^+$  exchanger (amiloride-sensitive component of  $\text{pH}_i$  recovery). When BDE cells were preincubated (60–80 min) with 1 mM DIDS to inhibit the  $\text{HCO}_3^-$ -dependent transport processes and perfused with amiloride at the moment of  $\text{NH}_4\text{Cl}$  removal (Table II), the nadir acidification was  $6.46 \pm 0.09$  and the recovery was further decreased to  $0.053 \pm 0.006$   $\text{pHU}/\text{min}$  ( $\text{jH}^+$   $4.21 \pm 0.52$   $\text{mM}/\text{min}$  at  $\text{pH}_i$  6.55). Two transport processes ( $\text{Na}^+$  dependent, DIDS inhibitable  $\text{HCO}_3^-$  transport) that could mediate the amiloride insensitive component of  $\text{pH}_i$  recovery from an acid load in the presence of  $\text{HCO}_3^-$ , are a  $\text{Na}^+/\text{HCO}_3^-$  symport and a  $\text{Na}^+$  dependent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. To discriminate between these two transporters, we studied the amiloride insensitive component of  $\text{pH}_i$  recovery from an acid load in cells (Table II) preincubated for 40 min in

a  $\text{Cl}^-$  free medium (equimolar substitution with gluconate). To exclude the influence of the  $\text{Na}^+/\text{H}^+$  exchanger, the cells were perfused with 1 mM amiloride at the moment of  $\text{NH}_4^+$  withdrawal. In these experimental conditions the basal  $\text{pH}_i$  was significantly higher ( $P < 0.01$ ) than in the presence of  $\text{Cl}^-$  ( $7.35 \pm 0.04$ ,  $n = 5$ ), the nadir acidification was  $6.88 \pm 0.05$  and  $\text{pH}_i$  recovered from the acid load at a rate of  $0.15 \pm 0.050$   $\text{pHU}/\text{min}$  ( $\text{jH}^+$   $6.92 \pm 2.42$   $\text{mM}/\text{min}$ ), at the same rate as in the presence of  $\text{Cl}^-$  and amiloride. This  $\text{Cl}^-$ -independence of the  $\text{pH}_i$  recovery mechanism excludes a  $\text{Na}^+$  dependent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger as an acid extruding mechanism in these normal BDE cells, as described in some other cell types (24–25) and indicates that recovery from an acute acid load in the presence of bicarbonate is driven by the activities of the  $\text{Na}^+/\text{H}^+$  exchanger and the  $\text{Na}^+/\text{HCO}_3^-$  symport.

*Effect of secretin on the rate of  $\text{pH}_i$  recovery from an acute acid load (Table I, Table II and Fig. 3).* BDE cells preincubated and perfused with 200 nM secretin ( $n = 10$ ) showed similar degrees of acidification and similar rates of  $\text{pH}_i$  recovery from the  $\text{NH}_4\text{Cl}$ -induced acid load (calculated at the  $\text{pH}_i$  value common to all experiments) (Fig. 3 B, Table I) as in control experiments (Fig. 3 A, Table I), indicating that secretin has no direct effect on the activity of the  $\text{Na}^+/\text{H}^+$  exchanger.

In the presence of bicarbonate, the rate of  $\text{pH}_i$  recovery from the acid load was also unaffected by secretin (Table II, Fig. 3, C and D). To evaluate more specifically the effect of secretin on  $\text{Na}^+/\text{HCO}_3^-$  symport, the same experiments were also performed by superfusing the cell with 1 mM amiloride at the moment of 20 mM  $\text{NH}_4\text{Cl}$  withdrawal (Table II and Fig. 3, E and F). With this protocol, secretin also has no effect on the activity of the  $\text{Na}^+/\text{HCO}_3^-$  symport.

*$\text{Cl}^-/\text{HCO}_3^-$  exchange.* Both the net  $\text{pH}_i$  increase and recovery ( $\delta\text{pH}_i$ ) and the rate of the  $\text{pH}_i$  increase and recovery after  $\text{Cl}^-$  removal and readmission respectively (Fig. 4) were the parameters used to evaluate the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger as previously described (20–25). After  $\text{Cl}^-$  removal (Fig. 4 C, control experiments)  $\text{pH}_i$  rose  $0.24 \pm 0.08$   $\text{pHU}$  at a



**Figure 3.** Effect of secretin on the rate of  $pH_i$  recovery from acute acid load. *A* and *B*. BDE cells preincubated (10 min) and perfused with 200 nM secretin (*B*,  $n = 10$ ), in absence of bicarbonate (Hepes), showed similar degrees of acidification and rates of  $pH_i$  recovery, after 20 mM  $NH_4Cl$  withdrawal, when compared with control experiments (*A*,  $n = 9$ ) perfused with albumin, the secretin carrier. *C* and *D*. BDE cells preincubated (10 min) and perfused with 200 nM secretin (*D*,  $n = 10$ ), in presence of bicarbonate (KRB), showed similar degree of acidification and similar rate of  $pH_i$  recovery, after 20 mM  $NH_4Cl$  withdrawal, as compared with control experiments (*C*,  $n = 9$ ) perfused with albumin. *E* and *F*. At the moment of  $NH_4Cl$  withdrawal, BDE cells were superfused with 1 mM amiloride in KRB. BDE cells pretreated and perfused with 200 nM secretin (*F*,  $n = 8$ ) showed no difference in the degree of acidification and in the rate of  $pH_i$  recovery from 20 mM  $NH_4Cl$  acid load with respect to the albumin-carrier controls (*E*,  $n = 8$ ).

