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Article

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Cellular bicarbonate protects rat duodenal mucosa from acid-induced injury

See related Commentary on pages 1743–1744.

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Secretion of bicarbonate from epithelial cells is considered to be the primary mechanism by which the duodenal mucosa is protected from acid-related injury. Against this view is the finding that patients with cystic fibrosis, who have impaired duodenal bicarbonate secretion, are paradoxically protected from developing duodenal ulcers. Therefore, we hypothesized that epithelial cell intracellular pH regulation, rather than secreted extracellular bicarbonate, was the principal means by which duodenal epithelial cells are protected from acidification and injury. Using a novel in vivo microscopic method, we have measured bicarbonate secretion and epithelial cell intracellular pH (pH_i), and we have followed cell injury in the presence of the anion transport inhibitor DIDS and the Cl^- channel inhibitor, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). DIDS and NPPB abolished the increase of duodenal bicarbonate secretion following luminal acid perfusion. DIDS decreased basal pH_i, whereas NPPB increased pH_i; DIDS further decreased pH_i during acid challenge and abolished the pH_i overshoot over baseline observed after acid challenge, whereas NPPB attenuated the fall of pH_i and exaggerated the overshoot. Finally, acid-induced epithelial injury was enhanced by DIDS and decreased by NPPB. The results support the role of intracellular bicarbonate in the protection of duodenal epithelial cells from luminal gastric acid.

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Introduction

The duodenal mucosa is regularly exposed to intermittent pulses of gastric acid, with luminal acidity varying rapidly between pH 2 and 7 (1). Without protective mechanisms in place, duodenal cells, as is the case with other cells in the upper gastrointestinal tract, are believed to irreversibly acidify in the presence of acidic luminal contents, injuring the epithelium (2). With the measurement of a neutral pH in the juxtamucosal mucus gel in the presence of luminal acid, the bicarbonate secretion hypothesis was developed, wherein bicarbonate, secreted by the epithelial cells, completely neutralized luminal acid diffusing through the mucus gel toward the epithelium (3). Correlation of bicarbonate secretion with mucosal protection from acid-related injury further bolstered this hypothesis (4–6). Recently, with the cloning of transport proteins and more sophisticated understanding of the bicarbonate secretory process, emphasis has been placed on cellular bicarbonate uptake and exit in the context of duodenal mucosal defense (7, 8). Furthermore, luminal acid upregulates other putative defense mechanisms, such as mucosal blood flow and mucus gel secretion, suggesting that bicarbonate secretion is part of a multicomponent defensive system (9–11). Nevertheless, the currently accepted primary duodenal mucosal defense factor is indeed secreted bicarbonate (12).

In our studies of duodenal epithelial pH_i measured in vivo, we made several observations, coupled with the analysis of others, that suggested that this hypothesis is not sufficient to fully account for duodenal cell protection. The first observation was that duodenal epithelial cells readily acidified when the mucosa was superfused with mildly acidic (e.g., pH 4.5) solutions (13), which indicated that acid was not completely neutralized in the mucus gel and could enter the epithelial cells. Second, after observing an increase of duodenal cell buffer capac-

ity in response to perfused acid, we hypothesized that intracellular, rather than secreted extracellular bicarbonate, played an important role in protecting duodenal cells from acid injury (14). Third, we found that bicarbonate secretion did not increase when luminal acid was present, casting further doubt on the role of extracellular bicarbonate in acute mucosal protection from acid (13). Fourth, Wormsley has argued that complete neutralization of luminal acid is possible only with a vast molar excess of bicarbonate, as is the case in general when a strong mineral acid is titrated with a volatile base (15). Fifth, on the basis of mathematical modeling of pH within the mucus gel exposed to secreted bicarbonate and luminal acid, Engel and colleagues have suggested that the dimensions of a neutral layer within the gel would be too thin to afford substantial mucosal protection (16). Finally, neutralization of acid with secreted bicarbonate generates high levels of carbon dioxide, leaving the duodenal cells with an additional volatile acid stress. This phenomenon also argues against the importance of extracellular acid neutralization per se as the predominant protective mechanism (17, 18).

To determine the relative importance of the bicarbonate secretion hypothesis, we first devised experiments that were designed to "uncouple" bicarbonate secretion from mucosal protection. We chose to use two inhibitors, 4,4′diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), both known to inhibit epithelial bicarbonate secretion (7, 19, 20), which were predicted to have divergent effects on pH_i regulation and mucosal protection. DIDS, by enhancing acidification and abolishing the pH_i overshoot following a pulse of perfused acid (14) behaves as if it were inhibiting a bicarbonate uptake mechanism, which was most likely the Na^+:HCO_3^- cotransporter (NBC1), thought to be present in duodenal epithelial cells (7). NPPB, an anion channel inhibitor, was chosen because it was predicted to inhibit bicarbonate exit across the epithelial cell apical membrane, as is the case when the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is dysfunctional (21, 22). Inhibition of apical bicarbonate exit may help preserve cellular buffering power by increasing intracellular bicarbonate concentration and hence buffering power, protecting the cells from acidification and consequent injury. If neutralization of gastric acid in the duodenal mucus gel is the primary mucosal defense mechanism, both inhibitors, by inhibiting bicarbonate secretion, should enhance mucosal injury equally. In contrast, if prevention of a marked reduction of intracellular bicarbonate/pH_i regulation is the prime protective mechanism, NPPB, which inhibits apical bicarbonate secretion, and DIDS, which inhibits basolateral bicarbonate uptake, should have opposite effects on duodenal cell pH_i and viability. To distinguish between these two mechanisms, we studied the effects of DIDS and NPPB on pH_i, bicarbonate secretion, and mucosal injury in the duodenum of living rats.

