We measured villous cell intracellular pH (pH_i) and solute diffusion between the bathing media and the epithelial cells in stripped, chambered mouse duodenum. Apical perfusion of a high CO_2 solution rapidly acidified the upper villous cells with recovery after its removal. Apical zoniporide (ZP) enhanced CO_2-induced acidification. Serosal ZP, dimethylamiloride (DMA) or stilbene anion transport inhibitors failed to alter CO_2-induced acidification, whereas serosal high CO_2 buffer acidified the upper villous cells. Serosal 5-hydroxytryptamine rapidly acidified the upper villous cells. All serosally-perfused fluorescent compounds stained the crypt area, but not the villi or villous cells. In contrast, intravenous carboxyfluorescein quickly diffused into the interstitial space of the entire mucosa, and mucosally perfused fluorescent compound rapidly penetrated the epithelial cell layer. In muscle-stripped duodenum mounted in a small-aperture perfusion chamber, serosal solutes can readily diffuse only to the crypt cell region, whereas access to the villous epithelial cells is diffusion-limited. In contrast, rapid villous cell responses to serosally applied solutes are best explained by neural reflexes. Limited viability of the villous cells and impaired structural stability of the villi further limit long-term, villous cell functional studies of mucosal preparations mounted in small aperture diffusion chambers.

**Keywords:** upper villous cells, fluorescence, CO_2, ion transport inhibitor, chemical diffusion, serosal perfusate, duodenum, diffusion barrier, carboxyfluorescein; dimethylamiloride, carbon dioxide; zoniporide

**INTRODUCTION**

One of the primary functions of the intestine is to transport large amounts of fluid, solutes, and other substances between the environment and the systemic circulation. Of the several model systems designed to study intestinal transport
function, the muscle stripped, chambered intestinal mucosal preparation enables the measurement of bidirectional transmucosal solute fluxes and short circuit current \( I_{sc} \), while enabling the polarized application of secretagogues and other substances, a useful feature not found in most other model transport systems based on the study of intact tissue.

The chambered system was developed for a relatively tight and flat epithelium obtained from an organism with tissues known to withstand prolonged *in vitro* incubations, such as frog skin (1). Application of this system to architecturally more complex, leakier, and less robust tissues such as mammalian intestine is not straightforward, in particular since solutes present in the solution exposed to the apical surface may easily penetrate the mucosa, and serosal solutes must traverse many structures that are not encountered *in situ* prior to reaching the villous epithelial cells. Supporting this concept are reports that compounds present in the serosal perfusate that do not work through neural mechanisms affect villous cell function only when present in high concentrations, over long pre-incubations, or when simultaneously perfused in the apical solution (2 - 5). These factors are likely to confound interpretation of the polarity of the effect of solutes perfused in the serosal and mucosal solutions in chambered intestinal preparations. Another factor confounding Ussing-chamber studies of intestine is that villous cell viability may be limited. Limited viability and structural stability could limit the number of functional tests that could be performed in this preparation. This problem is particularly evident in studies of duodenum, where frank villous damage is evident after 2 hrs or less of incubation of the chambered mucosa despite maintenance of transmucosal resistance and depolarization in response to forskolin (6, 7). For example, in rat duodena chambered for 180 min, the transmucosal potential difference, short circuit current, and resistance were stable despite increased apparent permeability coefficients \( P_{app} \) for propranolol and mannitol and 50% villous shortening, indicative of villous damage (6). To date, there have been no studies specifically addressing the accessibility of the villous epithelial cells to serosal solutes in chambered intestinal preparations.