maximal rate of  $0.125 \pm 0.06$  pHU/min ( $H^+$  flux =  $6.02 \pm 3.27$  mM/min,  $n = 15$ ). When  $Cl^-$  was readmitted, the cells recovered to baseline at a maximal rate of  $0.188 \pm 0.080$  pHU/min ( $H^+$  flux =  $11.82 \pm 5.34$  mM/min). The rate of  $pH_i$  change and the  $H^+$  fluxes measured during  $Cl^-$  readmission (i.e., at a higher  $pH_i$  than the basal values when  $Cl^-$  was acutely removed) was significantly higher ( $P < 0.01$ ) than when measured during  $Cl^-$  removal. In addition, if the  $H^+$  fluxes measured during acute  $Cl^-$  removal, in all the control experiments performed in the present study ( $n = 30$ ), were plotted against their corresponding  $pH_i$  values at the moment of  $Cl^-$  removal (Fig. 5 *A*), a significant direct correlation ( $r = 0.66$ ,  $P < 0.0002$ ) between  $pH_i$  and  $H^+$  flux was found. The same correlation ( $r = 0.60$ ,  $P < 0.0008$ ) was also found if the  $H^+$  fluxes measured in correspondence with  $Cl^-$  readmission were plotted against the correspondent  $pH_i$  values (Fig. 5 *B*). These findings suggest either that the higher gradient of  $HCO_3^-$  favors  $Cl^-/HCO_3^-$  exchange at the higher  $pH_i$  values, or that there is

an allosteric effect of  $pH_i$  on the activity of the exchanger, as demonstrated in other cell types (22, 24, 26–27).

The effect of  $Cl^-$  removal was completely abolished by 1 mM DIDS pretreatment (60–80 min,  $n = 6$ ; Fig. 4 *C*) indicating that the increase in  $pH_i$  induced by acute  $Cl^-$  removal depends on the transport of  $HCO_3^-$  across the cell membrane.

**Effect of secretin on the activity of the  $Cl^-/HCO_3^-$  exchanger (Table III).** When BDE cells were preincubated and perfused with 200 nM secretin, a maximal rate of alkalization was observed after  $Cl^-$  removal ( $0.43 \pm 0.41$  pHU/min,  $H^+$  flux =  $21.13 \pm 20.56$  mM/min, Fig. 4 *B*) that was significantly higher ( $P < 0.012$ ) than obtained in carrier-control experiments ( $0.11 \pm 0.05$  pHU/min,  $H^+$  flux =  $5.52 \pm 2.63$  mM/min,  $n = 15$ ) (Fig. 4 *A*). As illustrated in Fig. 6, following  $Cl^-$  removal, values for  $H^+$  flux in 8 out of 15 experiments in the secretin group were higher than the maximum value found in 15 control experiments (8 BDE cell preparations). This finding indicated that under the conditions of these experiments,

Table III. Effect of Secretin or DBcAMP on the Activity of the  $\text{Cl}^-/\text{HCO}_3^-$  Exchanger

	Secretin controls <i>n</i> = 15	Secretin* <i>n</i> = 15	DBcAMP controls <i>n</i> = 15	DBcAMP* <i>n</i> = 11
Basal pH	7.16±0.08	7.14±0.10	7.14±0.10	7.13±0.08
<i>Chloride removal</i>				
Delta pH	0.25±0.07	0.29±0.10	0.24±0.08	0.31±0.06 <sup>  </sup>
pHU/min max	0.11±0.05	0.43±0.41 <sup>‡</sup>	0.12±0.06	0.27±0.13 <sup>†</sup>
H Flux (mM/min)	5.52±2.63	21.13±20.56 <sup>‡</sup>	6.02±3.27	12.57±6.27 <sup>†</sup>
<i>Chloride readmission</i>				
pHU/min max	0.21±0.09	0.43±0.34 <sup>§</sup>	0.19±0.08	0.54±0.37 <sup>**</sup>
H Flux (mM/min)	12.67±5.66	27.25±21.86 <sup>§</sup>	11.82±5.34	34.79±26.40 <sup>**</sup>

The activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger was measured by the net  $\text{pH}_i$  increase (delta  $\text{pH}_i$ ) promoted by acute  $\text{Cl}^-$  removal and the rate of both  $\text{pH}_i$  increase after  $\text{Cl}^-$  removal and of  $\text{pH}_i$  recovery after  $\text{Cl}^-$  readmission in BDE cells cultured and perfused with  $\text{HCO}_3^-$ -enriched media. \* Activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger measured in BDE cells preincubated and perfused with 200 nM secretin (albumin solution) or 100  $\mu\text{M}$  DBcAMP. BDE cells preincubated and perfused with the secretin carrier (i.e., albumin) were used as secretin controls. Data are given as means±SD. <sup>‡</sup>  $P < 0.012$  vs. secretin controls. <sup>§</sup>  $P < 0.025$  vs. secretin controls. <sup>||</sup>  $P < 0.04$  vs. DBcAMP controls. <sup>†</sup>  $P < 0.003$  vs. DBcAMP controls. <sup>\*\*</sup>  $P < 0.005$  vs. DBcAMP controls.

~ 50% of BDE cells responded to secretin by increasing the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger.  $\text{pH}_i$  change (i.e.,  $\delta\text{pH}_i$ ) following acute  $\text{Cl}^-$  removal showed no statistical difference between secretin-treated cells and controls (0.29±0.10 vs. 0.25±0.07 pHU). However, when external  $\text{Cl}^-$  was readmitted, secretin-treated cells showed a maximal rate of  $\text{pH}_i$  recovery (0.43±0.34 pHU/min,  $\text{H}^+$  flux = 27.25±21.86 mM/min)

that was significantly higher than in control experiments (0.21±0.09 pHU/min,  $\text{H}^+$  flux = 12.67±5.66 mM/min,  $P < 0.025$ ).  $\text{H}^+$  flux values measured during acute  $\text{Cl}^-$  removal or  $\text{Cl}^-$  readmission, showed a significant correlation ( $\text{Cl}^-$  removal:  $r = 0.44$ ;  $P < 0.015$ ;  $\text{Cl}^-$  readmission:  $r = 0.54$ ;  $P < 0.004$ ) with their correspondent  $\text{pH}_i$  values in all the secretin experiments ( $n = 27$ ), as also seen in the control experiments,