Methods

Chemicals. 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acid, BCECF acetoxymethyl ester (BCECF/AM), DIDS, and propidium iodide (PI) were obtained from Molecular Probes Inc. (Eugene, Oregon, USA). HEPES, NPPB, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Krebs solution contained 136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES at pH 7.0. For acid perfusion, Krebs solution was adjusted to pH 2.2, 2.0, 1.8, or 1.0 with HCl and adjusted to isotonicity (300 mosm) by reducing the NaCl concentration accordingly. Each solution was prewarmed to 37°C with temperature maintained by a heating pad during the experiment. NPPB was dissolved with DMSO for stock solution. Krebs containing 0.1% DMSO was used as vehicle solution.

In vivo microscopic preparation and pH_i measurement. The methods used for animal preparation and fluorescent microscopy, adapted from the technique originally described by Weinlich and coworkers (23), are described elsewhere in detail (14).

Effects of DIDS and NPPB on pH_i with or without luminal acid. After loading BCECF, time was set as $t = 0$. To examine the effect of luminal acid on pH_i, the duodenal mucosa was perfused with pH 7.0 Krebs for 10 minutes, followed with pH 7.0 or 2.2 solutions for 10 minutes (acid challenge period), and followed by pH 7.0 for 15 minutes. To determine the effect of inhibition of NBC1 and CFTR Cl^- channel on pH_i, respectively, DIDS (0.5 mM) or NPPB (0.1 mM) was added with pH 7.0 or 2.2 perfusion during the 10-minute challenge period.

Preparation of the duodenal loop. In separate experiments, a duodenal loop was prepared and perfused in order to measure duodenal HCO_3^- secretion as described previously (13). In urethane-anesthetized rats, the stomach and duodenum were exposed, and the forestomach wall was incised 0.5 cm using a miniature electrocautery. A polyethylene tube (diameter 0.5 mm) was inserted through the incision until it was 0.5 cm caudal to the pyloric ring, where it was secured with a nylon ligature. The distal duodenum was ligated proximal to the ligament of Treitz and was then incised, through which another polyethylene tube was inserted and sutured into place. To prevent contamination of the perfusate with bile-pancreatic juice, the pancreaticobiliary duct was ligated just proximal to its insertion into the duodenal wall. The resultant closed proximal duodenal loop (perfused length, 2 cm) was perfused with prewarmed saline using a peristaltic pump at 1 ml/min. Input (perfusate) and effluent of the duodenal loop were circulated through a reservoir, bubbled with 100% O_2. Perfusate pH was kept at pH 7.0 with a pH-stat (PHM290 and ABU901; Radiometer Analytical SA, Lyon, France).

Bicarbonate secretion measurement by pH-stat. For pH-stat measurements, the amount of 0.01 *N* HCl added to maintain constant pH was considered equivalent to duodenal HCO_3^- secretion. For duodenal HCO_3^- meas-

urement, a 30-minute stabilization with pH 7.0 saline (t = –35 to –5) was followed by baseline measurements with pH 7.0 saline (t = –5 to 10). To examine the effects of DIDS and NPPB on acid-induced bicarbonate secretion, acid solution was perfused with a Harvard infusion pump at 1 ml/min for 10 minutes (t = 10 to 20) with or without DIDS (0.5 mM) or NPPB (0.1 mM). O_2 gas-bubbled pH 7.0 saline was recirculated with a peristaltic pump, whereas pH 2.2 saline was perfused by syringe pump. The duodenal loop solution was gently flushed with 5 ml of perfusate to rapidly change the perfusate composition at t = 10 and t = 20.

Measurement of acid-induced epithelial cell injury. To assess acid-induced epithelial cell injury in vivo and in situ, we used a novel method using PI, confirmed by conventional histologic evaluation.

PI method. PI labels the nuclei of nonviable cells with leaky cell membranes (24, 25) and was thus used to identify the damaged epithelial cells in the duodenal villus. In vivo microscopic preparation of duodenum was performed as described above. To obtain fluorescent images of PI, the Olympus Merlin system (Olympus Optical Co., Tokyo, Japan), consisting of a frame-transfer camera, grating monochromator, and image processor, was used. PI fluorescence was visualized by excitation at 535 nm and 590 nm emission (Chroma Technology Inc., Brattleboro, Vermont, USA). After loading BCECF to visualize the epithelium, pH 7.0 Krebs containing PI (1 μM) was perfused through the chamber for 10 minutes from t = 0 minutes. Acid solutions (pH 2.0, 1.8, or 1.0) containing PI (1 μM) were perfused for 5 minutes (t = 10–15 minute, challenge period), followed by pH 7.0 solution for 15 minutes (t = 15–30 minutes, recovery period). Images of PI fluorescence followed by images of BCECF fluorescence were recorded every 5 minutes. PI-positive dots corresponding to injured cell nuclei were counted in each image (microscopic field) observed with ×10 objective lens. The mean of the number obtained from three microscopic fields was defined as the number for the given time period. To examine the effect of DIDS or NPPB on the acid-induced injury, pH 2.0 solution containing DIDS (0.5 mM) or pH 1.8 containing NPPB (0.1 mM) was administered during the challenge period.

Localization of PI-positive dots was confirmed using frozen histologic sections. Immediately following microscopic analysis (t = 30 minutes in pH 7.0 and pH 1.8 groups and at t = 20 minutes in pH 1.0 group), the observed area of duodenum was excised and mounted in OCT compound (Miles Inc., Elkhart, Indiana, USA) at –20°C. Frozen cryostat sections (10 μm) were mounted on glass microscope slides. Sections were coverslipped using glycerol and observed by a confocal laser microscope (LSM-510; Carl Zeiss, Göttingen, Germany).

