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±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^+/H^+ exchange and Na^+/HCO_3^- cotransport were not affected by this hormone. After acute Cl^- removal, pH_i rose 0.24±0.08 pHU at a maximal rate of 0.125±0.06 pHU/min (H^+ flux rates = 6.02±3.27 mM/min) and recovered after Cl^- readmission (0.188±0.08 pHU/min; H^+ flux rates = 11.82±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-Dibutyryladenosine-3′-5′-cyclic monophosphate (DBcAMP). The Cl^- channel blocker 5-nitro-2′-(3-phenylpropylamino)-benzoate (NPPB, 10 μM) 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 · intracellular pH regulation · secretin · Cl^-/HCO_3^- exchanger · Cl^- channel

Introduction

Bile duct epithelial (BDE)[1] 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 α-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′-diiso-thiocyano-2,2′-disulphonic acid stilbene; DiI, 1,1-dioctadecyl-1,3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate; GGT, gamma-glutamyl transpeptidase; L-15, Leibowitz 15 cell culture medium; DBcAMP, N^6,2′-*O*-dibutyryladenosine-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'-diisothiocyano-2,2'disulfonic acid stilbene (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)-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, 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'-tetramethyl-indo-carbocyanine perchlorate (DiI) were from BioTechnology Inc. (Stoughton, MA). *N*(gamma-l-glutamyl)-4-methoxy-2-napthylamide 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 α-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_i) 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_i indicator as described from this laboratory (10). BDE cells on glass coverslips were loaded with BCECF-AM (12 μM) 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 μm) and morphology. The 490/440 flourescent intensity (Fi) ratio data were converted to pH_i values by using the nigericin (12 μm) calibration curve technique as described (18, 19). Over the pH range 6.4–7.6 pH_i, fluorescence ratio varied in a linear fashion with pH_o. Fluorescence intensity was found to exceed background autofluorescence by at least 40-fold.

Total and intrinsic intracellular buffering power. The intrinsic buffering power (βi) was determined as described (20–25) by evaluating the pH_i change induced by the administration and withdrawal of a known amount of base. Giving that the βi is related to pH_i (10, 20–25), we estimated the βi values at different pH_i, by exposing the cells to 30 or 20 mM NH_4Cl in Hepes, Na^+-free buffered solutions and then decreased the NH_4Cl concentration by 5 or 10 mM for each step to 0 mM. The βi was then calculated from the midpoint change in pH_i at each step. βi values were then plotted vs. pH_i using a best fit Enzefitter 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 βi, we also evaluated the βi/pH_i curves from experiments performed with cells preincubated (10 min) and perfused with 200 nM secretin ($n = 8$) or 100 μM DBcAMP ($n = 5$). As in other studies (10, 23) a single exponential function better describes the known βi/pH_i relationships. βi changed from 6.8 mM/pH unit at pH_i 7.5 to 22.1 mM/pH unit at pH_i 7 and to 72.1 mM/pH unit at pH_i 6.5 (controls, $n = 9$) and was not significantly influenced by secretin (6.5, 22.4, and 77.3 mM/pH unit at pH_i 7.5, 7, and 6.5 respectively) or by DBcAMP (7, 21.3, and 70.3 mM/pH unit at pH_i 7.5, 7, and 6.5 respectively) at every pH_i value. The values of βi 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). β_{tot} was calculated from the formula: $\beta_{tot} = \beta i + 2.303 \times (HCO_3^-)_i$ where intracellular (HCO_3^-) 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^+ 140, K^+ 5.9, Mg^{++} 1, Ca^{++} 1.25, Cl^- 142.2, SO_4 = 1, PO_4 = 1.2, Hepes 10, glucose 5, pH 7.4; the HCO_3^--CO_2 buffered solution (KRB) contained (in mM) Na^+ 140, K^+ 5.9, Mg^{++} 1, Ca^{++} 1.25, Cl^- 122.2, SO_4 = 1, PO_4 = 1.2, HCO_3^- 25, glucose 5, pH 7.4. Na^+ free and Cl^- 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^+-free Hepes-buffered solution (pH 7.4). Secretin was made up as a concentrated solution in the appropriate perifusion buffer containing 1% (wt/vol) BSA. The secretin containing solution was then infused (1:60 vol/vol dilution) into the perifusion 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_i 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 perifusion solution to a final concentration of 10 μM (0.1% DMSO in the perifusion solutions). Valinomycin was dissolved in DMSO to a final concentration of 5×10^{-8} 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±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±0.3 μm and 8.3±0.3 μm, 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±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±15.7% were positive (range 46–92%). When BDE cells were cultured (L-15 or α-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±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±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% (Na^+, or Cl^- free solutions, DIDS [1 mM], Amiloride [1 mM] or NPPB [10 μM] when also assessed by the calcein AM/ethidium homodimer method) (13).

pH_i regulation in BDE cells

Basal pH_i. BDE cells, isolated from normal rat liver, maintained a basal pH_i of 7.04±0.06 (n = 30) in a nominally bicarbonate-free, Hepes-buffered media. However basal pH_i was significantly higher (7.16±0.10, n = 44; $P < 0.001$) when BDE cells were cultured and perfused with media supplemented with 25 mM HCO_3^- and gassed with 95% O_2/5% CO_2, suggesting that a HCO_3^- loading mechanism is present. Indeed, when BDE cells in bicarbonate were preincubated with 1 mM DIDS (60–80 min), an inhibitor of HCO_3^- transport, basal pH_i significantly decreased (6.95±0.08, $P < 0.001$, n = 10). As shown in Fig. 2, acute exposure of cells to 1 mM amiloride (inhibitor of Na^+/H^+ exchange) produced a 0.11±0.04 pHU decrease in