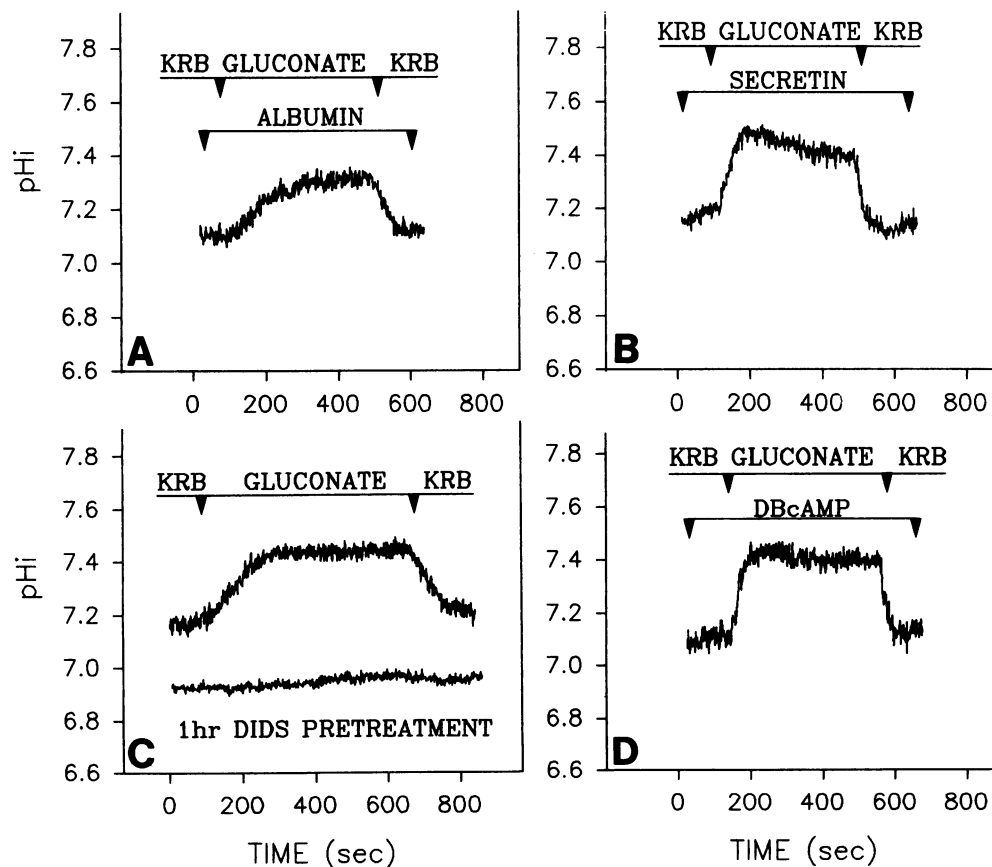
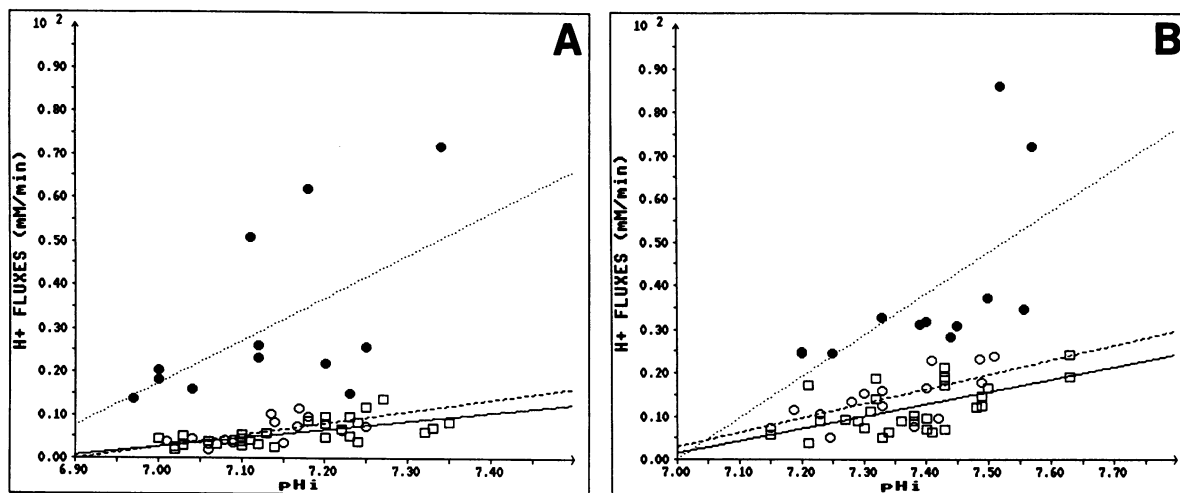


Figure 4. Effects of secretin or DBcAMP on  $\text{pH}_i$  changes promoted by acute  $\text{Cl}^-$  removal and readmission. A and B. BDE cells preincubated and perfused with 200 nM secretin (B,  $n = 15$ ), in presence of  $\text{HCO}_3^-$  (KRB), showed a maximal rate of alkalization after  $\text{Cl}^-$  removal (gluconate) and of  $\text{pH}_i$  recovery after  $\text{Cl}^-$  readmission that was significantly higher than those measured in albumin carrier-controls (A,  $n = 15$ ). C and D. BDE cells preincubated and perfused with 100  $\mu\text{M}$  DBcAMP (D,  $n = 11$ ), in presence of  $\text{HCO}_3^-$  (KRB), showed a maximal rate of alkalization after  $\text{Cl}^-$  removal (gluconate) and of  $\text{pH}_i$  recovery after  $\text{Cl}^-$  readmission that was significantly higher than those measured in control experiments (C,  $n = 15$ ). In C (lower  $\text{pH}_i$  tracing) 1 mM DIDS pretreatment (60–80 min,  $n = 6$ ), completely abolished the effect of  $\text{Cl}^-$  removal in controls, consistent with  $\text{HCO}_3^-$  transport across the cell membrane.



