

Membrane Localization of H⁺ and HCO₃⁻ Transporters in the Rat Pancreatic Duct

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ABSTRACT The pancreatic duct secretes alkaline fluid that is rich in HCO₃⁻ and poor in Cl⁻. The molecular mechanisms that mediate ductal secretion and are responsible for the axial gradients of Cl⁻ and HCO₃⁻ along the ductal tree are not well understood because H⁺ and HCO₃⁻ transport by duct cells have not been characterized or localized. To address these questions, we microdissected the intralobular, main, and common segments of the rat pancreatic duct. H⁺ and HCO₃⁻ transporters were characterized and localized by following intracellular pH while perfusing the bath and the lumen of the ducts. In intralobular ducts, Na⁺-dependent and amiloride-sensitive recovery from acid load in the absence of HCO₃⁻ was used to localize a Na⁺/H⁺ exchanger to the basolateral membrane (BLM). Modification of Cl⁻ gradients across the luminal (LM) and BLM in the presence of HCO₃⁻ showed the presence of Cl⁻/HCO₃⁻ exchangers on both membranes of intralobular duct cells. Measurement of the effect of Cl⁻ on one side of the membrane on the rate and extent of pH_i changes caused by removal and addition of Cl⁻ to the opposite side suggested that both exchangers are present in the same cell. In the presence of HCO₃⁻, intralobular duct cells used three separate mechanisms to extrude H⁺: (a) BLM-located Na⁺/H⁺ exchange, (b) Na⁺-independent vacuolar-type H⁺ pump, and (c) BLM-located, Na⁺-dependent, amiloride-insensitive, and 4',4'-diisothiocyanatostilbene-2,2'-disulfonic acid sensitive mechanism, possibly a Na⁺-dependent HCO₃⁻ transporter.

The main and common segments of the duct displayed similar mechanisms and localization of H⁺ and HCO₃⁻ transporters to the extent studied in the present work. In addition to the transporters found in intralobular ducts, the main and common ducts showed Na⁺/H⁺ exchange activity in the LM. Three tests were used to exclude a significant luminal to basolateral Na⁺ leak as the cause for an apparent luminal Na⁺/H⁺ exchange in an HCO₃⁻ secreting cells: (a) addition of amiloride and removal of Na⁺ from the LM had a profound effect on Na⁺/H⁺ exchange activity on the BLM and vice versa; (b) inhibition of all transporters in the BLM by bathing the duct in the inert hydrocarbon Fluorinert FC-75 did not prevent cytosolic acidification caused by removal of luminal Na⁺; and (c) luminal Na⁺ did not activate the basolateral Na⁺-dependent HCO₃⁻ transporter.

An Na⁺-independent, bafilomycin-sensitive H⁺ pumping activity was marginal in the absence of HCO₃⁻. Exposing all segments of the duct to CO₂/HCO₃⁻ significantly increased the appearance of H⁺ pumping in the plasma membrane. This

effect completely reversed on removal of HCO_3^- from the medium. Stimulation of the ducts with various agonists in the absence of HCO_3^- also induced H^+ pumping in the plasma membrane. All forms of H^+ pump activation were blocked by loading the duct cells with the Ca^{2+} buffer 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA). Direct measurement of $[\text{Ca}^{2+}]_i$ showed that HCO_3^- and all agonists reported to affect pancreatic fluid secretion increased $[\text{Ca}^{2+}]_i$ in microdissected intralobular ducts and the perfused common ducts. The overall studies provide the first detailed characterization and localization of H^+ and HCO_3^- transporters in the pancreatic duct; report the presence of several new, unsuspected transporters in the LM and BLM; and should allow better understanding of fluid and electrolyte secretion by the pancreatic duct.

INTRODUCTION

The exocrine pancreas secretes digestive enzymes and Cl^- -poor, HCO_3^- -rich fluid (Case, 1989; Kuijpers and De Pont, 1987). Most of the fluid is secreted by the ductal tree. The duct also determines the final electrolyte composition of the pancreatic juice (Shultz, 1987; Case and Argent, 1986). Fluid secretion by the duct requires an active vectorial transport of Cl^- and HCO_3^- in opposite directions. Some insight into the molecular mechanisms responsible for secretion by duct cells has been obtained recently. Isolated intralobular ducts in primary culture were used to demonstrate the presence of secretin-regulated Cl^- channels in the luminal membrane (LM) (Gray, Greenwell, and Argent, 1988) and cAMP-regulated maxi K^+ channels in the basolateral membrane (BLM) (Gray, Greenwell, Garton, and Argent, 1990). Nonselective cation channels in the AM and BLM were also found, although their role in fluid secretion has not been determined (Gray and Argent, 1990). Electrophysiological studies on microdissected and perfused intralobular ducts showed the presence of a Ba^{2+} -sensitive K^+ permeability in the BLM, as well as the effect of amiloride on membrane potential when added to the BLM, but not the AM of intralobular duct cells (Novak and Greger, 1988*a*; Novak and Greger, 1988*b*). Neither furosemide nor DIDS added to the BLM had an effect on the electrical properties of duct cells (Novak and Greger, 1988*b*). A microspectrofluorometric technique demonstrated the presence of Na^+/H^+ (Stuenkel, Machen, and Williams, 1988; Veel, Villanger, Holthe, Cragoe, and Raeder, 1992) and $\text{Cl}^-/\text{HCO}_3^-$ (Stuenkel et al., 1988) exchange mechanisms in intralobular ducts, although the membrane location of the transporters was not determined. All the above studies were performed with intralobular ducts. Similar studies on the cellular level with other segments of the duct have not been reported.

Based on the above studies with intralobular ducts from the rat, a model to account for ductal HCO_3^- and fluid secretion was proposed (Gray et al., 1988; Novak and Greger 1988*b*). The model includes a Cl^- channel and a $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the LM, as well as a K^+ channel, Na^+/H^+ exchanger, and Na^+ pump in the BLM. HCO_3^- is secreted by the luminal $\text{Cl}^-/\text{HCO}_3^-$ exchanger in exchange for Cl^- , which then leaves the cells through the AM Cl^- channels. The H^+ generated in the cytosol is extruded through the BLM by a Na^+/H^+ exchanger, and the Na^+ is exchanged for K^+ by the Na^+ pump. A variation of this model has been proposed in which H^+ efflux across the BLM is dominated by a vacuolar-type H^+ pump and not the Na^+/H^+

exchanger (Raeder, 1992). The later model is based on studies showing the presence of acidic vesicles in resting duct cells that disappear on stimulation with secretin (Veel, Buanes, Grotmol, Ostensen, and Raeder, 1991), and the coincidental appearance of BLM foldings when the cells are stimulated (Buanes, Grotmol, Landsverk, and Raeder, 1987; Buanes, Grotmol, Landsverk, Nafstad, and Raeder, 1988).

A general weakness of the proposed models is that H^+ and HCO_3^- transporters in pancreatic duct cells have not been thoroughly characterized, let alone localized to the AM or BLM. In addition, micropuncture studies showed dramatic decrease in luminal Cl^- and a reciprocal increase in luminal HCO_3^- along the ductal tree, (i.e., from intralobular to interlobular to main to the common duct) (Lightwood and Reber, 1977; Reber and Wolf, 1968; Case, Harper, and Scratcherd, 1969). This may indicate that different mechanisms mediate Cl^- absorption and HCO_3^- secretion in the different segments of the ductal tree. Hence, in the present studies, we used microdissected intralobular, main, and common ducts, perfused both luminal and basolateral sides, and measured intracellular pH (pH_i) in the presence and absence of HCO_3^- to characterize and localize the H^+ and HCO_3^- transport mechanism in these segments of the ductal tree. We also studied the CO_2/HCO_3^- and agonist-dependent appearance of H^+ pump activity in the plasma membrane of duct cells and the role of $[Ca^{2+}]_i$ in this activity. This also required studying the effect of various ductal secretagogues, including secretin and bombesin, on $[Ca^{2+}]_i$.

METHODS

Solutions

The solutions used in the present experiments were labeled A–G and had the following composition (in mM): solution A (NaCl, Hepes) contained 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 Hepes (pH 7.4 with NaOH); solution B (Na^+ -free, Hepes) was identical to solution A, except that 140 mM *N*-methyl-D-glucamine (NMG^+) Cl^- replaced 140 mM NaCl and pH was adjusted with Tris; solution C (Cl^- -free, Hepes) contained 140 Na^+ -gluconate (Glu), 5 KGlu, 1 $MgSO_4$, 4.6 Ca^{2+} -cyclamate, 10 glucose, and 10 Hepes (pH 7.4 with NaOH); solution D (NaCl, HCO_3^-) was similar to solution A, except that Hepes was reduced to 2.5 mM, NaCl to 115 mM, and included 25 mM $NaHCO_3$; solution E (Na^+ -free, HCO_3^-) was similar to solution B, except for the reduction of Hepes to 2.5 mM, $NMG Cl$ to 115 mM, and including 25 mM choline- HCO_3^- (this solution also continued 10 μM atropine to inhibit the cholinergic effect of choline [Muallem, Beeker, and Pandol, 1988]); solution F (Cl^- -free, HCO_3^-) was similar to solution C, except that Hepes was 2.5 mM, NaGlu was 115 mM, and $NaHCO_3$ was 25 mM; solution G (NaCl-free, HCO_3^-) contained 115 MNG^+ -Glu, 5 KGlu, 1 $Mg SO_4$, 4.4 Ca^{2+} -cyclamate, 10 glucose, 2.5 Hepes (pH 7.4 with $NMGOH$), 25 choline- HCO_3^- , and 10 μM atropine. All solutions containing HCO_3^- were equilibrated with 5% $CO_2/95\%$ air to pH 7.4 at room temperature. The osmolarity of all solutions was adjusted to 310 mosM with the major salt. Stock solutions of 100 mM amiloride, 100 mM DIDS, 50 mM H_2DIDS , 1 mM bafilomycin A1, 2 mM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF/AM), and 2 mM Fura 2/AM were made in DMSO and stored at $-20^\circ C$.

Microdissection of Pancreatic Ducts

Rats weighing 100–200 g were injected with Nembutal and killed by cervical dislocation. After death, an abdominal incision and a V-shaped cut up to the rib cage was made. The skin was cut and removed. A 2×2 -cm gauze was layered at the bottom of the rib cage. The liver was pulled

over toward the rib cage and the gauze was folded over the liver. The next stage varied depending on the desired preparation. Fig. 1A shows the entire ductal tree that was microdissected from the pancreas and cleared from acini to show the major parts of the ductal tree that were used in the present studies (see also illustrations in Waynforth, 1988).