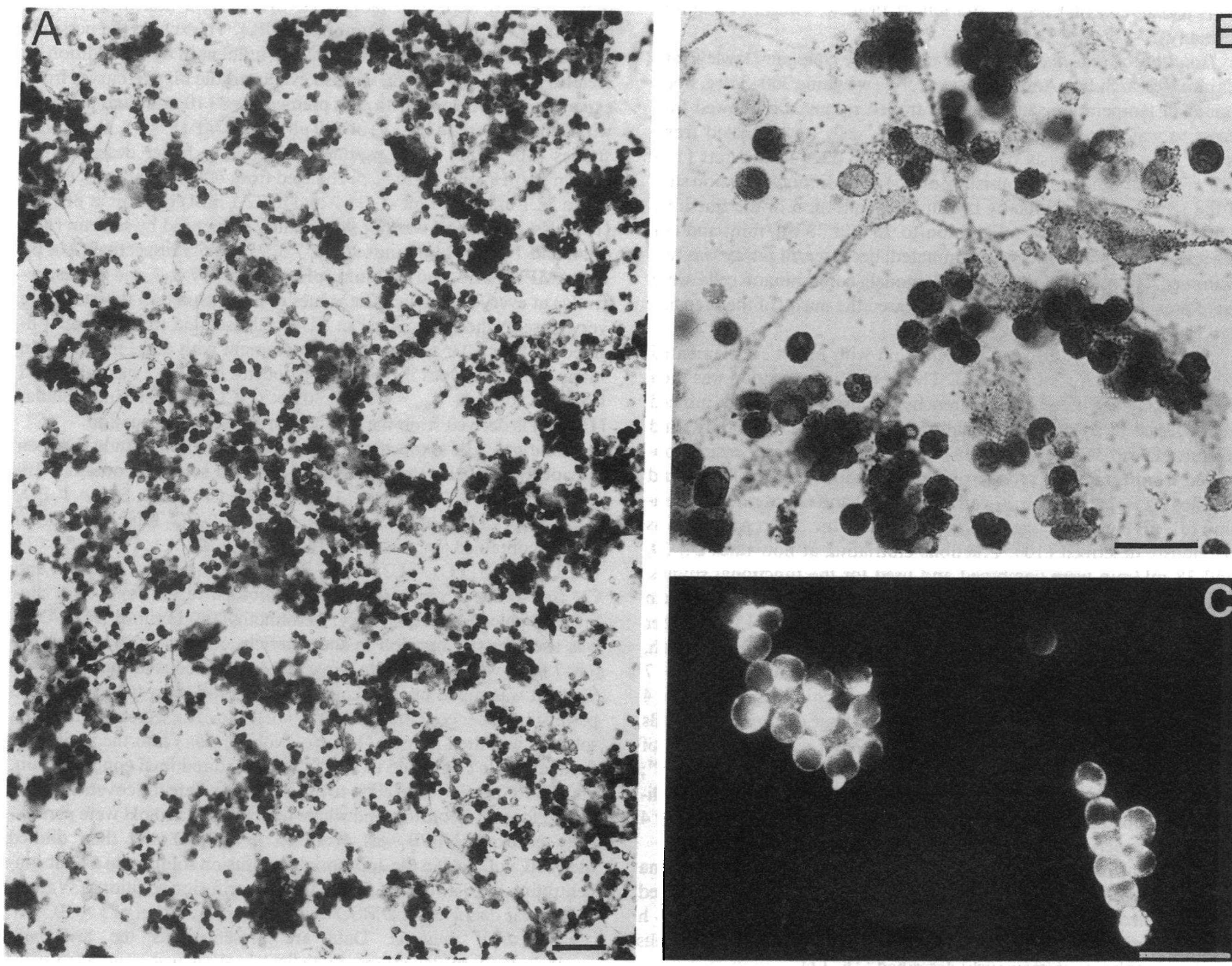

Figure 1. A. GGT cytochemistry of BDE cells cultured overnight (L-15 medium) on Matrigel covered coverslips. Bar, 50 μm. *B.* Clusters of overnight cultured BDE cells, positive for GGT. Bar, 20 μm. *C.* Clusters of freshly isolated BDE cells positive for cytokeratin 19 + 7. Bar, 20 μm.