The villous structure of the intestine, coupled with data obtained from *in vitro* and other studies, has led to the formulation of a compelling hypothesis in which secretory function was assigned to the crypt cells, whereas the upper villous cells mostly mediated solute absorption from the lumen (8). Recently, however a growing number of investigators, using microelectrodes, X-ray microanalysis, other related methods have questioned this hypothesis, since, under some circumstances, villous cells can secrete, and crypt cells can absorb (9 - 14). Despite these data, however, the ‘crypt-villous’ hypothesis has prevailed to the present (15, 16). A possible explanation for this apparent discrepancy is that Ussing chambered intestine, due to the aforementioned villous cell viability concerns, and due to the possibility that villous cells have limited access to compounds perfused in the serosal bath, may have compromised villous cell function and responses, which would impair their contribution towards overall epithelial transport function.
To address this apparent controversy, we adapted a well-described model of mounted, stripped duodenum (17 - 19) for the mouse. In order to selectively measure transport function of the upper villous cells, we mounted the stripped mucosa in chamber that enabled perfusion of the basolateral and mucosal surfaces, with simultaneous ratio fluorometric imaging, which enables measurement of epithelial intracellular pH (pH$_i$) in upper villous epithelial cells (20). Using this preparation, we tested the hypothesis that solutes perfused in the serosal solution had limited access to the villous epithelial cells whereas compounds perfused in the mucosal solution had ready access. We further hypothesized that existing mucosal neural elements present in the stripped mucosal preparation can be rapidly activated by compounds perfused in the serosal solution.

**MATERIALS AND METHODS**

**Chemicals and solutions**

2',7'-Bis(2-carboxyethyl)- 5(6)-carboxyfluorescein (BCECF) acid and its acetoxymethyl ester (BCECF-AM), 5(6)-chloromethyl SNARF®-1 acetate, 5(6)-carboxyfluorescein (CF), 5-N-hexadecanoyl-aminofluorescein (HAF) and 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS) were obtained from Molecular Probes (Eugene, OR). DMA, 5-hydroxytryptamine (5-HT), tetrodotoxin (TTX), acridine orange (AO), HEPES, 1,4-diazobicyclo-(2,2,2)-octane (DABCO) and other chemicals were obtained from Sigma Chemical (St. Louis, MO). A selective NHE-1 inhibitor, [1-(quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine (CP-597396; zoniporide, ZP) was kindly provided by Pfizer Inc. (Groton, CT). Krebs buffer solution used to perfuse the apical mucosa contained (in mM) 136 NaCl, 2.6 KCl, 1.8 CaCl$_2$ and 10 HEPES at pH 7.4. HEPES bicarbonate Ringer solution for the serosal perfusate contained (in mM) 120 NaCl, 4 KCl, 1.8 CaCl$_2$, 25 NaHCO$_3$, 10 glucose, and 10 HEPES, gassed with 95% O$_2$/5% CO$_2$ at 37 °C at pH 7.4. For serosal acid perfusion, HEPES bicarbonate Ringer solution was adjusted to pH 7.0 and 6.4 with 0.1 N HCl. The high CO$_2$ solution used for mucosal perfusion was made with 50 mM NaHCO$_3$/105 mM NaCl and 20 mM HCl/135 mM NaCl, pre-warmed to 37 °C, and vigorously mixed 1 min before perfusion, generating isotonic (310 mOsm) pH 6.4 saline solution, $P_{CO_2}$ = 260 Torr at 37°C, calculated with the Henry water solubility for CO$_2$ of 0.0321 mM/Torr at 37 °C and the first dissociation constant pK$_a$ of carbonic acid 6.1 at 37 °C (21 - 24). We confirmed that the pH of the mixed solution reached steady-state within 10 sec, whereas [CO$_2$] reached its equilibrium of carbonic acid, H$^+$ and HCO$_3^-$ by 1 min, and was stable for at least 10 min without stirring, as measured by pH and CO$_2$ electrodes (Lazar Research Laboratories, Inc., Los Angeles, CA). Each solution was pre-warmed to and maintained at 37°C using a water recirculating heating pad during the experiments. For stock solutions, DMA, ZP, TTX, CF and HAF were dissolved in DMSO; DIDS and AO were in distilled water, and stored at −20°C until use. 5-HT was dissolved in distilled water immediately before the experiment. For vehicle perfusion, the solution containing 0.1% DMSO was used.