Conventional histology. To compare PI staining in vivo described above with the alternative method, conventional hematoxylin-eosin (H&E) staining was performed on the paraffin-embedded sections. Duodenal loops were perfused with solutions of varying pH as described for the injury studies using the PI staining method, with the exception that PI was not included in the solutions. The acid challenge was 5 minutes long, followed by 15 minutes of perfusion with neutral solution, after which the duodenum was rapidly excised and fixed with 10% formalin. Vertical sections of the duodenum were H&E stained and observed using a light microscope with a ×20 or ×40 objective lens. Villous damage was assessed by counting the number of villi showing injury of the villus tip progressing down the villus, ranging from focal necrosis or bleb formation, ballooning, to frank sloughing. The percentage of villi displaying damage was assessed by a blinded observer in 100 villi in each of four sections per rat in two rats in each experimental group. A lesion score was calculated according to the method of Leung et al. (26): no injury = 0; focal necrosis = 1; ballooning = 2; sloughing less than 50% of villus length = 3; sloughing more than 50% of villous length = 4. In no section was sloughing of more than 50% of villous length observed; therefore, this degree of injury was not reported in Table 1. The score was multiplied by the percentage of villi with each type of damage relative to the total number of villi counted. The scores for each grade of injury were then summed to give the injury score. For example, for 100 villi, if 30 villi had no injury, 20 had focal necrosis, 10 had ballooning, and 3 had sloughing less than 50% of villous length, and 0 had sloughing more

Table 1
Effect of DIDS on acid-induced duodenal villous damage

Group/score	No injury (%)	Focal necrosis/ Bleb formation (%)	Ballooning (%)	Sloughing (%)	Lesion score
pH 7.0 + vehicle	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
pH 7.0 + DIDS	99.5 ± 0.3	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3
pH 2.2 + vehicle	99.5 ± 0.5	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3
pH 2.0 + vehicle	85.8 ± 3.0	8.8 ± 1.4	2.6 ± 0.7	0.8 ± 0.4	16.3 ± 2.3[A]
pH 2.0 + DIDS	58.3 ± 3.2	18.9 ± 1.7	10.5 ± 1.1	12.4 ± 0.9	77.0 ± 5.7[B]
pH 1.8 + vehicle	27.1 ± 2.1	20.6 ± 1.8	31.5 ± 1.0	22.0 ± 1.1	149.6 ± 3.4[A]
pH 1.8 + NPPB	46.4 ± 3.0	30.1 ± 2.8	16.4 ± 2.1	7.1 ± 0.7	84.3 ± 5.2[C]

The data depicted for each experimental group were obtained by examining 800 villi (four sections per rat, 100 villi per section, two rats) for the presence of injury, manifest as focal necrosis/bleb formation, ballooning of the villus tip, or sloughing. The mean percentage ± SEM of injured villi relative to the number of total villi counted are depicted. [A]P < 0.05 compared with pH 7.0 + vehicle; [B]P < 0.05 compared with pH 2.0 + vehicle; [C]P < 0.05 compared with pH 1.8 + vehicle.

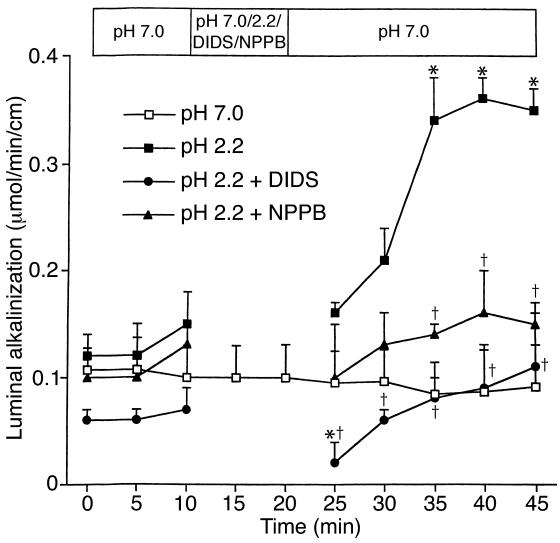


Figure 1
Effect of DIDS and NPPB on acid-induced bicarbonate secretion. Titratable alkalinity (bicarbonate secretion) was measured by the pH-stat method in duodenal loop perfusion experiments. Both DIDS and NPPB inhibit the increased bicarbonate secretion following acid exposure. All data are expressed as mean ± SEM from six rats. *$P < 0.05$ vs. pH 7.0 saline perfusion, †$P < 0.05$ vs. pH 2.2 saline perfusion.

than 50% of villous length, the injury score would be $30 \times 0 + 20 \times 1 + 10 \times 2 + 3 \times 3 + 0 \times 4 = 49$ out of a maximum of 400 and a minimum of 0.

Western blot analysis. An affinity-purified polyclonal Ab was raised in rabbits against a synthetic peptide derived from the N-terminus of pNBC1:pNBC1 (pancreatic variant of NBC1) (amino acids 1–19) coupled to an N-terminal cysteine. The affinity-purified polyclonal Ab to renal variant of NBC1, kNBC1, was raised against a synthetic peptide corresponding to amino acids 11–24, coupled to an N-terminal cysteine (27). Using these Ab's, we examined the expression of NBC1 variants in rat duodenum with Western blot analysis. Rat duodenum was disrupted at 0°C in a glass homogenizer with 100 ml TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl), containing 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin (buffer A). The homogenate was centrifuged at 300 *g* for 5 minutes and then at 4,000 *g* for 10 minutes. The supernatant was centrifuged at 150,000 *g* for 2 hours. The pellet was solubilized in buffer A and centrifuged at 150,000 *g* for 1 hour. The same procedure was used to isolate membrane proteins from rat pancreata and kidneys. The final pellet was solubilized in 1× Laemmli buffer, and proteins were separated by SDS-PAGE and were electrotransferred onto PVDF membrane (Bio-Rad Laboratories Inc., Hercules, California, USA) at 50 mA for 16–20 hours. Nonspecific binding was blocked by incubation for 1 hour in TBS containing 5% dry milk and 0.05% Tween 20 (Bio-Rad Laboratories Inc.). Primary pNBC1- or kNBC1-specific Ab's were used at a dilution of 1:1,000. Secondary horseradish peroxidase–conjugated mouse anti-rabbit Ab (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) was used at a dilution of 1:10,000. Bands were visualized using enhanced chemiluminescence (ECL) Western blotting kit and ECL Hyperfilm (Amersham Pharmacia Biotech, Keene, Pennsylvania, USA).