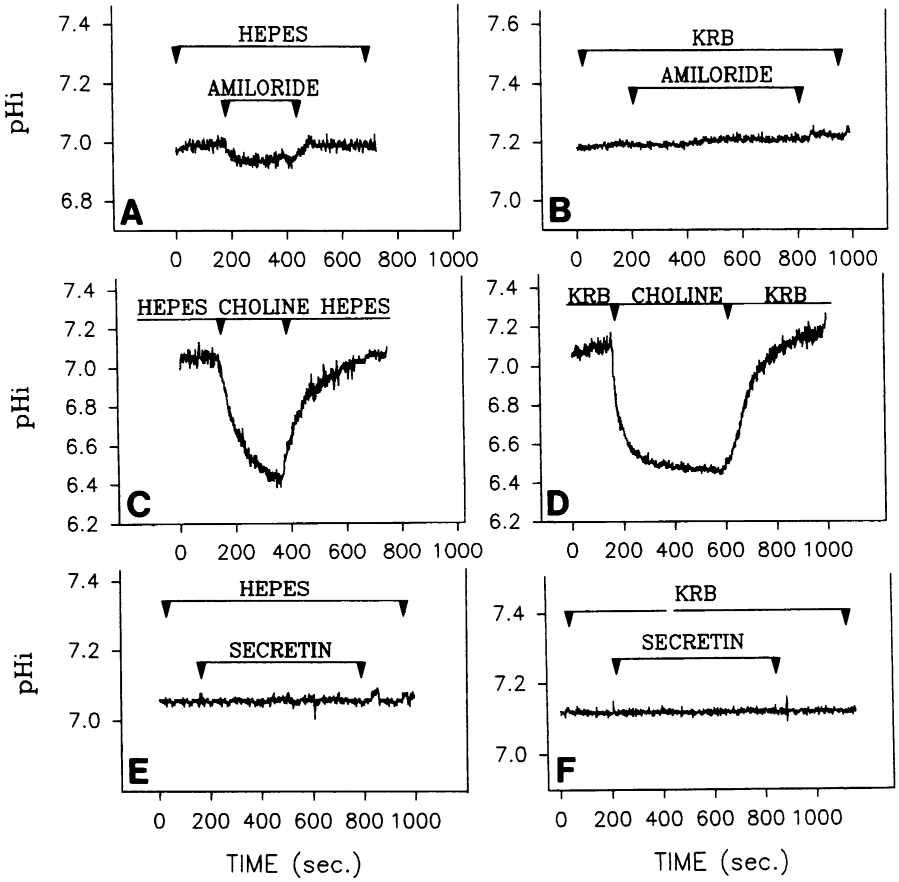

Figure 2. Effect of amiloride, sodium removal, and secretin on basal pH_i. *A.* Cells perfused with HCO_3^--free, Hepes buffered solution were acutely exposed to the Na^+/H^+ inhibitor, amiloride (1 mM; *n* = 4). Basal pH_i decreased and then returned to baseline after amiloride washout. (The tracing 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±0.08 pHU in the absence of bicarbonate (Fig. 2 *C, n* = 5) and by 0.71±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±0.06, *n* = 10) compared with carrier (i.e., albumin) controls (7.05±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±0.09) compared with the carrier controls (7.16±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 α-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±0.08), at a rate of 0.29±0.09 pHU/min at pH_i 6.60±0.04 (jH^+ 16.95±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±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	jH^+
			pHU/min	*mM/min*
Hepes controls (*n* = 10)	7.03±0.05	6.55±0.08	0.29±0.09 (pH_i 6.60)*	16.95±6.08
Hepes amiloride (*n* = 4)	7.02±0.05	6.48±0.04	0.05±0.02 (pH_i 6.51)*	3.75±1.33
Hepes secretin (*n* = 10)	7.02±0.06	6.56±0.07	0.28±0.10 (pH_i 6.61)*	16.78±6.9
Hepes albumin (*n* = 9)	7.05±0.14	6.58±0.07	0.27±0.12 (pH_i 6.63)*	15.30±7.91

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

Table II. Recovery from NH_4Cl Acid Load in KRB

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

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

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

In BDE cells cultured in HCO_3^- enriched media (αMEM) and perfused with bicarbonate buffered solutions (KRB), the pH_i decreased to 6.64±0.07 (n = 11) after NH_4Cl^- administration and withdrawal, and recovered at a rate of 0.204±0.073 pHU/min at pH_i 6.74 (jH^+ 11.60±4.05 mM/min, Table II). Removal of external Na^+ at the moment of NH_4Cl^- withdrawal produced a higher degree of acidification (nadir pH_i 6.42±0.04, n = 4) and the recovery to basal pH_i was completely inhibited, until external Na^+ was readmitted. Amiloride (1 mM) administered at the time of NH_4Cl^- withdrawal decreased the nadir acidification (6.55±0.08, Table II), and significantly ($P < 0.05$) decreased the rate of pH_i recovery to 0.13±0.05 (at pH_i 6.67, jH^+ 7.79±3.01 mM/min) with respect to controls, indicating that ~ 35% of the recovery is driven by the Na^+/H^+ exchanger (amiloride-sensitive component of pH_i recovery). When BDE cells were preincubated (60–80 min) with 1 mM DIDS to inhibit the HCO_3^--dependent transport processes and perfused with amiloride at the moment of NH_4Cl removal (Table II), the nadir acidification was 6.46±0.09 and the recovery was further decreased to 0.053±0.006 pHU/min (jH^+ 4.21±0.52 mM/min at pH_i 6.55). Two transport processes (Na^+ dependent, DIDS inhibitable HCO_3^- transport) that could mediate the amiloride insensitive component of pH_i recovery from an acid load in the presence of HCO_3^-, are a Na^+/HCO_3^- symport and a Na^+ dependent Cl^-/HCO_3^- exchanger. To discriminate between these two transporters, we studied the amiloride insensitive component of pH_i recovery from an acid load in cells (Table II) preincubated for 40 min in a Cl^- free medium (equimolar substitution with gluconate). To exclude the influence of the Na^+/H^+ exchanger, the cells were perfused with 1 mM amiloride at the moment of NH_4^+ withdrawal. In these experimental conditions the basal pH_i was significantly higher ($P < 0.01$) then in the presence of Cl^- (7.35±0.04, n = 5), the nadir acidification was 6.88±0.05 and pH_i recovered from the acid load at a rate of 0.15±0.050 pHU/min (jH^+ 6.92±2.42 mM/min), at the same rate as in the presence of Cl^- and amiloride. This Cl^--independence of the pH_i recovery mechanism excludes a Na^+ dependent Cl^--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 Na^+/H^+ exchanger and the Na^+-HCO_3^- symport.