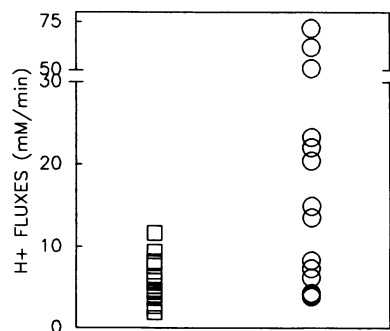


**Figure 5.** Plots of  $H^+$  flux values measured during acute  $Cl^-$  removal (*A*) or  $Cl^-$  readmission (*B*) and their correspondent  $pH_i$  values. The  $H^+$  fluxes measured in all the control experiments of the present study (squares and — lines,  $n = 30$ ) following  $Cl^-$  removal (*A*) showed a significant direct correlation ( $r = 0.66$ ,  $P < 0.0002$ ) with the  $pH_i$  values. The same correlation ( $r = 0.60$ ,  $P < 0.0008$ ) was also found if the  $H^+$  fluxes measured in correspondence with  $Cl^-$  readmission (*B*) were plotted against the correspondent  $pH_i$  values. The analysis of the exchanger activity/ $pH_i$  relationship was also performed for the secretin experiments ( $n = 27$ ) by separating secretin responders and nonresponders (i.e., values higher and lower than the maximum control value). The latter (open circles and - - - lines) showed ( $Cl^-$  removal:  $r = 0.62$ ,  $P < 0.012$ ;  $Cl^-$  readmission:  $r = 0.70$ ,  $P < 0.004$ ,  $n = 15$ ) a gradient and intercept (rate of  $H^+$  flux at  $pH_i 0$ ) of the linear regression line that was similar to the control experiments without secretin exposure, either after  $Cl^-$  removal (gradient = 26.9 vs. 20.5 mM/min; intercept = -186.9 vs. -130.5 mM/min) or after  $Cl^-$  readmission (gradient 33.4 vs. 27.2 mM/min; intercept -230.3 vs. -190.2 mM/min). In the secretin responders ( $n = 12$ ; closed circles and · · · lines) the significance of the correlation still persists ( $Cl^-$  removal:  $r = 0.60$ ,  $P < 0.037$ ;  $Cl^-$  readmission:  $r = 0.63$ ,  $P < 0.027$ ), and the secretin responders showed a gradient and an intercept of the regression line after  $Cl^-$  removal (gradient = 97.7 mM/min; intercept = -666.8 mM/min) or  $Cl^-$  readmission (gradient = 94.9 mM/min; intercept = -664.1 mM/min) that was significantly different ( $P < 0.04$ ) compared with both the secretin nonresponders and controls.

thus confirming that the activity of the  $Cl^-/HCO_3^-$  exchanger is, in presence of secretin, still directly related to the value of the  $pH_i$  at which it is measured. When an analysis of the exchanger activity/ $pH_i$  relationship was performed by separating secretin responders (values higher than the maximum control value) and nonresponders (values lower than the maximum control value), the nonresponders ( $Cl^-$  removal:  $r = 0.62$ ,  $P < 0.012$ ;  $Cl^-$  readmission:  $r = 0.70$ ,  $P < 0.004$ ;  $n = 15$ ) demonstrated a gradient and intercept ( $H^+$  flux at  $pH_i 0$ ) of the linear regression lines that was similar to the control experiments without secretin exposure, both after  $Cl^-$  removal (Fig. 5 *A*) and after  $Cl^-$  readmission (Fig. 5 *B*). In the secretin responders ( $n = 12$ ) the significance of the correlation still persists ( $Cl^-$  removal:  $r$

= 0.60,  $P < 0.037$ ;  $Cl^-$  readmission:  $r = 0.63$ ,  $P < 0.027$ ) but it is lower than controls and nonresponders, indicating that the activity of the exchanger is influenced by an additional variable, possibly related to the rate of hormone response. In addition, the secretin responders showed a gradient and an intercept of the regression lines after  $Cl^-$  removal (Fig. 5 *A*) or  $Cl^-$  readmission (Fig. 5 *B*) that was significantly different ( $P < 0.04$ ) compared with both secretin nonresponders and controls. The findings in these experiments suggest that the exchanger was set (regulated), in the presence of secretin, at a higher rate in the responders at any given  $pH_i$ .

*Effect of DBcAMP on basal  $pH_i$  and on the activity of the  $Cl^-/HCO_3^-$  exchanger (Table III).* Secretin increases the intracellular level of cAMP in BDE cells (11, 12), suggesting that the hormone's effect could be mediated by this cyclic nucleotide. However acute exposure to DBcAMP (100  $\mu M$ ), did not change basal  $pH_i$  either when cells were maintained in Hepes ( $n = 6$ ) or in KRB ( $n = 6$ ) media. Similarly when BDE cells were preincubated and perfused with 100  $\mu M$  DBcAMP, no significant difference in their basal  $pH_i$  was observed with respect to controls ( $7.13 \pm 0.07$ ,  $n = 19$  vs.  $7.16 \pm 0.10$ ,  $n = 44$ , KRB medium). Thus, like secretin, DBcAMP has no effect on basal BDE cell  $pH_i$ . In addition, the lack of effect of DBcAMP on basal  $pH_i$  in Hepes suggests that the activity of the  $Na^+/H^+$  exchanger is not directly influenced by this messenger. In contrast, the activity of the  $Cl^-/HCO_3^-$  exchanger was significantly stimulated by DBcAMP (Table III and Fig. 4 *D*). Both the maximal rate of alkalization after  $Cl^-$  removal ( $0.271 \pm 0.131$  pHU/min, respectively,  $H^+$  flux =  $12.57 \pm 6.27$  mM/min) and of  $pH_i$  recovery after  $Cl^-$  readmission ( $0.538 \pm 0.373$  pHU/min,  $H^+$  flux =  $34.79 \pm 26.40$  mM/min) were significantly higher ( $P < 0.003$  and  $P < 0.005$ , respectively) in BDE cells



**Figure 6.** Effect of secretin on the  $H^+$  flux values measured following acute  $Cl^-$  removal. BDE cells preincubated and perfused with 200 nM secretin (circles,  $n = 15$ ) showed, following acute  $Cl^-$  removal,  $H^+$  flux values (measured as in Methods) significantly higher ( $P < 0.012$ ) than those measured in carrier

controls (squares,  $n = 15$ ). Only 8 out of 15 experiments in the secretin group showed  $H^+$  fluxes higher than the maximum value found in the carrier controls, suggesting a physiologic heterogeneity in different populations of BDE cells or that secretin receptors are lost or internalized in some cells during the process of cell isolation.

preincubated (10 min) and perfused with 100  $\mu$ M DBcAMP (Fig. 4 D) when compared with controls (Fig. 4 C). The net  $pH_i$  increase following acute  $Cl^-$  removal was also significantly enhanced by DBcAMP treatment ( $0.31 \pm 0.06$  vs.  $0.24 \pm 0.08$ ,  $P < 0.04$ ).