Immunolocalization of NBC1. The duodenum from normal Sprague-Dawley rats was fixed by retrograde perfusion via the aorta with 4% paraformaldehyde, in 0.0375 M Na_2HPO_4 buffer, pH 6.2). Tissue blocks were cryoprotected with 30% sucrose, mounted on holders and frozen in liquid nitrogen. The pNBC1 and kNBC1 primary Ab's (1:100 dilution) were applied for 40 minutes. After several washes in PBS, pH 7.4, goat anti-rabbit IgG conjugated with Alexa 594 (1:500 dilution, Molecular Probes Inc.) was applied for 45 minutes. The slides were rinsed in PBS and mounted in Cytoseal 60 (Stephens Scientific, Riverdale, New Jersey, USA). A liquid-cooled PXL CCD camera (CH1; Photometrics, Osnabruck, Germany) coupled to a Nikon Microphot-FXA epifluorescence microscope, was used to capture and digitize the fluorescence images. The images were transferred to a Silicon Graphics Indy 5000 computer using ISEE 4.0 (c) software (Inovision Corp., Raleigh, North Carolina, USA), and printed on a Kodak 8650 PS color printer.

Statistics. All data from six rats in each group are expressed as the mean plus or minus SEM. Comparisons between groups were made by one-way ANOVA followed by Fischer least-significant difference test.

Results

Effect of DIDS and NPPB on acid-induced bicarbonate secretion. We first confirmed the effect of DIDS and NPPB on duodenal bicarbonate secretion by titration of the effluent of a perfused duodenal loop with a pH-stat, a measure of bicarbonate secretion. Figure 1 depicts that, following a 10-minute acid challenge, luminal alkalization gradually increased, consistent with increased bicarbonate secretion. DIDS and NPPB abolished the increase of bicarbonate secretion after acid exposure, confirming the importance of DIDS-and NPPB-sensitive transport processes in rat duodenal bicarbonate secretion.

Effect of DIDS and NPPB on pH_i with or without luminal acid. We then measured the effects of DIDS and NPPB on duodenal pH_i. Figure 2 depicts the effects of DIDS and NPPB on pH_i in the presence or absence of perfused luminal acid. Without luminal acid (Figure 2a), DIDS acidified duodenal epithelial cells, consistent with inhibition of cellular bicarbonate influx, whereas NPPB alkalinized the cells, consistent with inhibition bicarbonate exit. Divergent effects of DIDS and NPPB were also observed in the presence of perfused acid (Figure 2b). Perfused acid rapidly acidified the duodenal epithelial cells, followed by recovery of pH_i to baseline ($t = 25$). Following removal of the perfused acid, pH_i increased above the baseline value (overshoot) ($t = 30, 35$) as described previously (14). DIDS enhanced the intracellular acidification during the luminal acid challenge and abolished the overshoot following acid removal. These effects of DIDS are consistent with the

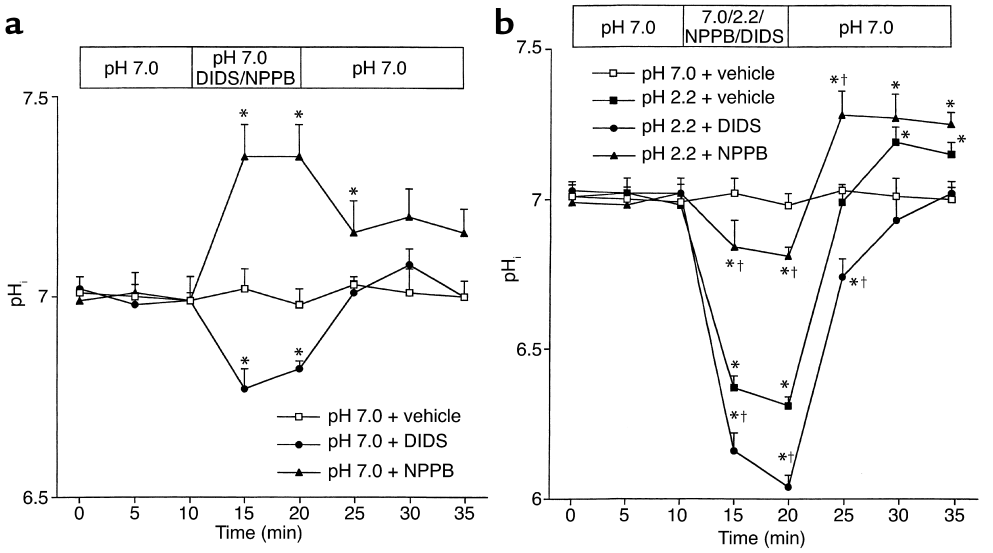


Figure 2
Effects of DIDS and NPPB on baseline pH_i and on pH_i during acid challenge of duodenal epithelial cells. (**a**) During perfusion with pH 7.0 solution, DIDS acidifies, whereas NPPB alkalinizes cells. (**b**) DIDS enhances, whereas NPPB attenuates acid-induced intracellular acidification during acid exposure. Note that DIDS abolishes, but NPPB enhances pH_i overshoot following acid challenge. *P < 0.05 vs. pH 7.0 Krebs group, †P < 0.05 vs. pH 2.2 Krebs group. All data are expressed as mean ± SEM from six rats.

inhibition of a bicarbonate influx process. In contrast, NPPB attenuated the decrease of pH_i during the luminal acid challenge and enhanced the overshoot of pH_i following acid removal, consistent with inhibition of cellular bicarbonate exit.