Effect of secretin on the rate of 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 pH_i recovery from the NH_4Cl^--induced acid load (calculated at the 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 Na^+/H^+ exchanger.

In the presence of bicarbonate, the rate of 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 Na^+/HCO_3^- symport, the same experiments were also performed by superfusing the cell with 1 mM amiloride at the moment of 20 mM NH_4Cl^- withdrawal (Table II and Fig. 3, *E* and *F*). With this protocol, secretin also has no effect on the activity of the Na^+/HCO_3^- symport.

Cl^-/HCO_3^- exchange. Both the net pH_i increase and recovery (δpH_i) and the rate of the pH_i increase and recovery after Cl^- removal and readmission respectively (Fig. 4) were the parameters used to evaluate the activity of the Cl^-/HCO_3^- exchanger as previously described (20–25). After Cl^- removal (Fig. 4 *C*, control experiments) pH_i rose 0.24±0.08 pHU at a

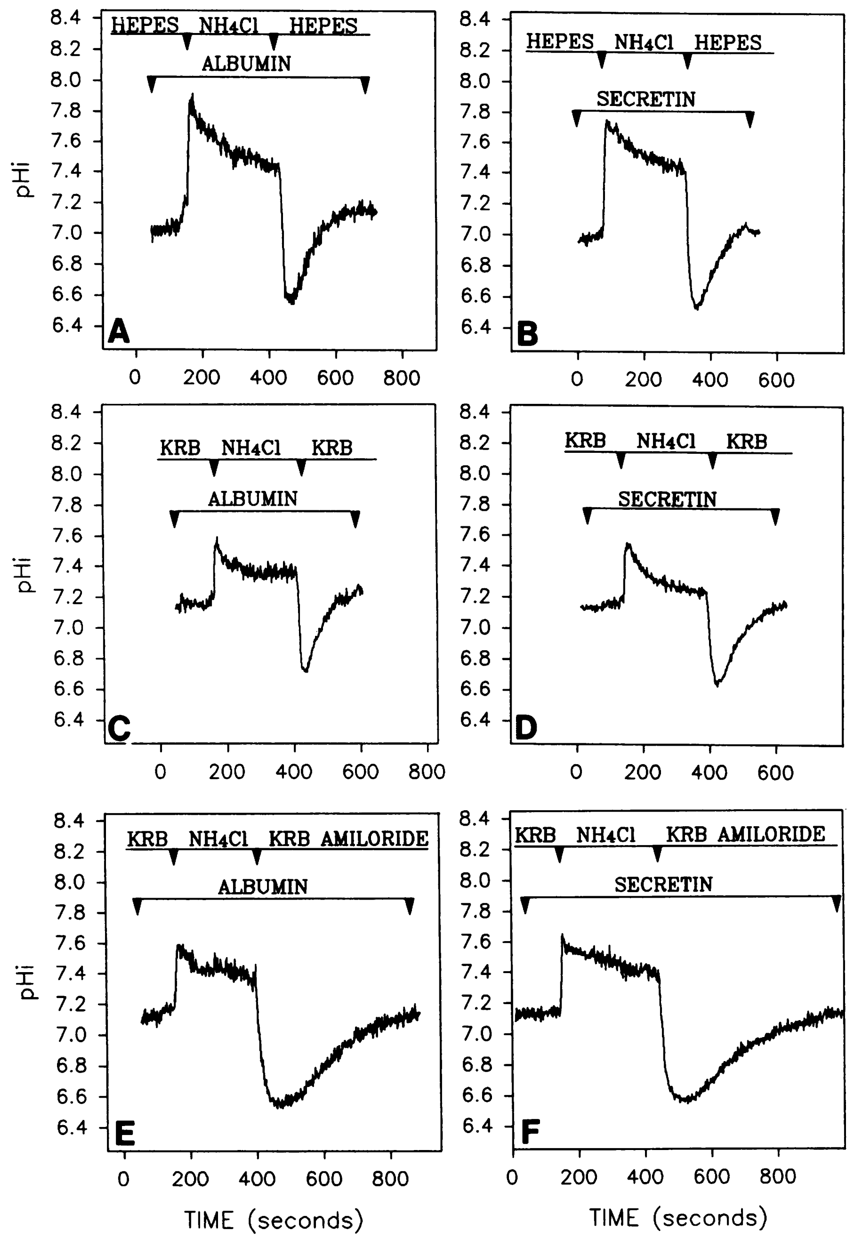

Figure 3. Effect of secretin on the rate of pH_i recovery from acute acid load. *A* and *B*. BDE cells preincubated (10 min) and perfused with 200 nM secretin (*B*, $n = 10$), in absence of bicarbonate (Hepes), showed similar degrees of acidification and rates of pH_i recovery, after 20 mM NH_4Cl withdrawal, 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±0.06 pHU/min (H^+ flux = 6.02±3.27 mM/min, $n = 15$). When Cl^- was readmitted, the cells recovered to baseline at a maximal rate of 0.188±0.080 pHU/min (H^+ flux = 11.82±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 alkalinization was observed after Cl^- removal (0.43±0.41 pHU/min, H^+ flux = 21.13±20.56 mM/min, Fig. 4 *B*) that was significantly higher ($P < 0.012$) then obtained in carrier-control experiments (0.11±0.05 pHU/min, H^+ flux = 5.52±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 Cl^-/HCO_3^- Exchanger