Thus DBcAMP reproduces the effects of secretin on  $pH_i$  regulation in BDE cells, stimulating the activity of the  $Cl^-/HCO_3^-$  exchanger without significant changes in the basal  $pH_i$ .

**Effect of the  $Cl^-$  channel blocker NPPB on the secretin stimulation of the  $Cl^-/HCO_3^-$  exchanger (Table IV).** In different cell types  $Cl^-/HCO_3^-$  exchangers may be functionally coupled with  $Cl^-$  channels (28–33). In BDE cells both cAMP- and  $Ca^{++}$ -regulated  $Cl^-$  channels have been recently described (34). In BDE cells pretreated and perfused with 200 nM secretin, 10  $\mu$ M NPPB (35) significantly decreased ( $P < 0.02$ ) the maximal rate of alkalization promoted by acute  $Cl^-$  removal ( $0.102 \pm 0.059$  pHU/min;  $H^+$  flux =  $4.92 \pm 2.80$  mM/min, Fig. 7 B) when compared with controls ( $0.247 \pm 0.180$ ;  $H^+$  flux =  $11.4 \pm 8.12$  mM/min, Table IV and Fig. 7 A). The rate of  $pH_i$  recovery following  $Cl^-$  readmission was also decreased in the presence of NPPB ( $0.163 \pm 0.064$  pHU/min;  $H^+$  flux =  $9.63 \pm 3.98$  mM/min) compared with its absence ( $0.333 \pm 0.159$  pHU/min;  $H^+$  =  $21.83 \pm 10.20$  mM/min;  $P < 0.005$ ). Furthermore the net  $pH_i$  increase following  $Cl^-$  removal did not increase significantly when cells were treated with secretin in the presence of NPPB compared with secretin exposure alone ( $0.23 \pm 0.10$  vs.  $0.30 \pm 0.08$  pHU). In addition, in the presence of NPPB alone, neither the net  $pH_i$  increase following  $Cl^-$  removal nor the rate of  $pH_i$  changes related to  $Cl^-$  removal and readmission showed significant differences compared with controls (Table III). These findings suggest that the  $Cl^-$  channel blocker NPPB blocks the effect of secretin on the activity of the  $Cl^-/HCO_3^-$  exchanger but not when the secretin stimulus is omitted.

**Effect of the  $Cl^-$  channel blocker NPPB on DBcAMP stimulation of the  $Cl^-/HCO_3^-$  exchanger activity (Table IV).** In BDE cells pretreated and perfused with 100  $\mu$ M DBcAMP, superfusion with 10  $\mu$ M NPPB ( $n = 8$ ) significantly ( $P < 0.03$ , Fig. 7 D) decreased the maximal rate of alkalization promoted by acute  $Cl^-$  removal ( $0.088 \pm 0.036$  pHU/min;  $H^+$  flux

=  $3.95 \pm 1.60$  mM/min;  $n = 8$ ) when compared with control studies ( $0.262 \pm 0.185$  pHU/min;  $H^+$  flux =  $12.55 \pm 9.41$  mM/min;  $n = 8$ , Fig. 7 C). The rate of  $pH_i$  recovery following  $Cl^-$  readmission was also decreased in the presence of NPPB ( $0.176 \pm 0.07$  pHU/min;  $H^+$  flux =  $10.01 \pm 5.3$  mM/min) but not in its absence ( $0.388 \pm 0.119$  pHU/min;  $H^+$  flux =  $27.72 \pm 19.37$  mM/min;  $P < 0.04$ ). The net  $pH_i$  increase following  $Cl^-$  removal showed no statistical difference when DBcAMP plus NPPB was perfused than when DBcAMP was present alone ( $0.23 \pm 0.07$  vs.  $0.28 \pm 0.09$  pHU). These findings suggest that the  $Cl^-$  channel blocker, NPPB, also prevents the stimulation of  $Cl^-/HCO_3^-$  activity by DBcAMP.

## Discussion

**$pH_i$  regulation in BDE cells.** This study identifies several ion exchange mechanisms that regulate intracellular pH in normal rat BDE cells as also observed previously when cells were isolated from bile duct obstructed rats (10). Thus despite the smaller size and lower yield of BDE cells from normal animals (8–10), these ion exchangers function in similar fashion when isolated by either method.

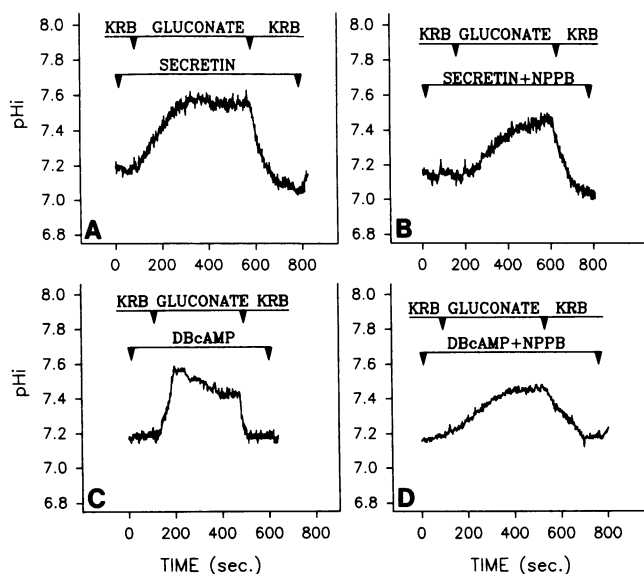
$Na^+/H^+$  exchange is the major acid extruding mechanism when  $HCO_3^-$  is omitted as demonstrated by inhibition of basal  $pH_i$  and spontaneous recovery from an acid load in the presence of amiloride, or during omission of sodium (Fig. 2, Table I). When  $HCO_3^-$  is present, the  $Na^+/H^+$  exchanger is minimally involved in basal  $pH_i$  maintenance (amiloride insensitivity), but is activated by an acid load, as suggested by amiloride inhibition of a portion of  $pH_i$  recovery (Table II). The  $Na^+/HCO_3^-$  symporter functions as an acid extruder, in the presence of  $HCO_3^-$  as indicated by: (a) A higher basal  $pH_i$  than in the absence of bicarbonate, (b) A drop in  $pH_i$  when  $Na^+$  is omitted (Fig. 2) or DIDS is added, and (c)  $Na^+$  and DIDS dependent but  $Cl^-$  independent  $pH_i$  recovery from an acid load (Table II). Both the latter as well as the failure of  $Cl^-$  omission to lower  $pH_i$  exclude the possibility that a  $Na^+$  coupled  $Cl^-/HCO_3^-$  exchanger functions as an acid extruder in BDE cells in contrast to some other cell types (20, 24, 25). Finally, a  $Cl^-/HCO_3^-$  exchanger is the major acid loading mechanism in BDE