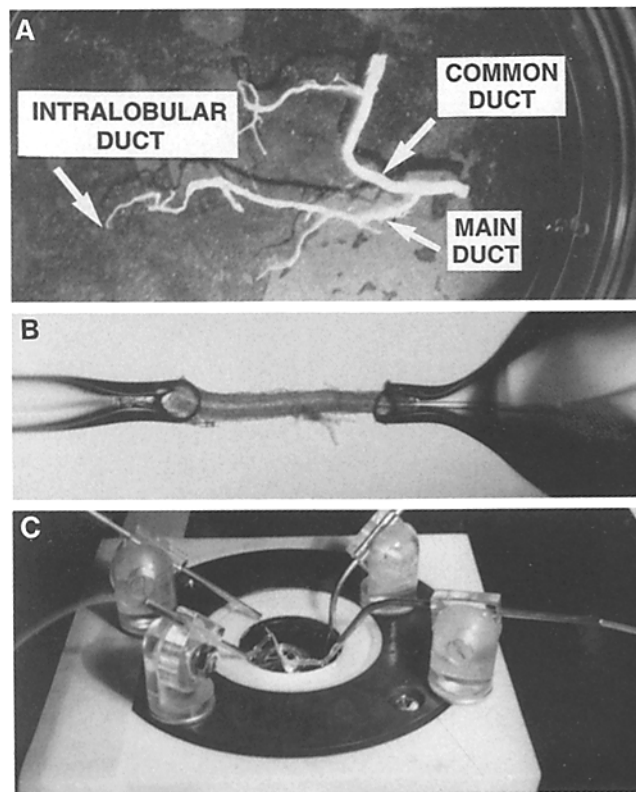


FIGURE 1. The experimental preparations used to localize H^+/HCO_3^- transporters in duct cells. The upper panel shows the ductal tree dissected out of a rat pancreas. The posterior and anterior main ducts are connected to the common duct. It is not possible to clearly see the intralobular portion of the duct at the magnification used ($\sim \times 5$). *B* shows a picture of an in vitro-perfused intralobular duct. The inner perfusion pipette can be seen in the holding pipette and in the lumen. The duct was placed in a perfusion chamber, and the lumen and bath were perfused. The picture shows the dilated lumen of the intralobular duct resulting from the perfusion. The bottom panel shows a portion of the common duct cannulated with a polyethylene tube (*C*, bottom right). The duct was always cannulated next to its entry to the intestine. The duct was held in place by a plastic holder (*C*, bottom left). To avoid blockage of flow, a small incision was made next to the holder. The bath perfusion tubing (*C*, upper right) and the vacuum aspiration outlet (*C*, upper left) are also shown.

To obtain a perfused common duct, the entry of the duct to the intestine was identified, and the duct was cleared from attached acini and as much as possible connective tissue. One or the two entries of the main duct were ligated to prevent leakage during perfusion. A lateral incision as close as possible to the intestine was made, and a cannula was inserted and ligated. The

cannula used to perfuse the main duct (see Fig. 1 C) was prepared from polyethylene tubing with an inner diameter (I.D.) and outer diameter (O.D.) of 0.48 and 0.64 mm, respectively. After securing the cannula, the duct was cut and removed into a dish containing solution A for dye loading through the lumen.

To obtain a perfused main duct, the common duct was cleaned sufficiently to identify the posterior and anterior entries of the main duct. Then the posterior main duct was cleared from acini and connective tissue and cannulated. The anterior main duct was cleared only if the first cannulation attempt failed. After cleaning, a lateral incision was made and a cannula was inserted. The cannula was inserted as far as possible to form a firm seal of the duct around the cannula. In this case, no ligation was necessary. The cannula was made from a 0.28-mm I.D. and 0.61-mm O.D. polyethylene tubing that was pulled to a tip of 100–120 μm I.D. and 220–240 μm O.D. The final shape of the cannula was conical, allowing the seal of the duct around the cannula.

To facilitate identification of the ductal tree so that intralobular ducts could be dissected, the lumen of the duct was filled with solution A containing several drops of food coloring. For this purpose, the entry of the common duct to the intestine was blocked with a hemostat. An incision was made in the common duct just above the entry of the anterior main duct and a cannula was inserted. About 1 ml of colored solution A was injected to the ductal tree. Injection was continued until dye was seen in intralobular ducts when viewed through a dissecting microscope. Similar staining of the vasculature resulted in different dye distribution and revealed a spiral folding of blood vessels around the ductal tree up to the region of intralobular ducts. The injected pancreas was removed to a cold solution A containing soybean trypsin inhibitor, and it was cut into pieces of ~5–10 mm long. One piece at a time was transferred to a dissecting dish containing the above solution maintained at 4°C. Ducts with stained lumen (and Fura 2 fluorescent when Fura 2/AM was used as described below) were dissected with sharpened forceps. The dye remained restricted to the ducts for ~30 min at 4°C, after which it could be clearly seen in acinar structures.

In some experiments, the pancreas was not cut, but the intralobular duct was identified by gross dissection down the colored ductal tree and was then cleared of acini. The middle panel of Fig. 1 shows how the interlobular duct was microperfused using the technique of Burg (1972) developed to study kidney tubules. The intralobular duct was transferred to a bath chamber constructed of black lucite. The duct was gently aspirated into the holding pipette and the inner perfusion pipette was advanced into the duct (Fig. 1 B, right side). The duct was secured in place by gentle aspiration into a second holding pipette (Fig. 1 B, left side). The intralobular duct lumen was perfused at a flow rate of ~50–100 nl/min. The main duct lumen was perfused at a flow rate of ~100 $\mu\text{l}/\text{min}$, and the common duct lumen was perfused at a flow rate of ~0.7 ml/min. In all experiments, the bath was perfused at a flow rate of 10–12 ml/min.

Dye Loading

Preliminary experiments showed that dye loading through the bath should be avoided, particularly with the common and main duct, but also in some segments of intralobular duct. What appeared to be small blood vessels remained attached to some duct segments. The cells in these segments were stained much more intensely than duct cells, especially with BCECF. Furthermore, the connective tissue that remained attached to all duct segments limited dye loading. These problems were solved by loading the dye through the lumen. After establishing luminal and bath perfusion, a small volume of solution A containing BCECF/AM or Fura 2/AM was perfused into the lumen while the bath was continually perfused. The luminal perfusion was stopped for 5–10 min until the fluorescence was between 50 and 200 times above background and then the dyes were washed by perfusing the lumen with solution A.

Since microdissection and perfusion of intralobular ducts were time consuming, to facilitate the $[Ca^{2+}]_i$ measurements, these segments were loaded with Fura 2 by an in situ application of luminal Fura 2/AM. About 1 ml of a solution containing 10 μ M Fura 2/AM was infused into the ductal tree through the common duct. After 10 min incubation at room temperature, the lumen was flushed with 10 ml solution A, and then with solution A containing food coloring. The pancreas was then removed into cold solution A for microdissection of intralobular ducts as described above.

After lumen loading of dyes, the fluorescence appeared uniform. Individual cells of the intralobular and the main ducts could be identified, and in these segments, the fluorescence appeared confined to the layer of duct cells. The thick layer of connective tissue prevented the identification of single cells in the common duct, but the fluorescence was uniform when dyes were loaded through the lumen, whereas areas of high fluorescence intensity appeared when dyes were loaded through the bath.

Measurement of pH_i

BCECF fluorescence was recorded from a stretch of 3–12 cells. No differences were noted in H^+ or HCO_3^- transport based on number of cells used to measure fluorescence. The fluorescence was measured at an emission wavelength of 530 nm with an inverted microscope attached to a dual excitation fluorimeter that provided alternating lights of wavelength 440 and 490 nm as previously described (Muallem, Zhang, Loessberg, and Star, 1992; Star, Zhang, Loessberg, and Muallem, 1992). With the common and main duct, fluorescence was measured with a 40 \times oil immersion objective, whereas with the intralobular duct, either 40 \times or 100 \times oil immersion objectives were used. The emitted light was directed by a dichroic mirror to a photomultiplier tube, and light intensity was measured at a resolution of 2/s. The fluorescence ratios of 490/440 were calibrated intracellularly by perfusing the ducts (lumen and bath) with solutions containing 145 mM KCl, 10 mM Hepes, 5 μ M nigericin, and 10 μ M tri-butyltin with pH adjusted to 6.2–7.6, as previously described (Muallem et al., 1992).

Measurement of $[Ca^{2+}]_i$

Two techniques were used to monitor changes in $[Ca^{2+}]_i$: (a) photon counting with the setup described above for BCECF (all duct segments), and (b) image acquisition and analysis (microdissected intralobular ducts). The recording system used to obtain the fluorescence images was similar to that used for fluorescence intensity measurements, except that the wavelengths of the excitation light were 340 and 380 nm. The emitted light was directed to a video scope camera, and the signal from eight consecutive frames at each wavelength was integrated before storage. The recording resolution was one set of images (340 and 380) every 6 s. The images were analyzed by a Photon Technology International Imagescan Program. The light or images monitored at 340 and 380 nm were used to calculate the fluorescence intensity or image ratio. The ratios were calibrated to obtain $[Ca^{2+}]_i$ as described before (Loessberg, Zhao, Luby-Phelps, Moss, Star, and Muallem, 1993).

RESULTS

Intralobular Ducts

A major portion of fluid and HCO_3^- secretion by the stimulated pancreas is mediated by the intralobular portion of the duct (Case, 1989; Case and Argent, 1986). Therefore, to test the models proposed to explain secretion by the pancreatic duct, we measured pH_i in microdissected and perfused intralobular ducts.

Localization of Na^+/H^+ Exchange

Fig. 2 shows that a Na^+/H^+ exchange mechanism can be found only in the BLM of the intralobular duct. The experiments were performed with solutions buffered with Hepes to minimize contribution of transporters other than the Na^+/H^+ exchanger to pH_i regulation (Muallem and Loessberg, 1990a, 1990b). In Hepes-buffered solutions, steady state pH_i in all segments of the ducts was similar and averaged 7.36 ± 0.02 ($n = 79$). Perfusing the lumen of the intralobular duct with a Na^+ -free solution had no effect on pH_i , whereas removal of Na^+ from the bath reduced pH_i to ~ 6.9 (Fig. 2) (average reduction 0.43 ± 0.03 pH units, $n = 4$). Even when the luminal solution contained Na^+ , removal of Na^+ from the bath caused a similar reduction in pH_i (second portion of Fig. 2). To maximize the rate of H^+ efflux and to detect the presence of other possible acid transporters, the cytosol was further acidified by a transient exposure of the cells to 20 mM NH_4^+ . On removal of NH_4^+ , pH_i was reduced to ~ 6.3 . Addition of Na^+ to the lumen had no effect on pH_i . In contrast,

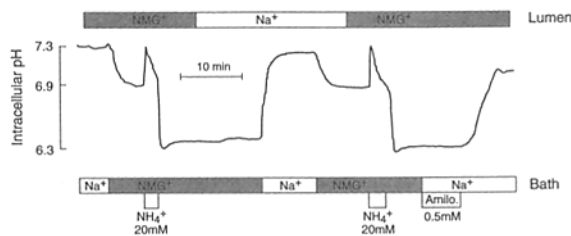


FIGURE 2. Na^+/H^+ exchange in the basolateral membrane of the intralobular pancreatic duct. All solutions used in this experiment were buffered with Hepes. The lumen of the intralobular duct was continuously perfused with solution A (NaCl),

which was also used to perfuse the bath. Where indicated, the luminal and bath solutions were changed to the Na^+ -free solution B (NMG⁺). To acidify the cytosol, the duct was transiently exposed to NH_4^+ by replacing 20 mM NMG⁺ with 20 mM NH_4^+ in the bath-perfusing solution B. To test for the sidedness of Na^+ -dependent H^+ efflux, the Na^+ -free solution B was replaced by perfusing the lumen and then the bath with the Na^+ -containing solution A. After a second acidification by short incubation with NH_4^+ , the duct was perfused with solution A (NaCl) containing 0.5 mM amiloride. Then amiloride was washed by perfusing the bath with solution A (NaCl).

addition of Na^+ to the bath resulted in an immediate and rapid increase in pH_i . The second portion of the Fig. 2 shows that this effect of Na^+ could be completely blocked by 0.5 mM amiloride. Thus, the intralobular duct expressed a Na^+/H^+ exchange mechanism only in the BLM.