**Animal studies**

**Measurement of upper villous cell pH$_i$ in the stripped duodenal mucosa**

All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee (VA IACUC). Male C57Bl6 mice weighing 20 - 25 g (Harlan, San Diego, CA, USA) were fasted for 1 hr, but had free access to water.
Under isoflurane anesthesia (1.5 - 2.0%) using a rodent anesthesia inhalation system, mice were placed supine on a rodent operating stage warmed with recirculating water (Summit Medical Systems, Bend, OR, USA). Body temperature was maintained at 36 - 37 °C by the stage and a pad warmed with recirculating water, monitored by a rectal thermistor. The abdomen was opened via midline incision, and duodenum was exposed. The pylorus was tightly ligated, and the duodenum was filled with 0.3 ml saline pre-warmed at 37 °C. The anterior wall of the duodenum was incised between the pylorus and the papilla of Vater using a thermal cautery (Geiger Medical Technologies, Inc., Monarch Beach, CA, USA). The exposed mucosa was incubated with 20 µM BCECF-AM in pH 7.4 Krebs buffer for 20 min to load the duodenal epithelial cells. The preloaded duodenum was removed and immediately placed in a Petri dish filled with ice-cold, oxygenated Ringer solution, and with the mucosal side pinned down between two rubber supports. Under a stereomicroscope (Zeiss, Jena, Germany), the serosa and muscle layers were stripped using a sharp scalpel blade and fine forceps. The stripped duodenal mucosa was mounted mucosal side down on a horizontal two-chamber perfusion apparatus (RC-50, Warner Instrument Corp., Hamden, CT), in which mucosal and serosal sides were separately perfused in the closed system, and set on the stage of an inverted fluorescence microscope (Zeiss Axiovert 200). The chamber temperature was kept at 37 °C by internal resistive elements with automatic temperature controller (TC-344B, Warner Instrument). Each of two PE-50 polyethylene tubes was connected with the insertion tube into the each side of the chamber as to enable rapid changes of the perfusate.

The mucosal side (apical chamber volume 20 µl; actual exposed volume 5 µl) was perfused with pH 7.4 Krebs solution in a glass syringe at a rate of 100 µl/min using a Harvard infusion pump (Model 11 plus, Harvard Apparatus, Holliston, MA). The serosal side (chamber volume 50 µl; actual exposed volume 15 µl) was perfused with pH 7.4 HEPES bicarbonate Ringer solution in a glass syringe at a rate of 200 µl/min using a Harvard infusion pump (Model 33) or a peristaltic pump (Fisher Scientific, Pittsburgh, PA). The high CO₂ solution for mucosal perfusion was made 1 min prior to perfusion. The stripped mucosa was perfused with mucosal Krebs and serosal HEPES bicarbonate Ringer solutions for a 10 min stabilization period prior to performing experimental interventions.