Immunolocalization of the electrogenic sodium bicarbonate cotransporter pNBC1. Since bicarbonate influx into duodenal cells by electrogenic sodium bicarbonate cotransport is thought to play an important role both in pH_i regulation following acute acid loading and in transepithelial bicarbonate secretion, we immunolocalized NBC1 protein variants in rat duodenum. For this purpose, rabbit affinity-purified polyclonal Ab's were raised against the specific N-terminus of pNBC1 and kNBC1 variants of NBC1 (27). The pNBC1 Ab recognized a major band at approximately 150 kDa on immunoblots using membrane fractions from rat duodenum (Figure 3). The specificity of the labeling was confirmed using the pNBC1 Ab preabsorbed with the immunizing peptide. Moreover, data from pancreatic membranes, which strongly express pNBC1 (approximately 150-kDa band), is shown for comparison. The size of the recognized protein in all tissues is larger than the predicted size of approximately 120 kDa, suggesting that pNBC1 is glycosylated or posttranslationally modified in other ways. In duodenum, a minor band of approximately 90 kDa was also detected. Unlike pNBC1, kNBC1 was not detected in the rat duodenum. The results of the immunocalization studies are shown in Figure 4. pNBC1 was highly expressed on duodenal epithelial cells on the basolateral membrane. In contrast, kNBC1 was not detectable (not shown). These results demonstrate that pNBC1 is the basolateral electrogenic sodium bicarbonate cotransporter in rat duodenum.

Detection of acid-induced epithelial injury with PI staining in vivo. Initial studies were performed to address the feasibility of using PI staining to measure duodenal epithelial injury in response to a brief pulse of luminal acid. Figure 5 depicts images of PI-stained mucosa generated in vivo (Figure 5, a–c) and images of corresponding frozen sections (Figure 5, d–f). Figure 5, a and d, depicts mucosae exposed to pH 7.0; Figure 5, b and e, depicts mucosae 15 minutes after a 5-minute exposure to pH 1.8; and Figure 5, c and f, depicts mucosae 5 minutes after a 5-minute exposure to pH 1.0. During perfusion with neutral solution, only rare PI staining was visible at t = 30 minutes (Figure 5a), though submucosal PI-positive structures were visible in sections (Figure 5d). The red appearance of the villi in (Figure 5a) was due to BCECF fluorescence observed through a 590-nm filter. Fifteen minutes after a 5-minute exposure to a pH 1.8 solution, brightly fluorescent PI-positive dots, corresponding to PI-stained nuclei, were observed over the villi (t = 30 minutes; Figure 5b). Five minutes after a 5-minute pH 1.0 challenge, numerous PI-positive nuclei were observed, producing diffuse PI-positive staining over the villous

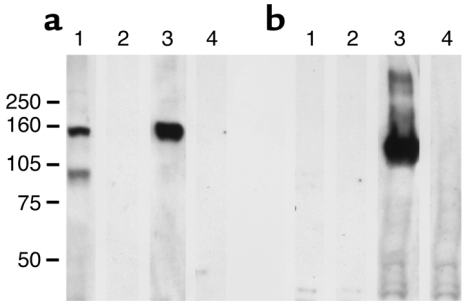


Figure 3
Western blot of rat duodenum membrane proteins probed with pNBC1-specific (**a**) and kNBC1-specific (**b**) Ab's. Rat duodenum membrane proteins (80 μg) were loaded in lanes 1 and 2 in both **a** and **b**. (**a**) Rat pancreas membrane proteins (25 μg)were loaded in lanes 3 and 4. (**b**) Rat kidney membrane proteins (100 μg) were loaded in lanes 3 and 4. The pNBC1-specific Ab and the kNBC1-specific Ab were used at dilution 1:1,000. For lanes 2 and 4, pNBC1 (**a**) and kNBC1 (**b**) Ab's preincubated with the corresponding specific peptide (1:100) were used.

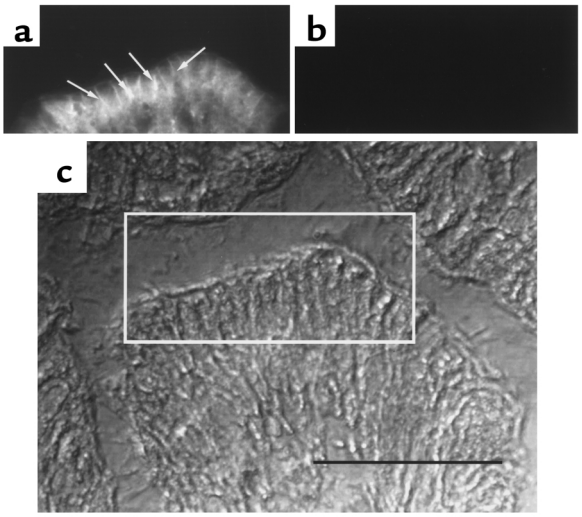


Figure 4
Immunolocalization of pNBC1 in rat duodenum. (**a**) pNBC1 is localized to the basolateral membranes of duodenal villus epithelial cells appearing as a cup-shaped structure (arrows). (**b**) When the pNBC1 Ab was preincubated with a specific peptide (1:100), staining was suppressed. (**c**) An image taken with Nomarski optics is included to provide orientation. The white rectangle encloses the same area shown in **a**. Calibration bar, 50 μm.

tips (t = 20 minutes; Figure 5c). Frozen sections of acid-exposed mucosa (Figure 5, e and f) revealed that PI-related fluorescence was localized to the nuclei of the villous tip epithelial cells and the nuclei of detached epithelial cells above the villi, but not the middle portion of the villus or the crypts (not shown). Villous ballooning was additionally observed after exposure to pH 1.0 (t = 20 minutes; Figure 5f).

Effect of DIDS and NPPB on acid-induced epithelial injury

In vivo PI staining. Having devised a means of reproducibly measuring acid-related injury by in vivo microscopy, we then measured injury in the presence of DIDS and NPPB to determine if bicarbonate secretion could be uncoupled from acid-related mucosal injury. Figure 6 depicts the time course of in vivo PI staining before and after a 5-minute pH 1.8 exposure. PI-positive cells gradually increased in number in the villous cells. In control studies, pH 7.0 Krebs perfusion slightly increased the number of PI-positive cells during a 30-minute experiment. To determine the effect of each inhibitor on cell viability, we used preperfusions of slightly different pH to best demonstrate their respective effects. A 5-minute exposure to pH 2.0 or pH 1.8 gradually increased the number of PI-positive cells (Figure 6, a and b). DIDS further increased the number of PI-positive cells, consistent with enhancement of the mucosal susceptibility to acid injury (Figure 6a). Conversely, NPPB reduced the number of PI-positive cells observed after a 5-minute perfusion with a pH 1.8 solution (Figure 6b).