Localization of Cl^-/HCO_3^- Exchange in Intralobular Duct

A Cl^-/HCO_3^- exchanger is believed to be the major HCO_3^- efflux mechanism in the luminal membrane (Gray et al., 1990; Novak and Greger, 1988b; Raeder, 1992). This was directly tested by measuring the effect of changing the Cl^- gradient across the LM and/or the BLM on pH_i . Fig. 3A shows that in the absence of HCO_3^- , the sequential removal of Cl^- from the BLM and the LM acidified rather than alkalinized pH_i by ~ 0.1 pH unit. In the presence of HCO_3^- , the removal of Cl^- from the BLM increased pH_i by ~ 0.15 (0.18 ± 0.02 , $n = 5$) pH_i units and subsequent removal of HCO_3^- from the LM increased pH_i to ~ 7.5 (7.53 ± 0.03 , $n = 5$) (Fig. 3B). This

finding was unexpected because all models assume that a $\text{Cl}^-/\text{HCO}_3^-$ exchanger is present only in the LM (Case and Argent, 1986; Gray et al., 1990; Novak and Greger, 1988b; Raeder, 1992; Shultz, 1987). Therefore, we first tested the effect of restoring Cl^- to the BLM on pH_i . Fig. 3 B shows that addition of Cl^- to the BLM resulted in a rapid reduction of pH_i from 7.5 to ~ 7.27 (7.25 ± 0.04 , $n = 5$). Subsequent addition of Cl^- to the LM restored the resting pH_i .

To further characterize the mechanisms responsible for the Cl^- -dependent HCO_3^- transport in the two membranes, we tested the effect of Na^+ and DIDS on these activities. Fig. 4 A shows that incubating the luminal and basolateral membranes with an Na^+ -free, HCO_3^- -containing solution resulted in reduction and stabilization of pH_i at ~ 6.7 . Removal of Cl^- from the BLM and then the LM, and the subsequent addition of Cl^- to the BLM and then the LM, produced the same changes in pH_i seen in the presence of Na^+ (Fig. 3), although at slower rates (Fig. 4 A). This is probably because of the pH_i dependency of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Green,

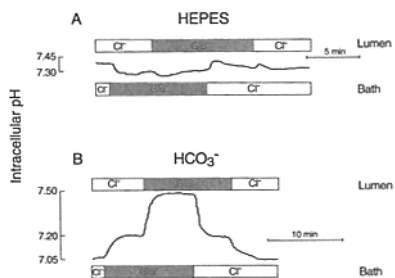


FIGURE 3. $\text{Cl}^-/\text{HCO}_3^-$ exchange in the luminal and basolateral membranes of the intralobular pancreatic duct. The two parts of the experiment were performed with the same intralobular duct, which was continuously perfused through the lumen and the bath. In panel A, all perfusion solutions were buffered with HEPES. The duct was perfused first with solution A

(Cl^-) on both sides. Where indicated, the bath and then the lumen solutions were changed to a Cl^- -free, HEPES-buffered solution C (Glu^-). Finally, the bath and then the lumen were perfused with solution A. In B, all solutions were buffered with HCO_3^- and equilibrated with 5% CO_2 . The HEPES-buffered solution A in the lumen and bath was changed to the HCO_3^- -buffered solution D (Cl^-). After stabilization of pH_i , the bath and then the lumen were perfused with a HCO_3^- -buffered, Cl^- -free solution F (Glu^-). Subsequently, the bath and then the lumen were exposed to Cl^- by perfusion with solution D (Cl^-).

Yamaguchi, Kleeman, and Muallem, 1990; Olnes, Tonnessea, Ludt, and Sandrig, 1987). Fig. 4 B shows that treating the duct with 0.5 mM DIDS for ~ 5 min at 37°C almost completely inhibited the effect of removal of Cl^- from the BLM and the LM on pH_i .

The experiments in Figs. 3 and 4 suggest that an Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism exists in both the LM and the BLM of the intralobular pancreatic duct. Fig. 3 B also suggests that the two exchangers in the opposing membranes affect the activity of each other. This is better illustrated in the experiments presented in Fig. 5. In these experiments, we compared the rate and extent of the pH_i changes caused by removal and addition of Cl^- in the presence and absence of Cl^- in the opposite side. Removal of Cl^- from the luminal side in the presence of Cl^- in the basolateral side slowly increased pH_i from ~ 7.1 to only 7.25. Addition of Cl^- to the LM under these conditions slowly restores the initial pH_i (not shown). Removal of Cl^- from the basolateral side while the lumen was perfused with

a Cl^- -free solution rapidly increased pH_i to ~ 7.55 . Hence, the presence of Cl^- in the BLM attenuated the pH_i increase caused by the removal of Cl^- from the LM. Addition of Cl^- to the lumen in the absence of Cl^- in the BLM rapidly reduced pH_i to ~ 7.25 . However, it is important to note that luminal Cl^- did not restore resting pH_i , and the addition of Cl^- to the bath was needed to reduce pH_i back to ~ 7.1 . In fact, in all experiments with the same protocol ($n = 4$ with intralobular ducts, $n = 3$ with the main duct, and $n = 8$ with the common duct), addition of Cl^- to the lumen only did not restore resting pH_i . This provides strong evidence for the operation of a Cl^-/HCO_3^- exchanger in the BLM of the pancreatic duct.

The second portion of Fig. 5 shows that removal of Cl^- from the BLM in the presence of Cl^- in the LM caused a slow and a small change in pH_i . Removal of Cl^- from the LM in the absence of Cl^- in the BLM resulted in a rapid and large increase

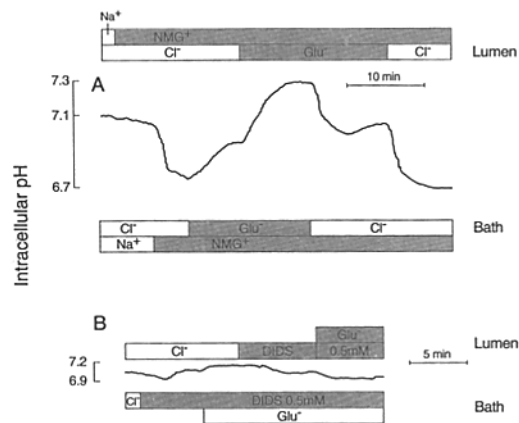


FIGURE 4. Properties of Cl^-/HCO_3^- exchange in intralobular duct. All solutions used in these experiments were buffered with CO_2/HCO_3^- . The experiments in *A* and *B* were performed with two separate intralobular ducts. The control period showed behavior similar to that in Fig. 3 *B*. In *A*, the lumen and bath were perfused with solution D (Na^+). Then Na^+ was sequentially removed from the lumen and the bath by perfusion with the Na^+ -free,

HCO_3^- -buffered solution E (NMG^+). To test the effect of Cl^- , the bath and then the lumen were perfused with the Na^+ - and Cl^- -free, HCO_3^- -buffered solution G (NMG^+/Glu^-). Finally, Cl^- was added to the bath and then the lumen by perfusion with solution E (Na^+ -free, Cl^- -containing). In *B*, the lumen and bath were perfused with solution D. Then the bath was exposed to solution D containing 0.5 mM DIDS, before perfusion with Cl^- -free solution F containing 0.5 mM DIDS. While this solution was maintained in the bath, the lumen was perfused with solution D and then solution F containing 0.5 mM DIDS.

in pH_i . Thus, the presence of Cl^- in the LM attenuated the pH_i changes caused by removal of Cl^- from the BLM. Finally, in the absence of Cl^- in the LM, the addition and removal of Cl^- from the BLM caused rapid and large changes in pH_i , whereas in the absence of Cl^- in the BLM, the addition and removal of Cl^- from the LM caused similar rapid and large changes in pH_i . The combined protocols in Fig. 5 clearly indicate that the exchanger in each membrane influences the activity of the exchanger in the opposite membrane.

Na⁺-dependent and -independent H⁺ Efflux

The experiments in Fig. 2 showed that in HEPES-buffered solutions, an Na^+/H^+ exchanger localized to the BLM dominates H^+ fluxes in intralobular ducts. The

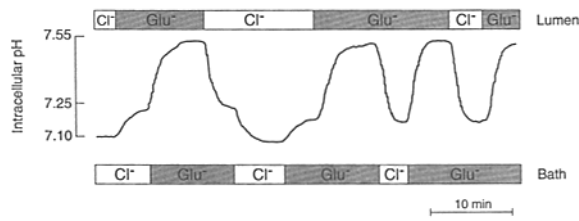


FIGURE 5. Localization of the luminal and basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchangers. All solutions used were buffered with $\text{CO}_2/\text{HCO}_3^-$. Cl^- indicates perfusion with the HCO_3^- -buffered, Cl^- -containing solution D. Glu^- indicates perfusion with the

HCO_3^- -buffered Cl^- -free Solution F. Where indicated, the luminal and bath solutions were changed between solution D and F to determine the effect of luminal Cl^- on bath $\text{Cl}^-/\text{HCO}_3^-$ exchange and bath Cl^- on luminal $\text{Cl}^-/\text{HCO}_3^-$ exchange.

influence of HCO_3^- on this exchanger and other H^+ and HCO_3^- transporters is illustrated in Fig. 6. Exposing the LM and then the BLM to a HCO_3^- -buffered solution resulted in the expected transient acidifications. Since the perfusion rate in each side of the duct was sufficiently high, a transient acidification caused by exposure of the BLM and AM to CO_2 could be observed. This became a convenient procedure to verify the successful perfusion of the luminal membrane of the duct.

In the presence of HCO_3^- , pH_i stabilized at $\sim 7.17 \pm 0.02$ ($n = 23$). Removal of Na^+ from the LM of ducts bathed in HCO_3^- -buffered solutions had no effect on pH_i , whereas removal of Na^+ from the BLM reduced pH_i to ~ 6.8 (Fig. 6). After an acid load with 20 mM NH_4^+ in the absence of Na^+ on both membranes, pH_i was reduced to ~ 6.1 , but then the ductal cells partially recovered pH_i , which increased to ~ 6.4 . This was different from the behavior observed in HEPES-buffered solution, where little Na^+ -independent H^+ efflux was observed. The addition of Na^+ to the LM had no effect on pH_i , while addition of Na^+ to the BLM resulted in a rapid and complete recovery of pH_i .