**Measurement of pH, in vivo**

The perfused, BCECF-loaded, stripped duodenal mucosa was alternately excited at 495 nm and 440 nm using a rapid wavelength-switching devise (DG-4; Sutter Instrument Co., Novato CA) with narrow-bandpass interference filters (Chroma Inc., Brattleboro, VT). Fluorescence of the microscopically observed chambered segment of duodenal mucosa at 515 nm emission was recorded with a cooled charge-coupled device video camera (Hamamatsu Orca-EN; Hamamatsu USA, Bridgewater, NJ). Fluorescence intensity of the selected area was measured by first capturing the image (20 msec for 495 nm and 100 msec for 440 nm) using an Apple G5 microcomputer and digitized, with the area of interest defined and intensity measured using image analyzer software (OpenLab; Improvion, Lexington, MA, USA). Images were captured and stored and then analyzed using OpenLab software (Improvion Inc., Lexington, MA). Each 495 nm/440 nm image pair was captured every 30 or 60 s. Readings were taken ~1 s before and after each measured time point. The paired readings needed to calculate a fluorescence ratio were thus taken at a maximum of 3 s apart. Images were analyzed by selecting three areas of interest in the upper villous region, which were followed throughout the experiment. *In vitro* calibration using an aqueous solution containing 0.2 µM BCECF acid were made as described previously (25, 26). pH, was calculated according to an *in vitro* calibration curve. The mean ratio from the three selected areas was defined as the fluorescence ratio at the time period. Since the viability of the duodenum in a chambered preparation is less than other intestinal segments (6, 7) and the fluorescence
retention in the villous cells is time-dependent, we removed the tissues from living animals, limited the overall length of the studies to ~30 min in this preparation, and continuously monitored tissue fluorescence, which highly correlates with histological damage (6). Tissue autofluorescence and dark current signal were small (~200) in comparison with the villous cell BCECF intensity measured at 495 nm (1500-4000), and were subtracted from the fluorescent signal. The fluorescence image of the BCECF-loaded, stripped mucosa (Fig. 1A) was indistinguishable from the image of the BCECF-loaded mouse mucosa obtained in vivo (Fig. 1B) and retained this appearance up to ~20 min after the mucosa was placed in the chamber. Note that no senescent or extruding cells were observed in the villi of the stripped preparation after starting the perfusion, presumably due either to removal of the existing dying cells by the force of the perfusate, or due to inadequate time for cell extrusion to occur (27).

Experimental protocols

Perfusion protocols

After a 10-min stabilization period, the time was set at t = 0. First, to examine the pH_i stability, concurrent with the villous cell viability, the mucosal side of the stripped duodenum was perfused with pH 7.4 Krebs buffer and the serosal side was perfused with pH 7.4 gassed HEPES bicarbonate Ringer solution up to 20 min, since the BCECF fluorescence retention in the upper villous cells is limited for ~30 min including the stabilization period (10 min) enough to acquire the intensity to calculate pH_i. To examine the effect of serosal acidification on upper villous cell pH_i, the serosal side was perfused with pH 7.4 Krebs buffer for 2 min (basal period, t = 0 to 2 min), followed by with pH 7.0 or 6.4 gassed HEPES bicarbonate Ringer solution for 2 min (challenge period, t = 2 to 4 min), then with pH 7.4 Krebs for 16 min (recovery period, t = 4 to 20 min). The mucosal side was perfused with a pH 7.4 Krebs buffer solution throughout the experiment. To examine the effect of mucosal acidification on upper villous cell pH_i, the mucosal side was superfused with pH 7.4 Krebs buffer for 2 min (basal period, t = 0 to 2 min), followed with pH 6.4 saline or pH 6.4 high CO_2 solution for 4 min (challenge period, t = 2 to 6 min), followed by pH 7.4 Krebs for 6 min (recovery period, t = 6 to 12 min). The serosal side was perfused with pH 7.4 gassed HEPES bicarbonate

Fig. 1. Fluorescent images of BCECF-loaded mouse duodenal villi. BCECF fluorescent image of the mouse stripped duodenal mucosa (left) strongly resembles BCECF-loaded mouse duodenum imaged in vivo (right). Note that BCECF is localized in the villous cells, not in the crypts. Bars = 100 μM.
Ringer solution throughout the experiments (t = 0 to 12 min), with or without inhibitor (as described below) during the 4 min (challenge period, t = 2 to 6 min).

To examine the effect of inhibition of NHE-1 on upper villous cell pH$_i$ during luminal high CO$_2$ exposure, a specific NHE-1 inhibitor ZP (10 µM) was combined with the high CO$_2$ solution in the mucosal perfusate or with pH 7.4 gassed HEPES bicarbonate Ringer solution in the serosal perfusate during the challenge period. ZP, in the concentrations used, did not affect the pH of the high CO$_2$ solution. A non-specific NHE-1 inhibitor DMA (0.1 mM), or a Na$^+$/HCO$_3$- cotransporter (NBC) inhibitor DIDS (1 mM) was perfused in the serosal solution during the challenge period.