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

The activity of the Cl^-/HCO_3^- exchanger was measured by the net pH_i increase (delta pH_i) promoted by acute Cl^- removal and the rate of both pH_i increase after Cl^- removal and of pH_i recovery after Cl^- readmission in BDE cells cultured and perfused with HCO_3^--enriched media.
* Activity of the Cl^-/HCO_3^- exchanger measured in BDE cells preincubated and perfused with 200 nM secretin (albumin solution) or 100 μM DBcAMP. BDE cells preincubated and perfused with the secretin carrier (i.e., albumin) were used as secretin controls. Data are given as means±SD. ‡ $P < 0.012$ vs. secretin controls. § $P < 0.025$ vs. secretin controls. ‖ $P < 0.04$ vs. DBcAMP controls. ¶ $P < 0.003$ vs. DBcAMP controls. ** $P < 0.005$ vs. DBcAMP controls.

~ 50% of BDE cells responded to secretin by increasing the activity of the Cl^-/HCO_3^- exchanger. pH_i change (i.e., δpH_i) following acute 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 Cl^- was readmitted, secretin-treated cells showed a maximal rate of pH_i recovery (0.43±0.34 pHU/min, H^+ flux = 27.25±21.86 mM/min) that was significantly higher than in control experiments (0.21±0.09 pHU/min, H^+ flux = 12.67±5.66 mM/min, $P < 0.025$). H^+ flux values measured during acute Cl^- removal or Cl^- readmission, showed a significant correlation (Cl^- removal: r = 0.44; $P < 0.015$; Cl^- readmission: r = 0.54; $P < 0.004$) with their correspondent pH_i values in all the secretin experiments (n = 27), as also seen in the control experiments,

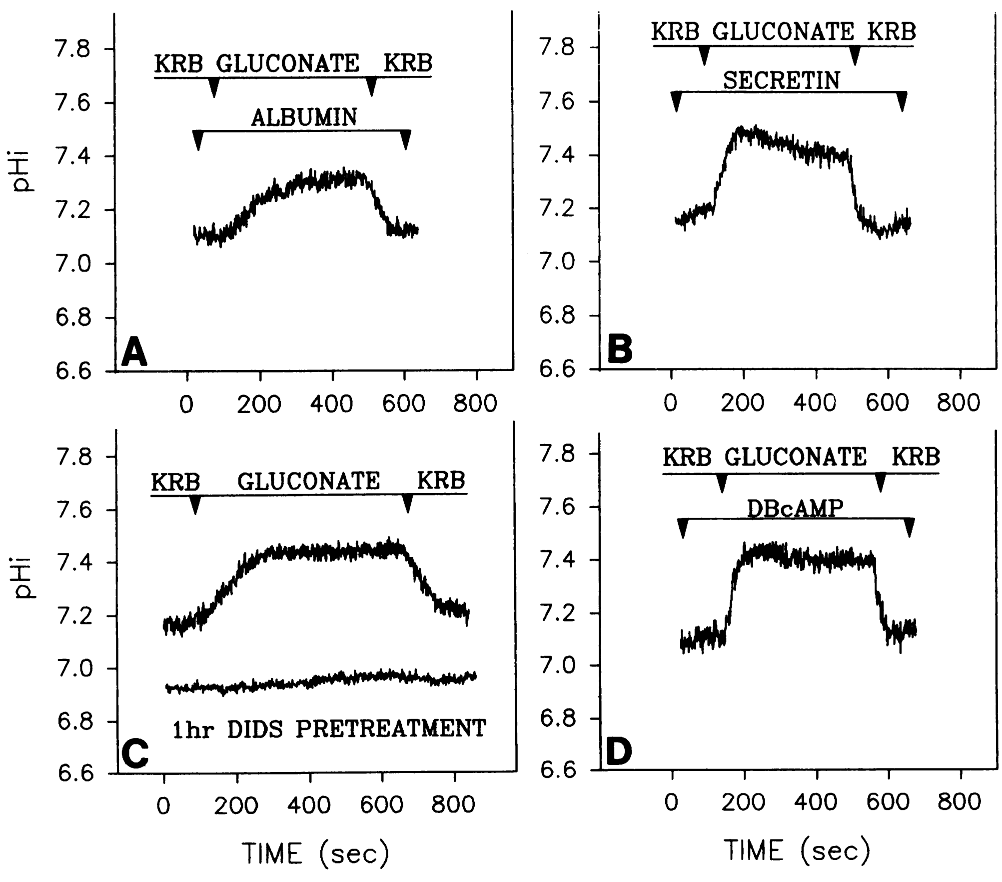

Figure 4. Effects of secretin or DBcAMP on pH_i changes promoted by acute Cl^- removal and readmission. *A* and *B*. BDE cells preincubated and perfused with 200 nM secretin (*B*, n = 15), in presence of HCO_3^- (KRB), showed a maximal rate of alkalinization after Cl^- removal (gluconate) and of pH_i recovery after 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 μM DBcAMP (*D*, n = 11), in presence of HCO_3^- (KRB), showed a maximal rate of alkalinization after Cl^- removal (gluconate) and of pH_i recovery after Cl^- readmission that was significantly higher than those measured in control experiments (*C*, n = 15). In *C* (lower pH_i tracing) 1 mM DIDS pretreatment (60–80 min, n = 6), completely abolished the effect of Cl^- removal in controls, consistent with HCO_3^- transport across the cell membrane.