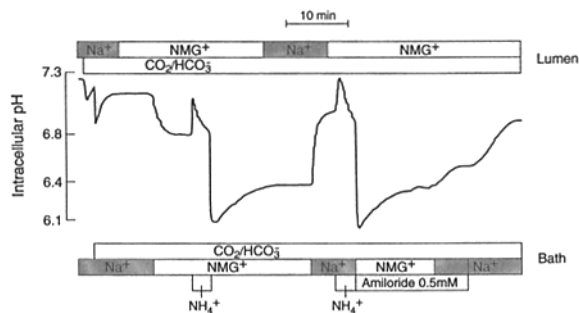


FIGURE 6. Recovery of intralobular duct cells from acid load in the presence of HCO_3^- . An intralobular duct perfused with solution A on both sides was exposed to a HCO_3^- -buffered solution D (lumen and then bath). All subsequent solutions were buffered with $\text{CO}_2/\text{HCO}_3^-$. After stabilization of pH_i , Na^+ was removed from the lumen

and then the bath by perfusion with Na^+ -free solution E (NMG^+). The cells were acidified by replacing 20 mM NMG^+ with 20 mM NH_4^+ in the bath solution. Addition of Na^+ to the lumen of acidified cells was achieved by perfusion with solution D (Na^+). Then, Na^+ was added to the bath by perfusion with solution D. After the second incubation with NH_4^+ , a Na^+ -free solution E containing 0.5 mM amiloride was used to remove NH_4^+ . This solution was replaced with the Na^+ -containing solution D which was also supplemented with 0.5 mM amiloride to demonstrate the Na^+ -dependent, amiloride-insensitive pH_i increase in the presence of HCO_3^- . Finally, the amiloride was removed by perfusing the bath with solution D.

To determine the contribution of the Na^+/H^+ exchanger to the Na^+ -dependent H^+ efflux, the cells were acidified again in the absence of external Na^+ on both sides of the duct, while including 0.5 mM amiloride in the solution used to wash the NH_4^+ . Amiloride did not prevent the partial recovery of pH_i after removal of NH_4^+ , excluding the possibility that contaminating Na^+ in intercellular spaces was responsible for this recovery. On the other hand, amiloride attenuated, but did not completely inhibit, H^+ efflux caused by addition of Na^+ to the BLM. Finally, removal of amiloride resulted in recovery of resting pH_i .

The results in Fig. 6 suggest the existence of Na^+/H^+ exchange and two additional H^+/HCO_3^- transport mechanisms in the presence of HCO_3^- : (a) an Na^+ -independent H^+ efflux, and (b) an Na^+ -dependent, but amiloride-insensitive pH_i increase. The nature of these mechanisms have been studied in the common duct, as demonstrated below. To prevent redundancy, similar characterizations are not shown here for the intralobular duct. However, it is clear that the Na^+ -dependent, amiloride-insensitive mechanism is present exclusively in the BLM of the intralobular pancreatic duct.

MAIN AND COMMON DUCTS

The HCO_3^- and Cl^- content of the fluid in the main and common portions of the duct is very different from that of the intra- and interlobular ducts (Reber and Wolf, 1968; Case and Argent, 1986). Thus, it was of interest to characterize and localize H^+/HCO_3^- transporters in the main and common ducts. Fig. 1 shows the portions of the main and common ducts used for the purpose of the present studies. These two segments are attached to each other and indeed showed identical properties of pH_i regulation to the extent tested in the present studies. Most of the studies were performed with the common duct because it could be dissected, cannulated, and perfused easier than the main duct. However, all H^+/HCO_3^- transporters that were identified in the common duct were demonstrated to exist and behave similarly in the main duct. This includes the BLM-located Na^+/H^+ and Cl^-/HCO_3^- exchangers, the LM-located Cl^-/HCO_3^- exchanger, and the H^+ pump. The most unexpected finding of a Na^+/H^+ exchanger in the luminal membrane is demonstrated for both ducts.

Localization of Na^+/H^+ Exchange

Fig. 7 shows the presence and relationship of the BLM and LM Na^+ -dependent H^+ efflux mechanisms in the main pancreatic duct. In Hepes-buffered solutions, removal of luminal Na^+ had a small effect on pH_i , while removal of bath Na^+ reduced pH_i from 7.38 ± 0.03 ($n = 3$) to 6.83 ± 0.04 ($n = 3$). After exposure to NH_4^+ in the absence of Na^+ on both sides of the duct, pH_i was reduced to ~ 6.3 , and then slightly increased and stabilized at ~ 6.35 . Noticeably, addition of Na^+ to the luminal side rapidly increased pH_i to $\sim 7.05 \pm 0.05$ ($n = 3$). Addition of Na^+ to the bath restored resting pH_i .

The second portion of Fig. 7 shows that removal of Na^+ from the bath in the presence of Na^+ in the lumen had no effect on pH_i . This is very different from the behavior observed in the intralobular duct, where removal of Na^+ from the bath in the presence or absence of luminal Na^+ similarly reduced pH_i (see Fig. 2). Removal

of luminal Na^+ in the absence of Na^+ in the bath resulted in the expected reduction in pH_i . After acidification by incubation with NH_4^+ , addition of Na^+ to the bath increased pH_i to $\sim 7.0 \pm 0.04$ ($n = 3$). However, bath Na^+ did not restore resting pH_i . The addition of Na^+ to the luminal solution was required to further increase and restore resting pH_i . All the Na^+ -dependent changes in pH_i in HEPES-buffered solutions illustrated in Fig. 7 with the main duct were blocked by amiloride (not shown). The amiloride sensitivity will be shown below in the common duct.

Finding a Na^+ -dependent H^+ efflux mechanism in the luminal membrane of a cell secreting HCO_3^- was completely unexpected, especially because no such activity was found in the intralobular segment of the ductal tree. An obvious explanation for this observation is that during duct microdissection and cannulation and/or perfusion, the tight junctions were damaged and became leaky to Na^+ ions. Hence, several protocols were used to demonstrate that separate Na^+ -dependent and amiloride-sensitive H^+ efflux activities exist in the LM and BLM of the main and common

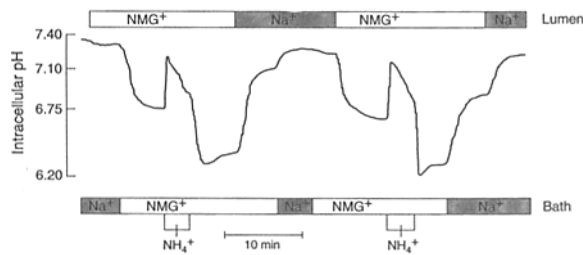


FIGURE 7. Luminal and basolateral Na^+ -dependent H^+ efflux in the main duct. All solutions in these experiments were buffered with HEPES. The lumen and bath of a cannulated main duct were perfused with solution A. Then Na^+ was sequentially removed from the

lumen and the bath by perfusion with the Na^+ -free solution B (NMG^+). The cytosol was further acidified by incubation with 20 mM NH_4^+ . NH_4^+ was removed by perfusion with solution B. After stabilization of pH_i , Na^+ was added to the lumen by replacing solution B with solution A (Na^+). Note the Na^+ -dependent pH_i increase. Subsequently, Na^+ was added to the bath by perfusion with solution A. In the second portion of the experiment, Na^+ was removed first from the bath and then from the lumen by perfusion with solution B (NMG^+). After exposure to NH_4^+ , Na^+ was added to the bath and then to the lumen by perfusion with solution A (Na^+).

ducts. The first of these protocols involved comparing the effect of Na^+ removal and inhibition of the Na^+/H^+ exchanger with amiloride in one membrane on the Na^+/H^+ exchange in the opposite membrane. The results of these protocols are recorded in Fig. 8. All the experiments in this figure were performed with HEPES-buffered solutions. Fig. 8A shows that in the presence of luminal Na^+ , the removal of Na^+ from the bath resulted in a small reduction in pH_i that recovered upon adding Na^+ back to the bath. However, in the absence of luminal Na^+ , the removal of Na^+ from the bath rapidly and markedly acidified the cytosol. Addition of Na^+ to the bath only partially recovered pH_i , and addition of Na^+ to the lumen further increased pH_i . The subsequent portion of Fig. 8A shows that adding amiloride to the lumen to inhibit the Na^+/H^+ exchanger in this membrane was sufficient to expose a cytosolic acidification on removal of Na^+ from the bath. Removal of amiloride from the Na^+ -containing luminal solution in the absence of bath Na^+ resulted in an increase in pH_i . Furthermore, in the presence of bath Na^+ and amiloride, removal of Na^+ from

the lumen was sufficient to trigger cytosolic acidification. Fig. 8 B shows the complementary protocol in which removal of bath Na^+ was required before removal of luminal Na^+ can cause cytosolic acidification, and that the recovery of pH_i of acidified cytosol upon addition of Na^+ to the lumen could be blocked by amiloride.

A second approach to demonstrating the presence of a luminal Na^+/H^+ exchange was to replace the bath solution with the inert fluorinated hydrocarbon Fluorinert FC-75 to prevent all transport events in this membrane (Fig. 9). This procedure was successfully used in localizing transporters in the kidney tubules (Strange and Spring, 1987; Star, 1990). In the control period (not shown), we verified that removal of Na^+ from the lumen in the presence of Na^+ in the bath had only a small effect on pH_i , similar to that in Fig. 8. Fig. 9 shows that when the bath solution was replaced with Fluorinert FC-75, removal of luminal Na^+ resulted in cytosolic acidification that was reversed on readdition of Na^+ . The effect was also observed when the luminal solutions contained HCO_3^- and equilibrated with CO_2 .

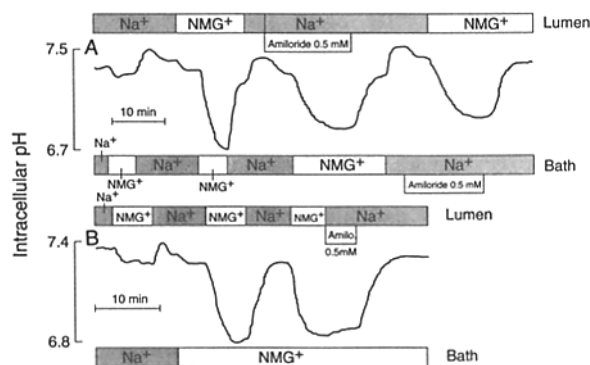


FIGURE 8. Localization of luminal and basolateral Na^+/H^+ exchangers in the common duct. The two experiments were performed with separate ducts and were designed to show the effect of Na^+ and/or amiloride in one side on the Na^+/H^+ exchange activity in the other side. Thus, all solutions were buffered with HEPES and were either solution A (Na^+) or Na^+ -free solution B (NMG^+).