Table IV. Effect of NPPB on Secretin and DBcAMP Stimulation of the Activity of the  $Cl^-/HCO_3^-$  Exchanger

	Albumin + NPPB <i>n</i> = 9	Secretin + NPPB <i>n</i> = 12	Secretin <i>n</i> = 12	DBcAMP + NPPB <i>n</i> = 8	DBcAMP <i>n</i> = 8
Basal pH	7.15 $\pm$ 0.07	7.13 $\pm$ 0.09	7.12 $\pm$ 0.07	7.11 $\pm$ 0.05	7.14 $\pm$ 0.08
<i>Chloride removal</i>					
Delta pH	0.22 $\pm$ 0.07	0.23 $\pm$ 0.10	0.30 $\pm$ 0.08	0.23 $\pm$ 0.07	0.28 $\pm$ 0.09
pHU/min max	0.09 $\pm$ 0.03	0.10 $\pm$ 0.06	0.25 $\pm$ 0.18*	0.09 $\pm$ 0.04	0.26 $\pm$ 0.18 <sup>§</sup>
H Flux (mM/min)	4.41 $\pm$ 1.45	4.92 $\pm$ 2.80	11.40 $\pm$ 8.12*	3.95 $\pm$ 1.60	12.55 $\pm$ 9.41 <sup>§</sup>
<i>Chloride readmission</i>					
pHU/min max	0.17 $\pm$ 0.06	0.16 $\pm$ 0.06	0.33 $\pm$ 0.16 <sup>‡</sup>	0.18 $\pm$ 0.07	0.39 $\pm$ 0.12 <sup>  </sup>
H Flux (mM/min)	10.31 $\pm$ 3.79	9.63 $\pm$ 3.98	21.83 $\pm$ 10.20 <sup>‡</sup>	10.01 $\pm$ 5.30	27.72 $\pm$ 19.37 <sup>  </sup>

The activity of the  $Cl^-/HCO_3^-$  exchanger measured in BDE cells superfused with the  $Cl^-$  channel blocker NPPB plus secretin or plus DBcAMP was compared with BDE cells treated with secretin or DBcAMP alone. Data of the secretin + NPPB or secretin-alone groups were also compared with an albumin (i.e., secretin carrier) + NPPB group. Data are given as means $\pm$ SD. \*  $P < 0.02$  vs. albumin + NPPB or secretin + NPPB (Anova one way). <sup>‡</sup>  $P < 0.005$  vs. albumin + NPPB or secretin + NPPB (Anova one way). <sup>§</sup>  $P < 0.03$  vs. DBcAMP + NPPB.

<sup>||</sup>  $P < 0.04$  vs. DBcAMP + NPPB.



**Figure 7.** Effect of the  $\text{Cl}^-$  channel blocker NPPB on secretin and DBcAMP stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchange activity. *A* and *B*. BDE cells preincubated and perfused with 200 nM secretin, superfused with 10  $\mu\text{M}$  NPPB (*B*,  $n = 12$ ) showed a maximal rate of  $\text{pH}_i$  increase following  $\text{Cl}^-$  removal (gluconate) and of  $\text{pH}_i$  recovery after  $\text{Cl}^-$  readmission significantly lower as compared with those obtained in BDE cells perfused with secretin alone (*A*,  $n = 12$ ). *C* and *D*. BDE cells preincubated and perfused with 100  $\mu\text{M}$  DBcAMP, superfused with 10  $\mu\text{M}$  NPPB (*D*,  $n = 8$ ) showed a maximal rate of  $\text{pH}_i$  increase following  $\text{Cl}^-$  removal (gluconate) and of  $\text{pH}_i$  recovery after  $\text{Cl}^-$  readmission significantly lower, compared with those obtained in BDE cell perfused with DBcAMP alone (*C*,  $n = 8$ ).

cells, as in other cell types (20–25), since  $\text{pH}_i$  increases after  $\text{Cl}^-$  removal and recovers after  $\text{Cl}^-$  readmission. Alternatively,  $\text{Na}^+/\text{H}^+$  exchanger could be activated by cell shrinkage resulting from  $\text{Cl}^-$  substitution with the nonpermeant gluconate (19, 36), or the membrane potential could hyperpolarize and stimulate electrogenic ion movements (37). However, DIDS completely inhibited (Fig. 4 *C*) the  $\text{pH}_i$  increase promoted by  $\text{Cl}^-$  removal, consistent with a primary involvement of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. In all cell types studied (20–26, 38, 39), the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger functions as an acid loader driven by the  $\text{Cl}^-$  gradient. Thus, this exchanger presumably functions as a counterpoint to the acid extruding systems, in regulation of  $\text{pH}_i$  in BDE cells.