Conventional histology. To help confirm the injury studies, routine histology was performed on sections obtained from duodenal mucosa exposed to the same conditions used for the PI experiments. As seen in Figure 7, exposure to pH 7.0 (Figure 7a), pH 2.2 (Figure 7b), or pH 2.0 (Figure 7c) perfusate did not produce microscopic damage to the villi. Focal necrosis, ballooning, and sloughing of villi represent increasing severity of injury with increasing $[H^+]$ (26, 28). Exposure to pH 2.0 perfusate (Figure 7c) produced no visible damage; the addition of 0.5 mM DIDS to the pH 2.0 perfusate was associated with focal necrosis of the villus tips (Figure 7e). More severe damage, with frank sloughing of the villous tips, was observed with pH 1.8 perfusion (Figure 7d). The injury was attenuated to ballooning by the presence of NPPB (Figure 7f). Thus, routine histologic examination confirmed that DIDS augmented acid-induced injury, whereas NPPB diminished injury. The percentage of the damaged villi is shown in Table 1. As seen in Table 1, DIDS alone did not increase the injury score, but increased the injury score in the presence of acid. NPPB in the presence of acid decreased the injury score, consistent with the data obtained with PI.

These results, which are consistent with the pH_i studies, further indicate that attenuation of a marked fall of pH_i/bicarbonate during an acid challenge is imperative for protection from mucosal injury. Furthermore, the divergent effects of DIDS and NPPB on mucosal injury, despite both abolishing bicarbonate secretion, would be predicted only if bicarbonate secretion was not a primary mucosal protective process.

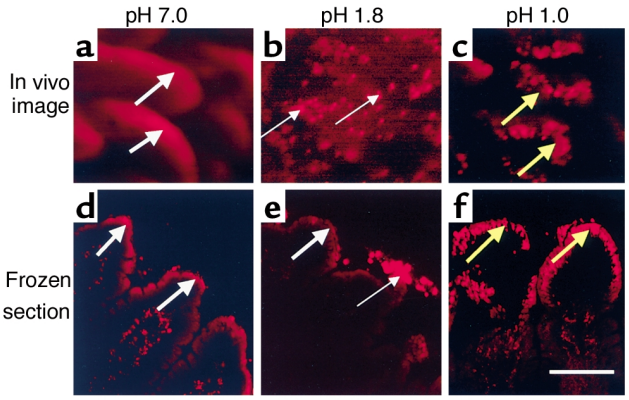


Figure 5
PI-positive staining in vivo and effect of acid. (**a** and **d**) A low number of visible PI-positive nuclei were visible 30 minutes after perfusion with pH 7.0 solution (t = 30 minutes). The red color of the villi is BCECF fluorescence observed through a 590-nm filter (thick arrows). (**b** and **e**) Fifteen minutes after a 5-minute pulse of pH 1.8 perfusate (t = 30 minutes), PI-positive dots are seen readily, localized to the villous tips (thin arrows). (**c** and **f**) Five minutes after a 5-minute pulse of pH 1.0 perfusate (t = 20 minutes), the villous tips have ballooned, and some are detached (yellow arrows). Note that all of the epithelial cells in the ballooned and detached villi are PI positive. Calibration bar, 100 μm. **a**, **d**, **e**, and **f** were printed so as to emphasize the faint staining of the villi.