When included, the concentration of amiloride was 0.5 mM. Note that removal of Na^+ from both sides was required to acidify the cytosol; addition of amiloride to lumen or bath was equivalent to removal of Na^+ from lumen or bath, respectively, and that addition of amiloride to bath did not prevent acidification caused by removal of Na^+ from lumen. Finally, addition of amiloride to the lumen in the absence of Na^+ in bath inhibited the luminal Na^+ -dependent alkalization (B).

Na^+ -dependent and -independent H^+/HCO_3^- Fluxes in the Main and Common Ducts

Additional evidence for luminal Na^+/H^+ exchanger in the common duct was obtained when we studied H^+/HCO_3^- transport in the presence of HCO_3^- . Fig. 10 shows that removal of Na^+ from one side of the membrane had a minimal effect on pH_i of ducts perfused with HCO_3^- -buffered solutions. Removal of Na^+ from the LM or BLM in the absence of Na^+ in the opposite side resulted in a similar rapid acidification by $\sim 0.47 \pm 0.02$ ($n = 7$) pH unit. After acidification by incubation with NH_4^+ in the presence of HCO_3^- , the cells partially recovered pH_i , which initially acidified to ~ 6.25 and then stabilized at 6.56 ± 0.03 ($n = 32$). Fig. 10 A shows that perfusing the luminal side with a solution containing Na^+ and 0.5 mM amiloride had minimal effect on pH_i . Addition of amiloride to the Na^+ -free bath solution did not

affect pH_i . However, removal of amiloride from the lumen, while still present in the bath at a concentration sufficient to maximally inhibit BLM Na^+/H^+ exchange in the presence of Na^+ resulted in cytosolic alkalization. Hence, this experiment shows that an amiloride-sensitive Na^+ -dependent H^+ efflux is present, and it is the only mechanism of H^+ efflux in the LM.

The influence of HCO_3^- on $\text{H}^+/\text{HCO}_3^-$ transport in the BLM is shown in Fig. 10 B. In the absence of luminal Na^+ , addition of a solution containing Na^+ , amiloride and H_2DIDS only slightly increased pH_i . Removal of H_2DIDS in the continuous presence of amiloride resulted in a pH_i increase close to the resting level. This

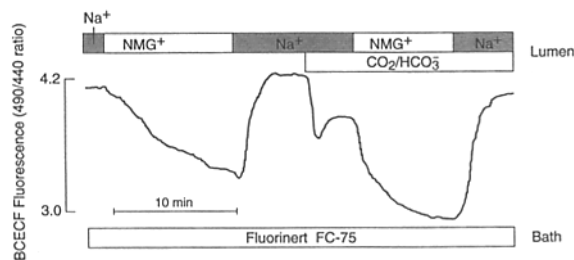


FIGURE 9. Na^+/H^+ exchange in the lumen of the common duct during inhibition of basolateral transporters. For the purpose of these experiments, the common duct was cannulated on both sides before removal from the animal. Hence, the luminal solution was not

allowed to exit the lumen into the perfusion chamber to prevent mixing with the Fluorinert. Rather, the solution flowing through the outlet tube was directed to waste. The height of the draining tube was adjusted to maintain the same flow rate as that normally achieved with the duct cannulated only on one side. The two sides-cannulated common duct was perfused with HEPES-buffered solution A on both sides and then exposed sequentially to an Na^+ -free solution B on the luminal side and then the basolateral side, and showed the pH_i changes in the first part of Fig. 8. Thus, cell pH did not acidify after removal of Na^+ from the lumen only. After perfusing both membranes with solution A and restoration of stable pH_i , the bath solution was aspirated while maintaining continuous perfusion of the lumen. About 1 ml of Fluorinert FC-75 was added to the bath. After ~2 min, the Fluorinert was removed by aspiration and replaced with fresh 1 ml Fluorinert. Duct cells bathed in Fluorinert maintained stable pH_i , which was followed for ~16 min before the first solution change. While bathed in Fluorinert, the lumen of the duct was perfused with a HEPES-buffered Na^+ -free solution B (NMG⁺) and then with solution A (Na^+). Subsequently, the lumen was perfused with $\text{CO}_2/\text{HCO}_3^-$ -buffered solution D (Na^+ -containing). After stabilization of pH_i , the lumen was perfused with the HCO_3^- -buffered, Na^+ -free solution E (NMG⁺) and finally with solution D. All the experiments were performed at room temperature, since temperature control of the perfusion chamber was achieved by controlling the temperature of the bath perfusion solution.

experiment was possible because the inhibitory effect of H_2DIDS is reversible (Wenzl, Sjaastad, Weintraub, and Machen, 1989). Hence, unlike the case in the luminal membrane (Figure 10 A), amiloride alone did not prevent cytosolic alkalization of previously acidified cytosol. This activity was observed in the presence of HCO_3^- , and it was blocked by 0.4 mM H_2DIDS (Fig. 10 B) and by 15–20 min of preincubation with 0.5 mM DIDS (not shown).

$\text{Cl}^-/\text{HCO}_3^-$ Exchange

The protocols used to demonstrate the presence of a $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism on both membranes of intralobular duct cells were used to show the presence

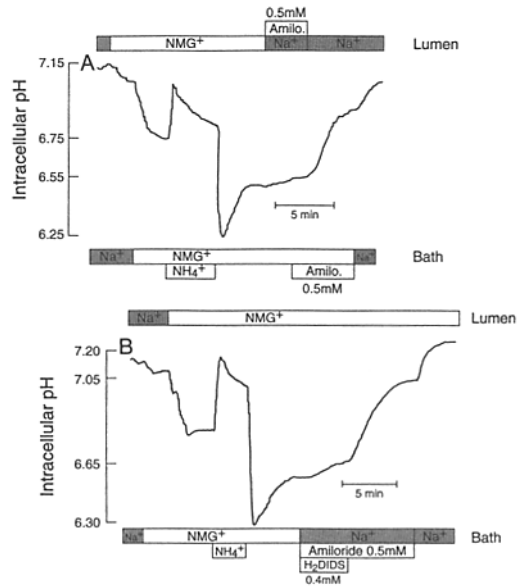


FIGURE 10. Localization of Na^+ -dependent HCO_3^- transport in the basolateral membrane. The two experiments were performed with the same duct. All solutions in both experiments were buffered with HCO_3^- . In *A*, after acidification by replacing solution D with solution A in Na^+ -free medium (NMG^+), the lumen was perfused with solution D (Na^+) containing 0.5 mM amiloride. Then the bath was perfused with solution E (NMG^+) containing 0.5 mM amiloride before removing the amiloride from the lumen. This experiment also shows that there was no significant leak of Na^+ from the lumen to the bath and all

effects of luminal Na^+ were blocked by amiloride. In *B*, after the acidification in Na^+ -free solution E (NMG^+), the bath was exposed to solution D (Na^+) that also contained 0.5 mM amiloride and 0.4 mM H_2DIDS . Removal of H_2DIDS by perfusing the bath with solution D containing amiloride resulted in pH_i increase. Finally, the bath was perfused with solution D.

and examine the behavior of the exchangers in the perfused main and common ducts. Fig. 11 shows the results of these experiments. The major findings are similar to those made with the intralobular duct. Thus, removal of Cl^- increased pH_i only in the presence but not absence of HCO_3^- (not shown). Removal of Cl^- from the lumen and then the bath (Fig. 11 *A*), or bath and then the lumen, (Fig. 11 *B*) was required

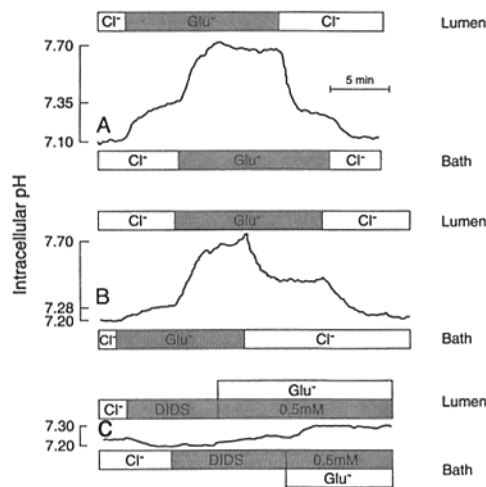


FIGURE 11. Cl^-/HCO_3^- exchange in the luminal and basolateral membranes of the common duct. All solutions were buffered with HCO_3^- and all experiments were performed with the same common duct. The protocol was the same as that in Fig. 5, and it entailed perfusing the luminal and basolateral membranes with solution D (Cl^-) or Cl^- -free solution F (Glu^-). The sequence of Cl^- removal and addition to the two sides was alternated in *A* and *B*. In *C*, the luminal and then the basolateral membranes were treated with 0.5 mM DIDS in solution D before exposure to a DISD-containing, Cl^- -free solution F.

for maximal cytosolic alkalinization. Addition of Cl^- to the lumen in the absence of Cl^- in the bath (Fig. 11 A) or to the bath in the absence of Cl^- in the lumen (Fig. 11 B), was not sufficient to restore resting pH_i . All the changes in pH_i caused by changes in cellular Cl^- gradient were blocked by DIDS (Fig. 11 C), and they could be observed in the absence of luminal and bath Na^+ (not shown).

H^+ PUMPING

Regulation of H^+ Pumping: Role of $\text{CO}_2/\text{HCO}_3^-$

While studying the effect of HCO_3^- on the Na^+ and Cl^- -dependent acid/base transporters, we noticed that exposing cells of intralobular ducts, the main or common ducts to $\text{CO}_2/\text{HCO}_3^-$, invariably and significantly increased the Na^+ -independent recovery from acid load. In many experiments, removal of Na^+ from both membranes was followed by rapid cellular acidification and subsequent partial recovery of pH_i . This is illustrated in Fig. 12. In addition, in the absence of Na^+ and

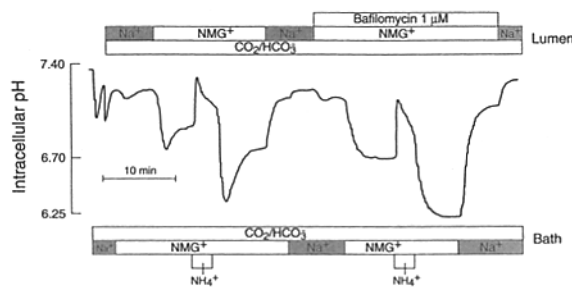


FIGURE 12. Inhibition of Na^+ -independent pH_i recovery from acid load by bafilomycin. In the control period, the bath and then the lumen of the common duct were perfused with a HCO_3^- -buffered solution D (Na^+) and then with Na^+ -free solution E (NMG^+). After incubation with NH_4^+ , the bath was

perfused with Na^+ -free solution E, which resulted in cytosolic acidification to pH_i of ~ 6.25 and recovery to pH_i of ~ 6.7 . The lumen and bath were then perfused with solution D (Na^+). After stabilization of pH_i , the lumen was perfused with Na^+ -free solution E (NMG^+) containing $1 \mu\text{M}$ bafilomycin. After ~ 5 min incubation with bafilomycin, the bath solution was changed to solution E. Incubation and removal of 20 mM NH_4^+ in the absence of luminal and bath Na^+ resulted in stable acidification. Finally, the bath and the lumen were perfused with solution D.

the presence of HCO_3^- , the cells partially recovered pH_i after acidification by exposure to NH_4^+ . Previous studies suggested that a vacuolar-type H^+ pump may be present in pancreatic intralobular duct cells (Veel et al., 1991, 1992; Raeder, 1992). Therefore, we tested the effect of bafilomycin A on this Na^+ -independent activity. The second portion of Fig. 12 shows that including $1 \mu\text{M}$ bafilomycin in the luminal membrane completely inhibited all Na^+ -independent H^+ efflux ($n = 3$). Bafilomycin was as effective when included in the bath solution ($n = 2$).