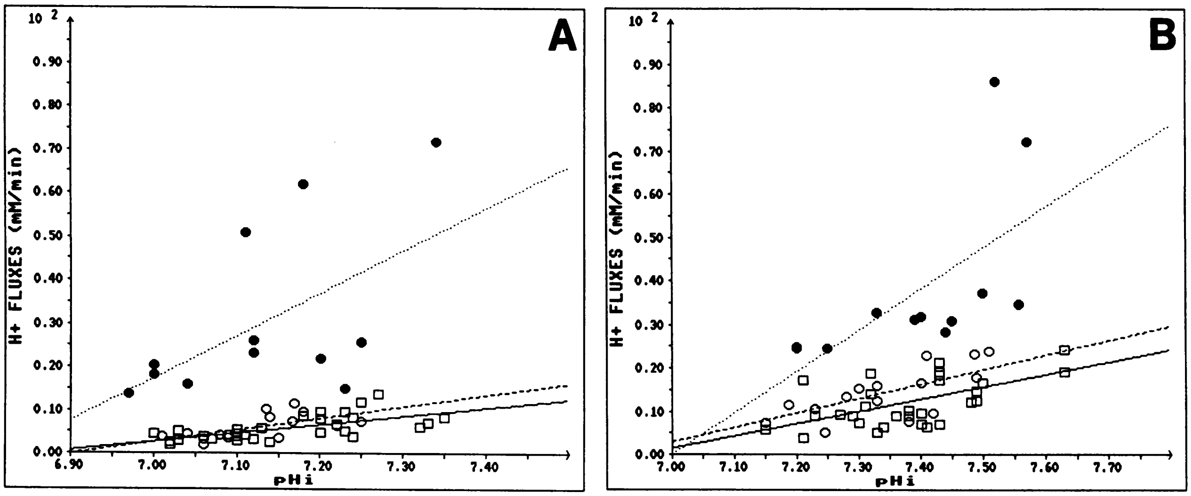

Figure 5. Plots of H^+ flux values measured during acute Cl^- removal (*A*) or Cl^- readmission (*B*) and their correspondent pH_i values. The H^+ fluxes measured in all the control experiments of the present study (squares and —— lines, $n = 30$) following Cl^- removal (*A*) showed a significant direct 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 then 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 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 then 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.

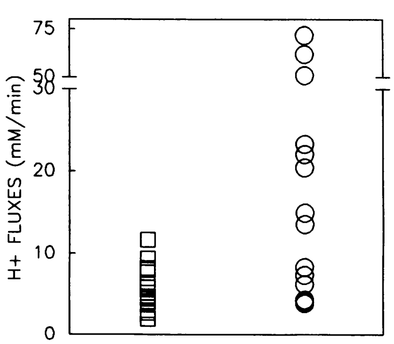

Figure 6. Effect of secretin on the H^+ flux values measured following acute Cl^- removal. BDE cells preincubated and perfused with 200 nM secretin (circles, $n = 15$) showed, following acute Cl^- removal, H^+ flux values (measured as in Methods) significantly higher ($P < 0.012$) than those measured 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 heterogenicity in different populations of BDE cells or that secretin receptors are lost or internalized in some cells during the process of cell isolation.

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 μM), 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 μM DBcAMP, no significant difference in their basal pH_i was observed with respect to controls (7.13±0.07, $n = 19$ vs. 7.16±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 alkalinization after Cl^- removal (0.271±0.131 pHU/min, respectively, H^+ flux = 12.57±6.27 mM/min) and of pH_i recovery after Cl^- readmission (0.538±0.373 pHU/min, H^+ flux = 34.79±26.40 mM/min) were significantly higher ($P < 0.003$ and $P < 0.005$, respectively) in BDE cells preincubated (10 min) and perfused with 100 μM 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±0.06 vs. 0.24±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 μM NPPB (35) significantly decreased ($P < 0.02$) the maximal rate of alkalinization promoted by acute Cl^- removal (0.102±0.059 pHU/min; H^+ flux = 4.92±2.80 mM/min, Fig. 7 *B*) when compared with controls (0.247±0.180; H^+ flux = 11.4±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±0.064 pHU/min; H^+ flux = 9.63±3.98 mM/min) compared with its absence (0.333±0.159 pHU/min; H^+ = 21.83±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±0.10 vs. 0.30±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 μM DBcAMP, superfusion with 10 μM NPPB ($n = 8$) significantly ($P < 0.03$, Fig. 7 *D*) decreased the maximal rate of alkalinization promoted by acute Cl^- removal (0.088±0.036 pHU/min; H^+ flux = 3.95±1.60 mM/min: $n = 8$) when compared with control studies (0.262±0.185 pHU/min; H^+ flux = 12.55±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±0.07 pHU/min; H^+ flux = 10.01±5.3 mM/min) but not in its absence (0.388±0.119 pHU/min; H^+ flux = 27.72±19.37 mM/min: $P < 0.04$). The net pH_i increase following Cl^- removal showed no statistical difference when DBcAMP plus NPPB was perifused than when DBcAMP was present alone (0.23±0.07 vs. 0.28±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	Secretin + NPPB	Secretin	DBcAMP + NPPB	DBcAMP
	n = 9	*n = 12*	*n = 12*	*n = 8*	*n = 8*
Basal pH	7.15±0.07	7.13±0.09	7.12±0.07	7.11±0.05	7.14±0.08
			Chloride removal		
Delta pH	0.22±0.07	0.23±0.10	0.30±0.08	0.23±0.07	0.28±0.09
pHU/min max	0.09±0.03	0.10±0.06	0.25±0.18*	0.09±0.04	0.26±0.18§
H Flux (mM/min)	4.41±1.45	4.92±2.80	11.40±8.12*	3.95±1.60	12.55±9.41§
			Chloride readmission		
pHU/min max	0.17±0.06	0.16±0.06	0.33±0.16‡	0.18±0.07	0.39±0.12‖
H Flux (mM/min)	10.31±3.79	9.63±3.98	21.83±10.20‡	10.01±5.30	27.72±19.37‖