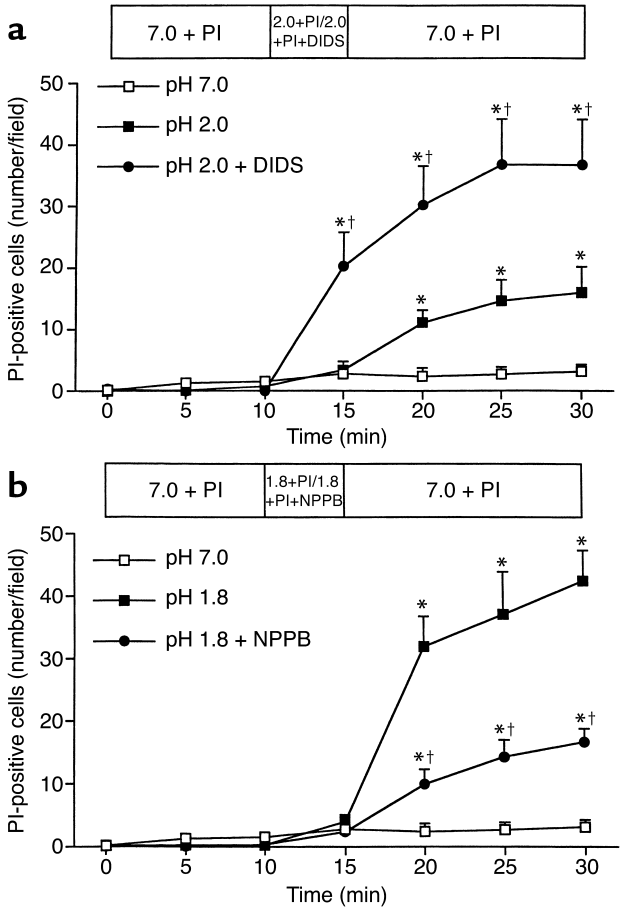


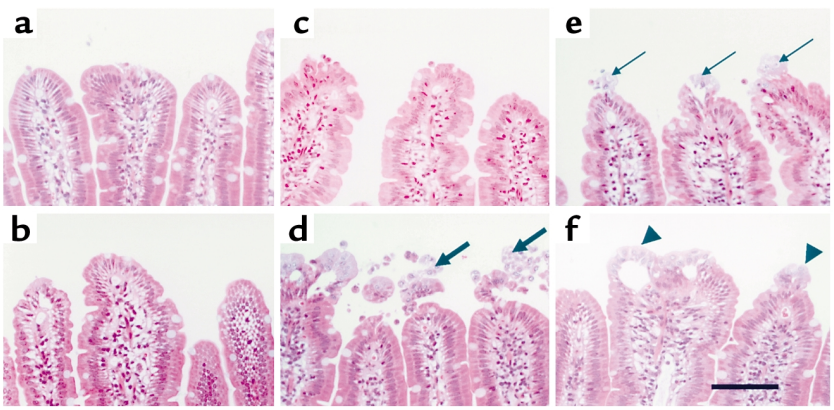
Figure 6
Effect of DIDS and NPPB on acid-induced duodenal injury (PI study). (**a**) Effect of DIDS. A 5-minute pulse of pH 2.0 injures the mucosa, as measured by a progressive increase of PI-positive nuclei. DIDS further increased the number of PI-positive cells induced by a pH 2.0 pulse. *P < 0.05 vs. pH 7.0 Krebs group, †P < 0.05 vs. pH 2.0 Krebs group. (**b**) Effect of NPPB. NPPB lessens mucosal injury caused by a 5-minute perfusion with pH 1.8 solution, as assessed by decreased number of PI-positive cells. *P < 0.05 vs. pH 7.0 Krebs group, †P < 0.05 vs. pH 1.8 Krebs group. All data are expressed as mean ± SEM from six rats.

Discussion

We studied the importance of intracellular bicarbonate/pH_i regulation in the protection of duodenal epithelial cells from luminal acid challenge. Both DIDS and NPPB abolished bicarbonate secretion after a luminal acid challenge. DIDS and NPPB, however, had divergent effects on pH_i at baseline (neutral perfusion) and during acid exposure. In complementary studies, DIDS exaggerated, whereas NPPB lessened, acid-induced epithelial injury. Immunolocalization studies identified pNBC1 in the duodenal epithelial cell basolateral membrane as a potentially important DIDS-sensitive bicarbonate influx pathway that plays a role in the prevention of duodenal epithelial injury. This is the first study showing that two inhibitors of bicarbonate secretion have opposite effects on pH_i and mucosal protection, suggesting that intracellular bicarbonate/pH_i regulation plays an important role in the protection of duodenal cells from a luminal acid load.

DIDS inhibits bicarbonate secretion in bicarbonate-secreting epithelia (19, 20). The acidifying effect of DIDS on pH_i, however, is less easily understood. DIDS inhibits several anion transporters thought to be present on duodenal epithelial cells. Although early studies identified an anion exchange process in membrane vesicles derived from the intestinal brush border (29), the AE2 anion exchanger isoform has been immunolocalized to the basolateral membrane of intestinal, including duodenal, epithelial cells (30). Given the known gradients for chloride and bicarbonate, AE2 would be predicted to mediate basolateral bicarbonate efflux in the absence of luminal acid. Moreover, inhibition of AE2 with DIDS would be predicted to alkalinize the cells, as was found in isolated murine duodenocytes (8), in contrast to the findings we report in the current study. Nevertheless, our study confirms the original measurement of epithelial cell pH_i in isolated *Necturus* duodenum, in which Kivilaakso and coworkers observed that 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene (SITS), an inhibitor closely related to DIDS, added to the serosal bath–acidified

Figure 7
Effect of DIDS and NPPB on acid-induced duodenal injury (H&E study). Injury was assessed in control and acid-exposed duodena with the use of conventional histologic sections. (**a**) Duodenal villi perfused with pH 7.0 solution, (**b**) pH 2.2 solution, (**c**) pH 2.0 solution, (**d**) pH 1.8 solution, (**e**) pH 2.0 + DIDS, and (**f**) pH 1.8 + NPPB. Note that DIDS increased mucosal injury, as manifest by focal necrosis seen in **e** (thin arrows), compared with no injury seen with pH 2.0 alone (**c**). The sloughing caused by pH 1.8 perfusion (thick arrows), seen in **d**, was not observed in the presence of NPPB (**f**), where ballooning (arrowheads), a more mild form of injury, was observed (H&E, ×400; calibration bar, 100 μm).



duodenal epithelial cells (2). An additional anion exchanger, downregulated in adenoma gene product (DRA), is present in the apical membrane of intestinal and colonic epithelial cells (31) and is believed to mediate intestinal and colonic fluid absorption. DRA, however, may only be variably sensitive to DIDS (32). The presence of NBC1 in duodenal epithelial cells has been demonstrated functionally previously and by PCR (7). Recently, NBC1 and a related electroneutral cotransporter NBCn1 were identified in the basolateral membrane of villus epithelial cells of murine duodenum (8). Using a pNBC1-specific Ab, our studies demonstrate that rat duodenal cells express the pNBC1 protein variant of the *NBC1* gene (33, 34) on their basolateral membrane. Furthermore, we were unable to detect the kNBC1 N-terminal variant in duodenal cells either by Western blot analysis or immunocytochemistry using a kNBC1-specific Ab. Finally, it is also possible that DIDS inhibited outwardly rectifying basolateral Cl^- channels (35, 36), with the resultant hyperpolarization inhibiting bicarbonate uptake via pNBC1. Therefore, the effect of DIDS on pH_i and on duodenal cell–mediated bicarbonate secretion is in keeping with its inhibition of basolateral bicarbonate influx via pNBC1 or a related, DIDS-sensitive bicarbonate uptake mechanism. Although it was not possible to reliably add DIDS to the basolateral side directly in the in vivo preparation used in this study, our results, taken in the context of previous studies of isolated duodenal preparations (2), are best explained by postulating that DIDS permeated through intercellular junctions and inhibited basolateral pNBC1. This explanation is further supported by the observation that the duodenum is a low-resistance, leaky epithelium that can be penetrated by numerous small organic compounds such as capsaicin and bradykinin (11, 37). Moreover enhancement of intracellular acidification during acid challenge and abolition of the overshoot after acid removal by luminal DIDS application are most consistent inhibition of bicarbonate uptake. We are unable, however, at the present time to exclude additional effects of DIDS on apical transport mechanisms in these experiments. We also note that even though DIDS is fluorescent, its fluorescence at the concentrations and wavelengths used is unlikely to interfere with BCECF-based pH_i measurements (38).