The HCO_3^- -induced appearance of H^+ pumping in the plasma membrane raised the question if this process is dynamic and can be reversed. This was tested in the experiment shown in Fig. 13. After equilibration in HCO_3^- -containing solutions and demonstrating Na^+ -independent H^+ efflux, HCO_3^- was removed from the lumen and the bath by perfusing with Hepes-buffered solutions. Removal of $\text{CO}_2/\text{HCO}_3^-$ caused pH_i to increase to ~ 7.7 , which was then stabilized at ~ 7.35 . After removal of Na^+ from both membranes and acidification by incubation with NH_4^+ , the duct cells

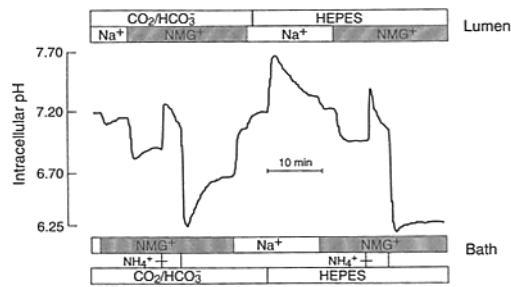


FIGURE 13. Reversal of CO_2/HCO_3^- -dependent H^+ pump activation. In the control period, perfusing the bath and the lumen with HCO_3^- -buffered solutions D (Na^+) and E (NMG^+) and transient incubation with 20 mM NH_4^+ were used to demonstrate Na^+ -independent H^+ efflux. Then, the

lumen and bath solutions were changed to a HEPES-buffered solution A (*Hepes*, Na^+). pH_i increased because of removal of CO_2 . After stabilization of pH_i , the same test of H^+ pump activity was used, except that HEPES-buffered solutions A (Na^+) and B (NMG^+) were used.

failed to show Na^+ -independent H^+ efflux. It is thus clear that incubating the duct in HEPES-buffered solutions for ~ 20 min (the time required to complete the protocol of transferring the duct from HCO_3^- to HEPES and acidifying the cytosol in the absence of Na^+) was sufficient to reverse the effect of CO_2/HCO_3^- on H^+ pumping.

Regulation of H^+ Pumping: Role of $[Ca^{2+}]_i$

It was previously reported that an increase in cell Ca^{2+} inhibited and delayed activation of H^+ efflux across the BLM of the outer medullary collecting duct (Hayes and Alpern, 1991). This effect could be prevented by loading the cells with BAPTA (Hayes and Alpern, 1991). Therefore, we attempted to determine whether the same applies to the pancreatic duct. Fig. 14 shows the effect of BAPTA loading on appearance of H^+ pumping in the pancreatic duct. In the control period, transferring the cells from HEPES to HCO_3^- -buffered solutions resulted in Na^+ -independent H^+ efflux, as shown in the first portion of Fig. 14. After stabilization of pH_i , the luminal solution was supplemented with 10 μM BAPTA/AM to load the duct cells with BAPTA. Such BAPTA loading completely prevented the CO_2/HCO_3^- -dependent appearance of H^+ pumping in the plasma membrane.

Fluid and electrolyte secretion by the pancreatic duct is stimulated by the cAMP stimulating agonist secretin (Ashton, Argent, and Green, 1991) and by the Ca^{2+}

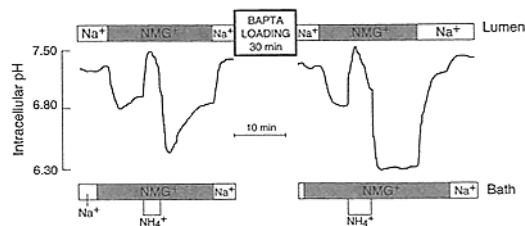


FIGURE 14. Inhibition of CO_2/HCO_3^- -dependent H^+ pump activation by BAPTA. All solutions were buffered with HCO_3^- , and both parts of the experiment were performed with the same duct. In the control period, H^+ pump activity was

demonstrated by following recovery from acid load in the absence of Na^+ . Then the lumen was perfused with solution D ($NaCl$, HCO_3^-) containing 10 μM BAPTA/AM. After 2 min, lumen perfusion was halted while bath perfusion at 37°C continued uninterrupted. After 30 min of incubation with BAPTA/AM, the lumen was perfused with solution D for ≥ 5 min before any solution change. Finally, incubation in Na^+ -free solution E (NMG^+) and exposure to NH_4^+ was used to measure Na^+ -independent H^+ efflux as in the control period.

mobilizing agonist carbachol (Stuenkel and Hootman, 1990). Previous studies suggested that secretin stimulates the fusion of H⁺ pumps containing intracellular vesicles with the plasma membrane (Veel et al., 1991). Therefore, in the next set of experiments, we determined the effect of the agonists on H⁺ pumping in HEPES-buffered solutions and the role of [Ca²⁺]_i in these effects. Fig. 15 confirms that in HEPES-buffered solutions, duct cells do not have plasma membrane H⁺ pumping activity. Stimulation of ducts maintained in HEPES-buffered solutions with secretin (trace b₁) or carbachol (trace c₁) before acidification with NH₄⁺ led to the appearance of H⁺ pumping in the plasma membrane. When the same ducts were loaded with BAPTA, both secretin (trace b₂) and carbachol (trace c₂) failed to induce H⁺ pumping in the plasma membrane.

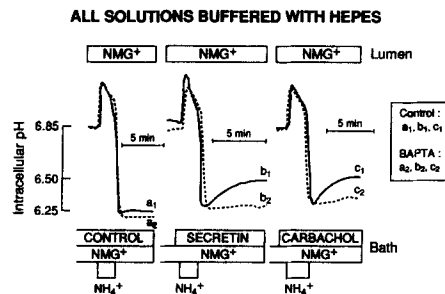
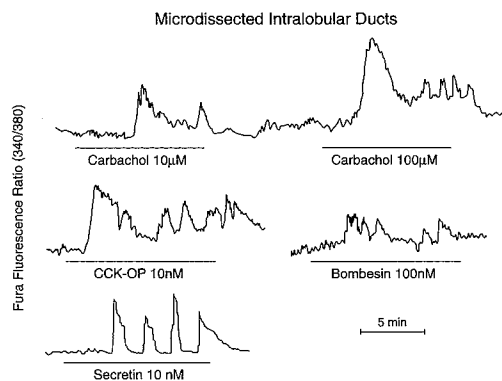


FIGURE 15. Inhibition of agonist-dependent H⁺ pump activation by BAPTA. All solutions were buffered with HEPES and the three experiments were performed with separate ducts. Each figure shows only the portion of the experiment that tested for H⁺ pump activity. The general protocol was to perfuse the lumen and then the bath with a Na⁺-free solution

B (NMG⁺) and then to expose the bath to solution B, where 20 mM NH₄⁺ replaced 20 mM NMG⁺. The recovery of pH_i after removal of NH₄⁺ with solution B (NMG⁺) was used to estimate H⁺ pump activity. After the control period, the lumen and bath were perfused with solution A and the cells were loaded with BAPTA as described in the legend to Fig. 14. After completion of BAPTA loading, H⁺ pump activity was measured in HEPES-buffered solutions as in the control period. Experiments a₁ and a₂ are H⁺ pump activity before (solid line) and after (dashed line) BAPTA loading. In experiments b₁ and b₂, the duct was stimulated with 10 nM secretin for ~10 min before completion of the solution changes needed for measurement of H⁺ pumping. The agonist was removed before BAPTA loading, and the cells were stimulated again with the same concentration of secretin before measurement of H⁺ pump activity in BAPTA-loaded cells. In experiments c₁ and c₂, where indicated, the duct was stimulated with 0.1 mM carbachol before measurement of H⁺ pump activity.

EFFECT OF HCO₃⁻ AND AGONISTS ON [Ca²⁺]_i

The inhibition of HCO₃⁻ and agonist-mediated H⁺ pump induction by BAPTA suggested that agonists capable of stimulating fluid and electrolyte secretion can also increase [Ca²⁺]_i of common duct cells. Previous studies showed that carbachol (Stuenkel and Hootman, 1990; Evans, Ashton, Elliott, Green and Argent, 1991) and cholecystokinin (CCK) (Smith, Yelamarty, Kramer, and Cheung, 1993) increase [Ca²⁺]_i of the cells in microdissected intralobular ducts. In the present studies, we confirm and extended these findings. Fig. 16 shows that in addition to carbachol and CCK, bombesin and secretin also increase [Ca²⁺]_i of cells in microdissected intralobular ducts. For these experiments, high agonist concentrations were needed to stimulate [Ca²⁺]_i changes that were oscillatory in nature. [Ca²⁺]_i oscillations are



ducts was not perfused. The bath was perfused with solution A (Hepes-buffered NaCl). Where indicated, the ducts were stimulated with 10 and 100 μ M carbachol, 10 nM CCK8, 100 nM bombesin, or 10 nM secretin.

usually seen at low agonist concentrations (Berridge, 1993). At present, it is not clear why microdissected ducts respond as if the agonists do not have free access to their receptors.

The effect of HCO_3^- and agonists on $[Ca^{2+}]_i$ of cells in the common duct or any perfused duct has not been examined before. To verify that $[Ca^{2+}]_i$ changes may be important in modulating H^+ pumping and fluid secretion of these cells, continuously perfused common ducts were used to measure the effect of HCO_3^- and agonists on $[Ca^{2+}]_i$. Fig. 17 shows that all agonists capable of increasing $[Ca^{2+}]_i$ in the intralobular duct stimulated a $[Ca^{2+}]_i$ increase in cells of the common duct. Figure 17 (bottom) shows that exposing the cells to CO_2/HCO_3^- also causes a transient increase in $[Ca^{2+}]_i$, further implicating a $[Ca^{2+}]_i$ change as the mediator in H^+ pump appearance

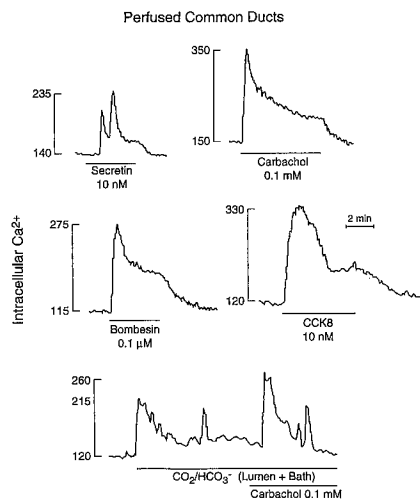


FIGURE 17. Effect of CO_2/HCO_3^- and agonists on $[Ca^{2+}]_i$ of perfused common ducts. All experiments were performed in separate ducts. The lumen and bath were perfused with solution A (Hepes-buffered NaCl). Where indicated, the ducts were stimulated with 10 nM secretin, 0.1 mM carbachol, 0.1 μ M bombesin, or 10 nM CCK8. To test the effect of CO_2/HCO_3^- on $[Ca^{2+}]_i$, the lumen and bath perfusates were changed from Hepes to CO_2/HCO_3^- -buffered solutions (bottom). After ~ 10 min incubation and while still in CO_2/HCO_3^- , the duct was stimulated with 0.1 mM carbachol. Carbachol was added to the bath solution. Similar effects were observed in at least three other experiments.

in the plasma membrane. Interestingly, the $\text{CO}_2/\text{HCO}_3^-$ -induced change in $[\text{Ca}^{2+}]_i$ did not prevent the ability of carbachol to cause a further increase in $[\text{Ca}^{2+}]_i$. The relationship between HCO_3^- and agonist-dependent $[\text{Ca}^{2+}]_i$ increases, and H^+ pump activation needs to be explored extensively in future studies.