**Effect of secretin on  $\text{pH}_i$  regulation in BDE cells.** The major findings in this study relate to the effects of secretin on these  $\text{pH}_i$  regulatory mechanisms. Secretin had no effect on basal  $\text{pH}_i$  either in the presence or in the absence of  $\text{HCO}_3^-$  containing media and had no effect on the rate of  $\text{pH}_i$  recovery when BDE cells were challenged with an acid load, suggesting that secretin does not directly modify either of the two acid extruding mechanisms,  $\text{Na}^+/\text{H}^+$  exchanger, or  $\text{Na}^+/\text{HCO}_3^-$  symport. The *in vivo* insensitivity of the secretin-stimulated bicarbonate secretion to amiloride, both in pig (40) and in the isolated guinea pig liver (41), is consistent with these findings, which further indicate that the mechanism of bicarbonate secretion stimulated by secretin is not the consequence of an intracellular alkalization as originally proposed. Rather, as shown in this study, secretin stimulates the rate of alkalization and of  $\text{pH}_i$  recovery induced by  $\text{Cl}^-$  removal and readmission, respectively, findings consistent with an increase in the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchange mechanism (Fig. 4, Table III).  $\text{Cl}^-$ /

$\text{HCO}_3^-$  exchangers mediate bicarbonate excretion in many different epithelia, including duodenum (42), pancreatic ducts (28–30, 39), urinary bladder (33), small intestine (43), and the cortical collecting tubule of the kidney (44).

It is not clear why secretin-stimulated BDE cells maintain their basal  $\text{pH}_i$  despite the enhanced activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Although a decrease in  $\text{pH}_i$  would result from an increase in activity of this acid-loading mechanism, it could be counterbalanced by an increase in activity of the acid extruders. However, neither the  $\text{Na}^+/\text{H}^+$  exchange nor the  $\text{Na}^+/\text{HCO}_3^-$  symport were stimulated by secretin when their activity was evaluated during recovery from an acute acid load. However stimulation of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger by secretin may occur only when  $\text{pH}_i$  increases above basal values. This would be consistent with the known  $\text{pH}_i$  dependence of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity, which in various cell types (20–27) is activated only at  $\text{pH}_i$  values above the cell's basal set point. Thus, when the activity of the  $\text{Na}^+/\text{H}^+$  or  $\text{Na}^+/\text{HCO}_3^-$  symport was evaluated during acid loading, the  $\text{pH}_i$  was at a lower range where activation of the  $\text{Cl}^-/\text{HCO}_3^-$  by secretin is not possible. Theoretically, the  $\text{Na}^+/\text{H}^+$  exchanger or the  $\text{Na}^+/\text{HCO}_3^-$  symport could be inhibited by the acid loading, a possibility ruled out for the  $\text{Na}^+/\text{H}^+$  exchanger, which was activated by lowering  $\text{pH}_i$  as in other cell types (10, 25). However, in rabbit renal cortex basolateral membrane vesicles,  $\text{Na}^+/\text{HCO}_3^-$  symport is much more active at basal than at lower or higher  $\text{pH}_i$  values (45). In BDE cells, basal  $\text{pH}_i$  remained unchanged when cells were perfused in  $\text{HCO}_3^-$  media with amiloride and secretin, excluding a counter  $\text{pH}_i$  regulatory response from the  $\text{Na}^+/\text{H}^+$  exchanger, but not from the  $\text{Na}^+/\text{HCO}_3^-$  symport, which is the major acid extruding mechanism in the BDE cell. Unfortunately attempts to inhibit  $\text{Na}^+/\text{HCO}_3^-$  symport, by omitting  $\text{Na}^+$  (choline or *N*-methyl-D-glutamine substitution) lowered  $\text{pH}_i$  to levels (approx. 6.4) where the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is inactive and probably unresponsive to any effects of secretin. Raising  $\text{pH}_i$  artificially above the basal  $\text{pH}_i$  by perfusing cells in high  $\text{pH}$ /bicarbonate media might theoretically resolve this issue, but, in the absence of  $\text{Na}^+$  no increase of  $\text{pH}_i$  was observed (data not presented).

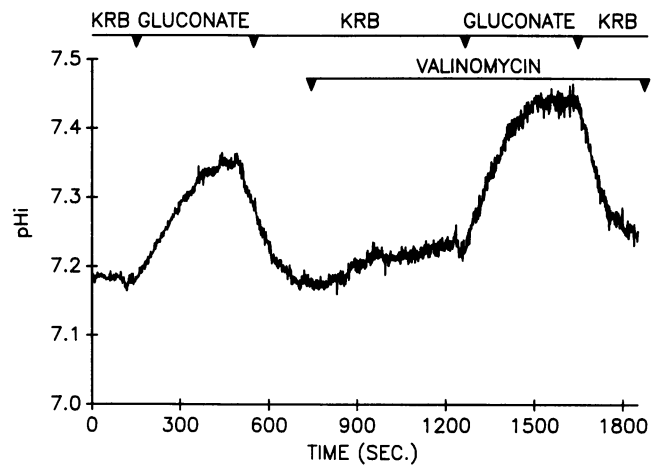
The findings of the present study are consistent with  $\text{HCO}_3^-$  excretion occurring via an apical localized  $\text{Cl}^-/\text{HCO}_3^-$  exchanger coupled to a  $\text{HCO}_3^-$  entry mechanism at the basolateral domain driven by the  $\text{Na}^+/\text{HCO}_3^-$  symport. This hypothesis is consistent with the mechanism of  $\text{HCO}_3^-$  excretion previously proposed for rabbit and guinea pig gallbladder epithelium (46, 47). Alternatively,  $\text{H}^+$  extrusion via a  $\text{H}^+$ -ATPase, could counteract the secretin-stimulated bicarbonate excretion. Such a mechanism has been suggested for the pig where secretin-stimulated bicarbonate biliary secretion, *in vivo*, is significantly inhibited by dicyclohexylcarbodiimide (48), a known  $\text{H}^+$ -ATPase inhibitor.

**Effect of DBcAMP on  $\text{pH}_i$  regulation in BDE cells.** Secretin increases intracellular cAMP concentrations in BDE cells isolated from both normal (11) and bile duct-ligated rat liver (12). Secretin also increases both the cAMP and the  $\text{IP}_3$  intracellular levels in pancreas (49) and in BDE cells (12). Biliary bicarbonate secretion is also stimulated, in the guinea pig, by a secretin analogue (i.e. [Tyr<sup>10,13</sup>, Phe<sup>22</sup>, Trp<sup>25</sup>]secretin) that increases the intracellular levels of cAMP but not  $\text{IP}_3$  (12). Since DBcAMP reproduced the effects of secretin and stimulated the activity of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in rat BDE cells without significant modification of the basal  $\text{pH}_i$ , it is likely that the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is influenced by

the levels of the intracellular messenger cAMP and could be regulated by hormones acting through the cAMP-dependent protein kinase A pathway. Secretin also regulates  $\text{HCO}_3^-$  excretion in pancreatic ductular cells using cAMP as second intracellular messenger (28–32, 49). Thus, cAMP appears to stimulate  $\text{HCO}_3^-$  excretion via  $\text{Cl}^-/\text{HCO}_3^-$  exchange in epithelia that add base to the luminal side of the cell, including BDE as well as duodenum (42, 50), pancreatic duct cells (28–32) and the cortical collecting tubules (44). In contrast, cAMP decreases the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (51) in gallbladder epithelium, which secretes acid into the lumen.