We developed a method in which cell injury could be assessed dynamically in vivo by using fluorescence microscopic methodology to detect PI staining. As shown previously, acid-induced mucosal injury in rat duodenum is a function of perfusate pH and exposure time (28). Although only a few previous studies have used PI staining to assess injury in vivo (24, 39, 40), we chose this technique since it provides a useful method for dynamically quantifying the luminal response to acid perfusion in vivo. Our results compare favorably with the data of Livingston et al., where a conventional histological approach was used to demonstrate duodenal cell injury during luminal perfusion with solutions of pH less than 2.0 (28). The gradual increase in the number of PI-positive cells during perfusion with neutral solutions reflects, in part, the spontaneous turnover of villous tip cells (41). Acidic solutions of pH 2.0 and 1.8 were chosen for the current duodenal cell injury studies since these pH values are close to the physiological range and were based on the ability to produce a reproducible increase of nuclear staining without engendering immediate or severe cell necrosis. As seen in the conventional histologic sections, and in agreement with previous studies (26, 28), early villus injury progresses from blebbing of the apical membrane to ballooning of the villus tips through sloughing. The overall injury scores confirmed the data obtained with the PI technique that DIDS enhanced injury, whereas NPPB decreased injury. Conventional injury studies hence confirmed that DIDS enhanced, whereas NPPB lessened, the amount of visible acid-related damage. The demonstration that the observed damage is preceded by irreversible cellular acidification would further support the hypothesis concerning the etiology of duodenal epithelial injury. We did not measure pH_i in damaged cells in our system since pH_i measurements in the presence of injury are unreliable due to dye leakage and since the lower limit of reliability with BCECF is approximately pH 6.2. In the absence of acid perfusion, DIDS mildly and reversibly acidified the epithelial cells, similar to the effects of perfusion with pH 4.5 solution, which is not injurious (13, 28). The lack of injury with DIDS at neutral pH, which was predicted from the pH_i data, lends further confidence to the correlation between pH_i and injury measured with PI and conventional histology.

Recent studies of CFTR indicate that this channel plays a major role in bicarbonate secretion (21, 42, 43). This channel, present in pancreatic ducts, duodenal epithelial cells, and Brunner's glands (44), is closely associated with epithelial HCO_3^- secretion. NPPB, a blocker of the CFTR Cl^- channel, inhibits bicarbonate secretion stimulated by cGMP agonists in rats (45) and by cAMP in mice (42), suggesting that bicarbonate exits from the cells through the CFTR itself or through CFTR-associated transporters. The mechanism by which bicarbonate is secreted across epithelia is controversial. CFTR may serve as a HCO_3^- conductance in human airway epithelia (46); alternatively, it may alter the electrical gradient for electrogenic bicarbonate entry, consistent with data obtained in studies of mouse pancreatic ducts (47–49). The former mechanism is compatible with the results of the current study. Specifically, NPPB, by inhibiting CFTR function, diminishes bicarbonate secretion by inhibiting exit across the apical membrane. The absence of bicarbonate exit might reset intracellular buffering at a higher level, protecting the cells from acidification due to acid loads. This contention is substantiated by the observation that duodenal epithelial cells obtained from patients with cystic fibrosis (CF) have impaired pH_i recovery from alkaline loads in chloride-free medium (50). Furthermore, our

results are also consistent with the presence of a CFTR-like, NPPB-sensitive bicarbonate-selective anion channel in the apical membrane, as has been described recently for guinea pig gallbladder (51).

One of the most interesting aspects of this study was that DIDS, by presumably inhibiting bicarbonate uptake across the basolateral membrane, exaggerates perfused acid-induced epithelial cell injury measured by PI staining, whereas NPPB, which presumably inhibits bicarbonate exit across the apical membrane, protects the cells from acid-induced injury. The importance of this observation does not lie in the mechanism of action of each inhibitor, but rather in that a compound such as NPPB can, on the one hand, inhibit bicarbonate secretion while, on the other hand, can protect duodenal cells from acidification and acid-induced injury. This decoupling of bicarbonate secretion and mucosal protection contradicts previous animal and human studies (4, 6, 52), although selective inhibition of apical bicarbonate exit may be an unusual means by which transepithelial bicarbonate secretion can be altered.

The lack of duodenal ulcer disease among patients with CF may be explained by similar logic. Although gastroduodenal pH is low and pancreatic and duodenal bicarbonate secretion is subnormal in patients with CF (53–55), these patients have a paradoxical low incidence of peptic ulcer disease (56–58). This low incidence of peptic ulcer disease is not ascribable to an absence of upper gastrointestinal acid-peptic disease, since severe acid-peptic esophageal injury is common (59, 60). Furthermore, CF patients, who are frequently endoscoped for upper gastrointestinal symptoms, have characteristic duodenal mucosal abnormalities (61), and can be infected with *Helicobacter pylori* (62). These observations further support our "intracellular bicarbonate" hypothesis: diminished apical bicarbonate exit with intact basolateral bicarbonate influx increases the steady state intracellular bicarbonate/pH_i in the vulnerable duodenal epithelial cells in these patients, protecting them from injury due to luminal acid.

In conclusion, we have presented data that support our hypothesis that intracellular pH regulatory mechanisms are an important means by which duodenal epithelial cells are protected from luminal acid, part of an intricate defense system that includes secretion of HCO_3^- from the pancreas and possibly Brunner's glands, and mucus from the epithelial cells and Brunner's glands. As a potent bicarbonate influx pathway, basolateral pNBC1 is likely to be a component of the protective mechanism. The protective effect of NPPB during luminal acid loading may serve as a useful model for studying the cellular processes responsible for preventing ulcers in CF patients.

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