DISCUSSION

The bulk of pancreatic fluid and electrolyte secretion is elaborated by the pancreatic ductal system. This process is poorly understood mainly because few studies addressed the nature and membrane localization of the transporters mediating the secretion on the cellular level. The limited studies reported to date used only intralobular ducts. However, it is clear that other portions of the ductal tree must determine the final electrolyte composition of the pancreatic juice because the Cl^- and HCO_3^- content of this fluid is dramatically different when sampled from the intralobular and the main duct (Case and Argent, 1986). Furthermore, the models proposed to explain fluid and electrolyte secretion in intralobular duct (Gray et al., 1988; Novak and Greger, 1988b; Raeder, 1992) are unsatisfactory because they cannot explain several features of fluid secretion. For example, the $\text{Cl}^-/\text{HCO}_3^-$ exchange and the Cl^- shunt pathway provide a mechanism for HCO_3^- secretion but not for net Cl^- absorption. It is doubtful that a $\text{Cl}^-/\text{HCO}_3^-$ exchanger can establish the final concentration of Cl^- and HCO_3^- found in the pancreatic juice. In the rat, the species used in the present studies, the alkaline pancreatic juice contains ~ 70 mM Cl^- and 80 mM HCO_3^- (Mangos and McSherry, 1971). In other species, such as the guinea pig and man (Padfield, Garner, and Case, 1989; Case and Argent, 1989), HCO_3^- can be as high as 140 mM and Cl^- can be as low as 20 mM. To establish these gradients across the LM of the rat duct by $\text{Cl}^-/\text{HCO}_3^-$ exchange, steady state pH_i during secretion should be ~ 7.7 or internal Cl^- below 10 mM. In other species, the situation is even more problematic. When tested (Green et al., 1990; Kurtz and Golchini, 1987), HCO_3^- was found to be a competitive inhibitor with external Cl^- , and it should stop the exchange before the final concentrations of Cl^- and HCO_3^- in the rat pancreatic juice can be achieved. Hence, it seems necessary to reexamine the mechanism of ductal fluid and electrolyte secretion.

The $\text{H}^+/\text{HCO}_3^-$ Transporters

Ion substitution and blocker sensitivities were used to demonstrate the presence of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange, Na^+ -dependent HCO_3^- transport, and a vacuolar-type H^+ pump in the plasma membrane of duct cells. These transporters were found in all segments of the duct examined, i.e., intralobular, main, and common parts of the ductal tree. Hence, in the absence of HCO_3^- , recovery from acid load required external Na^+ , and it was inhibited by amiloride. These criteria were used before (Muallem and Loessberg, 1990; Stuenkel et al., 1987) and in the present studies to indicate Na^+/H^+ exchange activity. Another H^+ transporter that was observed only in the presence of HCO_3^- is the H^+ pump that was characterized as Na^+ -independent and bafilomycin blockable cytosolic alkalinization in acid loaded cells (Figure 12).

Na^+ -independent, DIDS-sensitive, pH_i changes resulting from modification of cellular Cl^- gradient were used to demonstrate $\text{Cl}^-/\text{HCO}_3^-$ exchange in duct cells.

The exchangers in duct cells required HCO_3^- and did not accept OH^- , as was found for the Cl^-/HCO_3^- exchanger in pancreatic acinar cells (Muallem and Loessberg, 1990). This finding can explain why fluid secretion by isolated intralobular ducts required the presence of HCO_3^- (Ashton, Argent, and Green, 1990). An Na^+ -dependent HCO_3^- transport with properties similar to those found here, which include Na^+ -dependent, amiloride insensitivity, and DIDS sensitivity was reported before in several cell types (Boron and Boulpaep, 1983; Alpern, 1985; Wenzl et al., 1989; Muallem and Loessberg, 1990), and it was attributed to a Na^+ coupled HCO_3^- transport. It is possible that similar coupled cotransporters exist in intralobular (Fig. 6) and common ducts (Fig. 10). However, further studies are needed to exclusively identify this activity.

Localization of the Transporters

A constant finding of the present studies was the BLM localization of the newly described Na^+ -dependent HCO_3^- transporter in all segments of the duct. The best evidence for a BLM localization of this transporter was obtained with the intralobular duct because this segment lacked any apparent Na^+ -dependent H^+ or HCO_3^- transporter in the LM (Fig. 6). It was thus possible to show that 0.5 mM amiloride completely blocked recovery from acid load in the absence (Fig. 2), but not in the presence (Fig. 6), of HCO_3^- . In the case of the main and common ducts (Fig. 10), it was necessary to rely on the extent of inhibition by amiloride of the Na^+ -dependent alkalization of acidified cytosol to conclude that Na^+ -dependent HCO_3^- transport exists in the BLM, but not the LM.

In the presence of HCO_3^- , all segments of the duct demonstrated H^+ pump activity. However, we could not determine the sidedness of the pump because bafilomycin was equally effective in blocking the pump when added to the lumen or the bath. Previous studies have shown that stimulation of the ducts with secretin led to disappearance of acid-containing vesicles from the cytoplasm (Veel et al., 1991) and a concomitant large increase in the BLM surface area (Buanes et al., 1987, 1988). This was taken to suggest a BLM localization of the H^+ pump. Although this is expected to be the case for a HCO_3^- -secreting epithelia, direct evidence for BLM localization of the H^+ pump is still required.

Although Cl^-/HCO_3^- exchange activity has been demonstrated in the intralobular duct (Stuenkel et al., 1988), its sidedness has not been determined. The present studies are the first to localize this activity and to show that both membranes of duct cells have a Cl^-/HCO_3^- exchanger. While it was not unexpected to find a luminal Cl^-/HCO_3^- exchange, we did not expect to find an exchange activity in the BLM. Because the pancreatic duct is a Cl^- -absorbing epithelia and most Cl^- transport is transcellular (Case and Argent, 1989), the transepithelial Cl^- permeability is low. Indeed, in unstimulated ducts, the contribution of Cl^- conductance to LM and transepithelial potentials is marginal (Novak and Greger, 1988a, 1991). This makes it unlikely that a Cl^- leak from the lumen to bath or from bath to lumen was responsible for the apparent Cl^-/HCO_3^- exchange in the BLM. Furthermore, removal of Cl^- from the bath only always increased pH_i , and addition of Cl^- to the lumen in the absence of Cl^- in the bath never reduced pH_i to resting levels (Figs. 3–5 and 11).

$\text{Cl}^-/\text{HCO}_3^-$ exchange in the luminal and BLM was found in all segments of the duct. Furthermore, the experiment in Fig. 5 using intralobular ducts, and similar experiments performed with the main and common ducts (not shown), suggest that the two exchangers exist in the same or in $\text{H}^+/\text{HCO}_3^-$ -coupled neighboring cells. In the case of the intralobular and main ducts, single cells could be identified and used for single-cell recording to localize the exchangers to the same cell. However, duct cells are relatively small and closely packed, and BCECF has a high quantum yield so that it was not possible to exclude the contribution of neighboring, uncoupled cells to the recorded fluorescence. A complementary approach to single-cell recording was to determine the rate and extent of alkalinization caused by the sequential removal of Cl^- from the two membranes. If the BLM and LM exchangers were present on separate uncoupled cells, the sequence of Cl^- removal should have no effect on the extent of the pH_i increase. Only if the exchangers exist in the same or coupled cells and affect the activity of each other should the sequence of Cl^- removal determine the extent of alkalinization. Indeed, removal of Cl^- from each side had a markedly different effect, depending on whether Cl^- was present or absent from the other side of the duct (Fig. 5).

An Na^+/H^+ exchange activity in intralobular ducts has been reported (Stuenkel et al., 1988; Veel et al., 1992). Furthermore, addition of amiloride to the BLM, but not the LM, depolarized the membrane potential of intralobular duct cells (Novak and Greger, 1988a). This was interpreted as inhibition of a BLM-located Na^+/H^+ exchanger that acidified the cell, and as a consequence inhibited K^+ channels (Novak and Greger, 1988a). In the present studies, we provide direct evidence for the BLM localization of the Na^+/H^+ exchanger in intralobular duct. We also show the presence of the changer in the BLM of the main and common duct cells. In addition, we did not find any Na^+ -dependent H^+ or HCO_3^- transport in the luminal membrane of the intralobular duct.

A completely unexpected finding was an Na^+/H^+ exchange activity in the luminal membrane of the main and common ducts, but not in intralobular ducts. An apical Na^+/H^+ exchanger seems out of place in a portion of the duct where HCO_3^- concentration is the highest during stimulation of fluid secretion. Because most transductal Na^+ transport is believed to be paracellular, and the tight junctions of duct cells are permeable to Na^+ (Case and Scratcherd, 1974; Kuijpers, De Pont, and Westerhoff, 1989), a possible explanation for an apparent luminal Na^+/H^+ exchange that had to be considered is high luminal to basolateral Na^+ leak. Hence, we sought to develop independent evidence to verify the presence of a luminal Na^+/H^+ exchange activity. The first approach was to prevent Na^+ from reaching the BLM and to inhibit all transporters at this membrane by bathing the duct in Fluorinert FC-75. Fluorinert FC-75 also blocks any fluid flow from the LM to the BLM. Bathing the duct in Fluorinert F-75 did not prevent the acidification and subsequent alkalinization in the presence or absence of HCO_3^- , initiated by removal and addition of luminal Na^+ , respectively.