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±SD. * $P < 0.02$ vs. albumin + NPPB or secretin + NPPB (Anova one way). ‡ $P < 0.005$ vs. albumin + NPPB or secretin + NPPB (Anova one way). § $P < 0.03$ vs. DBcAMP + NPPB. ‖ $P < 0.04$ vs. DBcAMP + NPPB.

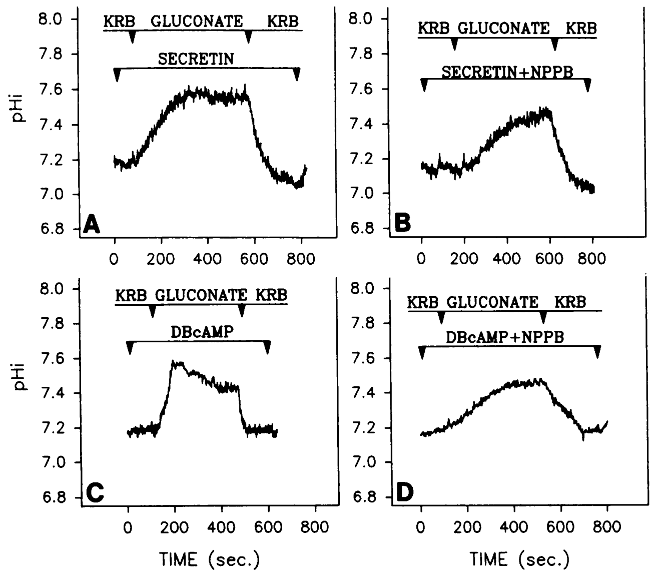

Figure 7. Effect of the Cl^- channel blocker NPPB on secretin and DBcAMP stimulation of Cl^-/HCO_3^- exchange activity. *A* and *B*. BDE cells preincubated and perfused with 200 nM secretin, superfused with 10 μM NPPB (*B*, $n = 12$) showed a maximal rate of pH_i increase following Cl^- removal (gluconate) and of pH_i recovery after 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 μM DBcAMP, superfused with 10 μM NPPB (*D*, $n = 8$) showed a maximal rate of pH_i increase following Cl^- removal (gluconate) and of pH_i recovery after 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 pH_i increases after Cl^- removal and recovers after Cl^- readmission. Alternatively, Na^+/H^+ exchanger could be activated by cell shrinkage resulting from 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 pH_i increase promoted by Cl^- removal, consistent with a primary involvement of the Cl^-/HCO_3^- exchanger. In all cell types studied (20–26, 38, 39), the Cl^-/HCO_3^- exchanger functions as an acid loader driven by the Cl^- gradient. Thus, this exchanger presumably functions as a counterpoint to the acid extruding systems, in regulation of pH_i in BDE cells.

Effect of secretin on pH_i regulation in BDE cells. The major findings in this study relate to the effects of secretin on these pH_i regulatory mechanisms. Secretin had no effect on basal pH_i either in the presence or in the absence of HCO_3^- containing media and had no effect on the rate of 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, Na^+/H^+ exchanger, or Na^+/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 alkalinization as originally proposed. Rather, as shown in this study, secretin stimulates the rate of alkalinization and of pH_i recovery induced by Cl^- removal and readmission, respectively, findings consistent with an increase in the activity of the Cl^-/HCO_3^- exchange mechanism (Fig. 4, Table III). Cl^-/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 pH_i despite the enhanced activity of the Cl^-/HCO_3^- exchanger. Although a decrease in 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 Na^+/H^+ exchange nor the Na^+/HCO_3^- symport were stimulated by secretin when their activity was evaluated during recovery from an acute acid load. However stimulation of the Cl^-/HCO_3^- exchanger by secretin may occur only when pH_i increases above basal values. This would be consistent with the known pH_i dependence of Cl^-/HCO_3^- exchanger activity, which in various cell types (20–27) is activated only at pH_i values above the cell's basal set point. Thus, when the activity of the Na^+/H^+ or Na^+/HCO_3^- symport was evaluated during acid loading, the pH_i was at a lower range where activation of the Cl^-/HCO_3^- by secretin is not possible. Theoretically, the Na^+/H^+ exchanger or the Na^+/HCO_3^- symport could be inhibited by the acid loading, a possibility ruled out for the Na^+/H^+ exchanger, which was activated by lowering pH_i as in other cell types (10, 25). However, in rabbit renal cortex basolateral membrane vesicles, Na^+/HCO_3^- symport is much more active at basal than at lower or higher pH_i values (45). In BDE cells, basal pH_i remained unchanged when cells were perifused in HCO_3^- media with amiloride and secretin, excluding a counter pH_i regulatory response from the Na^+/H^+ exchanger, but not from the Na^+/HCO_3^- symport, which is the major acid extruding mechanism in the BDE cell. Unfortunately attempts to inhibit Na^+/HCO_3^- symport, by omitting Na^+ (choline or *N*-methyl-D-glutamine substitution) lowered pH_i to levels (approx. 6.4) where the Cl^-/HCO_3^- exchanger is inactive and probably unresponsive to any effects of secretin. Raising pH_i artificially above the basal pH_i by perfusing cells in high pH/bicarbonate media might theoretically resolve this issue, but, in the absence of Na^+ no increase of pH_i was observed (data not presented).