**Effect of NPPB on the secretin and DBcAMP stimulation of the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger.** Both cAMP- and  $\text{Ca}^{++}$ -regulated  $\text{Cl}^-$  channels have been described, in preliminary studies, in isolated rat BDE cells (34). In pancreatic duct cells, secretin is thought to act by opening cAMP regulated  $\text{Cl}^-$  channels which then increases the gradient favoring  $\text{HCO}_3^-$  excretion via the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (28–32). These  $\text{Cl}^-$  channels have been associated (52–54) with expression of the cystic fibrosis transmembrane conductance regulator, whose mutation results in a characteristic defect ion  $\text{Cl}^-$  channel regulation (52–54). Therefore the inhibition of both the secretin and DBcAMP induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchange activity by NPPB suggests that their effects are mediated by opening of  $\text{Cl}^-$  channels (35). Although toxic effects of NPPB on the exchanger cannot be definitively excluded, nonspecific effects seem unlikely since NPPB did not affect the control activity of this exchanger (Table III) (Table IV).

When external chloride is removed, the  $\text{Cl}^-/\text{HCO}_3^-$  exchange is reversed and changes in  $\text{pH}_i$  now depend on the in-to-out chloride gradient and the intrinsic activity of the exchanger. However,  $\text{Cl}^-$  channels in rat BDE cells are inactive during basal conditions (55); thus in the presence of NPPB (alone or plus secretin or DBcAMP) the initial intracellular  $\text{Cl}^-$  concentration and the in-to-out  $\text{Cl}^-$  gradient after acute  $\text{Cl}^-$  removal should be the same as in controls. Furthermore, during  $\text{Cl}^-$  readmission the  $\text{Cl}^-$  gradient (out to in) should be essentially the same in all our experimental groups independently of the open state of the  $\text{Cl}^-$  channels. Changes in  $\text{pH}_i$  during  $\text{Cl}^-$  removal and readmission were both stimulated by secretin or DBcAMP but blocked by NPPB. Assuming that nonspecific toxic effects of NPPB did not occur, this finding suggests that secretin and DBcAMP activate  $\text{Cl}^-$  channels, but that the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is also activated as a secondary consequence of the opening of the  $\text{Cl}^-$  channel. This conclusion, e.g., that there is a link between activation of  $\text{Cl}^-$  channels and  $\text{Cl}^-/\text{HCO}_3^-$  exchange activity, is based on the known depolarizing effect of  $\text{Cl}^-$  removal and the effect of such a change in the membrane potential on the activity of the  $\text{Na}^+/\text{HCO}_3^-$  symport. Depolarization activates electrogenic  $\text{Na}^+/\text{HCO}_3^-$  symport in most tissues. This in turn would increase the entrance of  $\text{HCO}_3^-$  into the cells, activating the  $\text{HCO}_3^-/\text{Cl}^-$  exchanger secondarily at its  $\text{HCO}_3^-$  or pH sensitive sites as discussed above. That  $\text{HCO}_3^-$  entry via  $\text{Na}^+/\text{HCO}_3^-$  symport is stimulated electrogenically after  $\text{Cl}^-$  removal is suggested by the effect of Valinomycin (Fig. 8), a potassium ionophore that depolarizes cells. Following exposure of the bile duct epithelial cells to Valinomycin, chloride removal resulted in an enhanced rate of alkalization consistent with electrogenic  $\text{HCO}_3^-$  entry, presumably via a  $\text{Na}^+/\text{HCO}_3^-$  symport, or  $\text{HCO}_3^-$  conductive pathway. These findings essentially exclude that the exchanger is stimulated only as a consequence of the increased out-to-in  $\text{Cl}^-$  gradient generated by  $\text{Cl}^-$  channel opening as observed in



**Figure 8.** Effect of chloride removal on the rate of increase of  $\text{pH}_i$  in BDE cells before and after depolarizing the cell with valinomycin ( $5 \times 10^{-8}$  M). Note the more rapid alkalization and higher  $\text{pH}_i$  induced by chloride removal when the cells were depolarized, presumably reflecting stimulation of electrogenic bicarbonate entry. Example is 1 of 7 experiments.

pancreatic duct cells (28–32), since the acute  $\text{Cl}^-$  removal maneuver should lower the rate of alkalization instead of enhancing it as observed in our secretin and DBcAMP treated cells. Intracellular  $\text{Cl}^-$  concentrations would be diminished as a consequence of enhanced  $\text{Cl}^-$  efflux through the open  $\text{Cl}^-$  channels. A direct effect of secretin and DBcAMP on  $\text{Cl}^-$  channels is also supported by recent preliminary studies where patch clamp recordings have identified low conductance  $\text{Cl}^-$  channels that are activated by secretin in rat BDE cells by cAMP dependent mechanisms (56).

In summary, these studies demonstrate the presence of several ion transport mechanisms in normal BDE cells that function as acid extruders and acid loaders, thereby maintaining intracellular pH. Secretin stimulates the  $\text{Cl}^-/\text{HCO}_3^-$  exchange presumably by the action of protein kinase A and protein phosphorylation as suggested in other epithelia (28–32, 42, 50). Our findings are consistent with secretin stimulation of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger as a secondary consequence of  $\text{Cl}^-$  channel activation. It is presumed that the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is present on the apical membrane facing the bile duct lumen, based on knowledge of the physiologic properties of this epithelium, which secretes  $\text{HCO}_3^-$  into bile in response to secretin stimulation. However, the precise location of these transporters remains to be determined in these isolated nonpolarized cell preparations.

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