The second approach was to show that addition of amiloride to the LM was equivalent to Na^+ removal from the lumen (Fig. 8A). Thus, removal of Na^+ from the LM always resulted in a small cytosolic acidification. If the LM has no Na^+/H^+ exchange, this can be the case if bath Na^+ poorly accesses the BLM. This does not

appear to be the case because in the absence of luminal Na^+ , removal and addition of Na^+ to the bath solution resulted in rapid and large pH_i changes. Addition of amiloride to the luminal solution acidified the cytosol similar to removal of luminal Na^+ in the presence of bath Na^+ . If the luminal membrane has no Na^+/H^+ exchange, this is possible only if amiloride was freely permeable through the tight junctions and reached a concentration of 0.5 mM at the BLM in the face of continuous perfusion with an amiloride-free solution. In this case, removal of Na^+ from the bath in the continuous presence of amiloride in the lumen should have resulted with slow and small cytosolic acidification. This was not the case. In addition, if the Na^+/H^+ exchanger was present only in the BLM, then including amiloride only in the bath solution should have been sufficient to acidify the cytosol similar to removal of Na^+ from the BLM while including Na^+ and amiloride in the LM. Addition of amiloride only to the bath acidified the cytosol to the same extent as the addition of amiloride only to the lumen.

The last evidence for a luminal Na^+/H^+ exchange came from testing the effect of HCO_3^- on recovery from acid load (Fig. 10A). In these experiments, addition of Na^+ and amiloride to the lumen had no effect on pH_i . If the tight junctions were permeable to Na^+ , then pH_i should have increased because of Na^+ -dependent HCO_3^- transport present in the BLM and not inhibitable by addition of amiloride to this membrane (see Fig. 10B). Finally, including amiloride in the Na^+ -free bath solution did not prevent the recovery from acid load upon removal of amiloride from the Na^+ -containing luminal solution.

The evidence in Figs. 8–10 forces us to conclude that the luminal membrane of the main and common ducts has an amiloride-sensitive, Na^+ -dependent H^+ efflux, and thus, Na^+/H^+ exchange activity.

Regulation of H^+ Pumping

Exposure of all ductal segments to solutions containing CO_2/HCO_3^- always resulted in appearance in the plasma membrane of a H^+ pump activity. These findings are reminiscent of those reported in the turtle urinary bladder (Gluck, Cannon, and Al-Awqati, 1982; Cannon, van Adelsberg, Kelly, and Al-Awqati, 1985; van Adelsberg and Al-Awqati, 1986) and the proximal and collecting tubules of the kidney (Schwartz and Al-Awqati, 1985), where exposure of the cells to solutions containing CO_2/HCO_3^- led to insertion of H^+ pumps in the plasma membrane. In the kidney and turtle bladder, the pumps were inserted in the luminal membrane. If H^+ pump insertion in the plasma membrane was responsible for the appearance of H^+ pump in the pancreatic duct, it is more likely that the H^+ pumps were inserted in the BLM. Since the duct is a HCO_3^- -secreting epithelia, a BLM insertion will be of physiological significance (Raeder, 1992). Furthermore, this can explain the appearance of foldings in the BLM with stimulation of ductal secretion (Buanes et al., 1987, 1988).

Stimulation of the ducts with secretin and carbachol also increased H^+ pumping. A common property for all mode of stimulation is that they could be prevented by loading the ducts with BAPTA to buffer any changes in $[Ca^{2+}]_i$. In this respect, the insertion of H^+ pumps by CO_2/HCO_3^- in the turtle bladder was preceded by an increase in $[Ca^{2+}]_i$, and could be prevented by loading the cytosol with Ca^{2+} chelators (Cannon et al., 1985; van Adelsberg and Al-Awqati, 1986). Thus, it is possible that all

forms of H^+ pump insertion in the pancreatic duct are mediated by an increase in $[Ca^{2+}]_i$. Further support for this notion was obtained when we found that CO_2/HCO_3^- and all agonists known to affect pancreatic (Gardner and Jensen, 1986; Hootman and Williams, 1987) or ductal secretion (Case and Argent, 1989) increased $[Ca^{2+}]_i$ in intralobular, main, and common ducts. This includes an effect of secretin and bombesin on $[Ca^{2+}]_i$. Previous studies reported the effect of carbachol (Stuenkel and Hootman, 1990; Evans et al., 1991) and CCK (Smith et al., 1993) on $[Ca^{2+}]_i$ of intralobular duct cells. However, an effect of secretin and bombesin could not be demonstrated (Stuenkel and Hootman, 1990; Evans et al., 1991). The reason for the differences in the two studies are not clear. Lack of luminal perfusion cannot be the reason because the agonist similarly affected $[Ca^{2+}]_i$ of luminally perfused (Fig. 17) and nonperfused (Fig. 16) ducts. However, previous studies were performed with isolated duct cells maintained in culture for 1 wk (Stuenkel and Hootman, 1990) or with intralobular duct segments maintained in culture for 24–48 h (Ashton et al., 1991). In both cases, the ducts were subjected for some time to collagenase digestion. This may have affected the $[Ca^{2+}]_i$ signaling.

Additional conclusions from our findings is that pancreatic ductal secretion may be simultaneously regulated by $[Ca^{2+}]_i$ and cAMP. Secretin is a major secretagogue of ductal fluid and electrolyte secretion (Case and Argent, 1989; Raeder, 1992). It increases cellular cAMP (Case, Johnson, Scratcherd, and Sherratt, 1972; Arkle, Lee, Cullen, and Argent, 1986), as well as the activity of the cAMP activates Cl^- channels (Gray et al., 1988; Gray, Plant, and Argent, 1993) in the luminal membrane, which is essential for ductal secretion (Kopelman, Durie, Gaskin, Weizman, and Forstner, 1985; Ashton et al., 1991). On the other hand, the present studies show that secretin also stimulates an increase in $[Ca^{2+}]_i$ that may be in the form of $[Ca^{2+}]_i$ oscillations (Fig. 16). The changes in $[Ca^{2+}]_i$ may stimulate insertion of H^+ pumps in the BLM. Studies *in vivo* suggest that H^+ pump-mediated H^+ efflux is required for ductal fluid secretion (Grotmol, Buanes, and Raeder, 1986; Veel et al., 1992). Thus, the two signaling systems activated by secretin may be used to control events in separate membranes, Cl^- channels in the LM (cAMP) and H^+ pumping in the BLM (Ca^{2+}).

Physiological Significance

Three transporters localized to the BLM can participate in H^+ efflux (Na^+/H^+ exchanger and H^+ pump) or HCO_3^- influx (Na^+ -dependent HCO_3^- transport) during HCO_3^- secretion across the apical membrane of the duct. Based on transport rates measured in acid-loaded isolated ducts, it seems that the Na^+/H^+ exchanger dominates H^+ efflux across the BLM. If these differences in H^+ transport rates and capacities can be extended to physiological condition, then the Na^+/H^+ exchange should mediate most H^+ efflux during stimulation of HCO_3^- secretion. However, on the bases of their measurements of ductal secretion *in vivo* and Na^+/H^+ exchange rates *in vitro*, Raeder and co-workers (Veel et al., 1992; Raeder, 1992) argued against a significant role for the Na^+/H^+ exchanger in ductal fluid secretion. One of their major arguments is that maximal rate of H^+ efflux by the exchanger in isolated ducts can remove only a small percent of the cytosolic H^+ generated during HCO_3^- secretion by the intact pancreas (Veel et al., 1992). This seems a problematic argument because, in isolated ducts, the rate of H^+ efflux by H^+ pumps is lower than

that by the exchanger, and thus should be less likely to mediate BLM H^+ efflux during ductal HCO_3^- secretion. On the other hand, as a primary active mechanism, the pump can support a steeper H^+ gradient than a secondary active transport such as the Na^+/H^+ exchanger. Therefore, it is possible that in intralobular ducts, the exchanger mediate most of H^+ efflux. As the fluid in the duct becomes more alkaline and rich in HCO_3^- , the role of the H^+ pump in H^+ efflux across the BLM increases.

The Na^+ -dependent HCO_3^- transport may play a modulatory role in ductal fluid secretion. As an electrogenic process (Boron and Boulpaep, 1983; Muallem and Loessberg, 1990a), it is sensitive to the membrane potential (Boron and Boulpaep, 1983). As discussed before for other cell types (Alpern, 1985; Boron and Boulpaep, 1983; Muallem and Loessberg, 1990a), in resting cells, when the membrane potential is between -60 and -80 mV (Novak and Greger, 1988a, 1991), the cotransporter can mediate HCO_3^- efflux across the BLM. On stimulation, the membrane potential of rat duct cells depolarizes to ~ -40 to -20 mV (Novak and Greger, 1988b, 1991) where the cotransporter can mediate HCO_3^- influx. Such an influx, together with Na^+/H^+ exchange, will alkalize the cytosol and stimulate the Cl^-/HCO_3^- exchanger at the LM. Indeed, during stimulation of H^+ secretion by the parietal cells, the Na^+/H^+ exchanger is activated to increase pH_i , which is required for activation of Cl^-/HCO_3^- exchange (Muallem, Blissard, Crago, and Sachs, 1988).

The only HCO_3^- efflux mechanism in the LM identified in the present studies is the Cl^-/HCO_3^- exchanger. This exchanger probably mediates most of HCO_3^- efflux in intralobular ducts. As argued in the beginning of this discussion, it is difficult to explain HCO_3^- efflux in the main and common ducts by an electroneutral, 1:1 Cl^-/HCO_3^- exchange. It is possible that other HCO_3^- efflux mechanisms in the LM of the main duct were not found because the ducts were perfused with solutions containing 25 mM HCO_3^- , pH 7.4, and were not stimulated. During stimulation of secretion, the lumen is exposed to ~ 80 mM HCO_3^- , which by itself may activate additional mechanism. Addressing this problem needs further studies.

The two transporters that are not likely to be active during ductal HCO_3^- secretion are the BLM Cl^-/HCO_3^- exchanger and the luminal Na^+/H^+ exchanger found in distal portions of the pancreatic duct. Obviously, their physiological role is not known at present. One function can be a housekeeping role, analogous to the case in the kidney proximal tubule where luminal Na^+/H^+ exchanger is responsible for H^+ secretion, and BLM exchanger is believed to be responsible for regulation of pH_i . In this case, one would expect to find luminal Na^+/H^+ exchangers in all segments of the duct. The fact that luminal exchanger is found only in the distal portion of the ductal tree leads us to speculate that these transporters may be important during resting periods in keeping the duct lumen acidic. Duct cells not only secrete, but also appear to respond to CO_2/HCO_3^- by activation/insertion of ion transporters needed for HCO_3^- secretion, such as K^+ channels (Novak and Greger, 1991) and H^+ pumps (present studies). It is possible that duct cells are sensitive to luminal CO_2/HCO_3^- as a form of positive feedback mechanisms. In this case, it will be advantageous or even necessary for the ducts to keep the lumen acidic by performing net H^+ efflux when acinar cells do not secrete digestive enzymes to prevent uncontrolled HCO_3^- and fluid secretion. The luminal Na^+/H^+ exchanger can perform this task. Since the fluid

in the main and common ducts contain the highest HCO_3^- concentration, it is not unexpected that this segment and not the intralobular duct has the capacity to secrete acid to the lumen.

In summary, the present studies identified and localized the major H^+ and HCO_3^- transporters in the pancreatic duct and described some regulatory features of the H^+ pump. Although these studies are not sufficient to firmly support a specific mechanism of ductal HCO_3^- and fluid secretion, they can be a framework for future studies of this important physiological activity of the pancreas.

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