The findings of the present study are consistent with HCO_3^- excretion occurring via an apical localized Cl^-/HCO_3^- exchanger coupled to a HCO_3^- entry mechanism at the basolateral domain driven by the Na^+/HCO_3^- symport. This hypothesis is consistent with the mechanism of HCO_3^- excretion previously proposed for rabbit and guinea pig gallbladder epithelium (46, 47). Alternatively, H^+ extrusion via a 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 dicyclohexylcarbodimide (48), a known H^+-ATPase inhibitor.

Effect of DBcAMP on 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 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. [$Tyr^{10,13}$, Phe^{22}, Trp^{25}]secretin) that increases the intracellular levels of cAMP but not IP_3 (12). Since DBcAMP reproduced the effects of secretin and stimulated the activity of Cl^-/HCO_3^- exchanger in rat BDE cells without significant modification of the basal pH_i, it is likely that the activity of the Cl^-/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 HCO_3^- excretion in pancreatic ductular cells using cAMP as second intracellular messenger (28–32, 49). Thus, cAMP appears to stimulate HCO_3^- excretion via Cl^-/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 Cl^-/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 Cl^-/HCO_3^- exchanger. Both cAMP- and Ca^{++}-regulated 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 Cl^- channels which then increases the gradient favoring HCO_3^- excretion via the Cl^-/HCO_3^- exchanger (28–32). These Cl^- channels have been associated (52–54) with expression of the cystic fibrosis transmembrane conductance regulator, whose mutation results in a characteristic defect ion Cl^- channel regulation (52–54). Therefore the inhibition of both the secretin and DBcAMP induced stimulation of Cl^-/HCO_3^- exchange activity by NPPB suggests that their effects are mediated by opening of 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 Cl^-/HCO_3^- exchange is reversed and changes in pH_i now depend on the in-to-out chloride gradient and the intrinsic activity of the exchanger. However, 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 Cl^- concentration and the in-to-out Cl^- gradient after acute Cl^- removal should be the same as in controls. Furthermore, during Cl^- readmission the Cl^- gradient (out to in) should be essentially the same in all our experimental groups independently of the open state of the Cl^- channels. Changes in pH_i during 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 Cl^- channels, but that the Cl^-/HCO_3^- exchanger is also activated as a secondary consequence of the opening of the Cl^- channel. This conclusion, e.g., that there is a link between activation of Cl^- channels and Cl^-/HCO_3^- exchange activity, is based on the known depolarizing effect of Cl^- removal and the effect of such a change in the membrane potential on the activity of the Na^+/HCO_3^- symport. Depolarization activates electrogenic Na^+/HCO_3^- symport in most tissues. This in turn would increase the entrance of HCO_3^- into the cells, activating the HCO_3^-/Cl^- exchanger secondarily at its HCO_3^- or pH sensitive sites as discussed above. That HCO_3^- entry via Na^+/HCO_3^- symport is stimulated electrogenically after 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 alkalinization consistent with electrogenic HCO_3^- entry, presumably via a Na^+/HCO_3^- symport, or HCO_3^- conductive pathway. These findings essentially exclude that the exchanger is stimulated only as a consequence of the increased out-to-in Cl^- gradient generated by Cl^- channel opening as observed in pancreatic duct cells (28–32), since the acute Cl^- removal maneuver should lower the rate of alkalinization instead of enhancing it as observed in our secretin and DBcAMP treated cells. Intracellular Cl^- concentrations would be diminished as a consequence of enhanced Cl^- efflux through the open Cl^- channels. A direct effect of secretin and DBcAMP on Cl^- channels is also supported by recent preliminary studies where patch clamp recordings have identified low conductance Cl^- channels that are activated by secretin in rat BDE cells by cAMP dependent mechanisms (56).

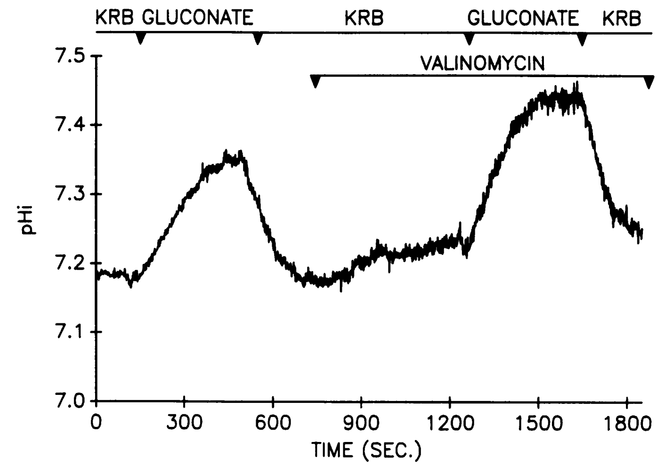

Figure 8. Effect of chloride removal on the rate of increase of pH_i in BDE cells before and after depolarizing the cell with valinomycin (5×10^{-8} M). Note the more rapid alkalinization and higher pHi induced by chloride removal when the cells were depolarized, presumably reflecting stimulation of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

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 Cl^-/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 Cl^-/HCO_3^- exchanger as a secondary consequence of Cl^- channel activation. It is presumed that the Cl^-/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 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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