Furthermore, we tested the hypothesis that solutes in the serosal perfusate may indirectly alter villous epithelial function by neuronal mechanisms. For example, inclusion of 5-HT (0.1 mM) in the serosal, but not the mucosal perfusate rapidly stimulates TTX-inhibitable, 5-HT$_4$ receptor-dependent anion secretion in stripped mouse duodenum (4, 28), suggestive of an intact neurally-mediated mechanism. We thus examined the effect of 5-HT (0.1 mM) perfused in the serosal solution on upper villous cell pH$_i$ from t = 2 to 12 min. For the 5-HT studies, TTX (10 µM) or vehicle (0.1 % DMSO) was perfused in the serosal solution during the stabilization period (t = -10 to 0 min) and throughout the experiment (t = 0 to 12 min).

Diffusion of serosal fluorescence into the stripped mucosa

Perfusion of fluorescent molecules

To determine if chemical compounds perfused in the serosal solution distributed into the villous core of the stripped duodenal mucosa, fluorescent compounds of molecular size similar to that of ion transport inhibitors (MW 300 - 600) were perfused with the serosal solution, with fluorescence observed from the mucosal side. To observe the upper villous, the exposed duodenal mucosa was first incubated with 5(6)-chloromethyl SNARF®-1 acetate (20 µM) in pH 7.4 Krebs buffer for 20 min in order to load the upper villous cells. 5(6)-chloromethyl SNARF®-1 acetate is a brightest and long lasting intracellular fluorescent dye among the SNARF®-1 compounds when loaded in the duodenal villous cells in rat and mice. Furthermore SNARF®-1 is a single excitation/dual emission dye (29), enabling simultaneous acquisition of 580 nm/640 nm emission image pairs using a Dual-View™ image splitter (Optical Insights, Santa Fe, NM) with narrow-bandpass excitation (488 nm) and emission filters (Chroma). We first used three green fluorescent compounds at 10 µM, similar to the concentration used for cellular dye loading. Compounds included a cell-impermeant CF (MW 376.32), a hydrophilic compound (AO, MW 301.82) and a lipophilic fluorescent compound (HAF, MW 585.74). The ester form of CF, CF diacetate, is taken up into cells and stains the cytosol, whereas CF is cell impermeant (30). AO stains the nucleus by binding to nucleic acids, and HAF stains the plasma membrane due to the affinity of the acyl chain with the cellular plasma membrane (25, 31).

After the 10-min stabilization as described above, time was set as t = 0. The fluorescent compounds were perfused over the serosa for 10 min, followed by washout with HEPES-bicarbonate Ringer solution for 10 min. SNARF red fluorescence was used to focus on the upper villous cells, followed by acquisition of the green fluorescent image (495 nm excitation) in the same focal plane. Images were recorded every 1 min and readings were taken ~5 s before and after each measured time point. After the experiment, the mucosa was used for the preparation of frozen cryostat sections as described below.

To further study transmucosal diffusion of small molecules, we examined the effects of time and concentration on the diffusion of fluorescence into and across the stripped mucosa. CF (10 or 100 µM) was perfused in the serosal solution for 60 min with villous images recorded every 10 min as described above. In order to measure serosa-to-mucosa (s-to-m) transmucosal dye movement, the
mucosal perfusate was collected every 10 min for fluorescence measurement. Additionally, CF (10 µM) was perfused in the mucosal solution in order to determine fluorescence permeation in the opposite direction (m-to-s). CF concentration in the perfusate was calculated according to the concentration-fluorescent intensity calibration curve at 495 nm excitation at constant pH 7.4, in order to avoid pH effects on fluorescent intensity. At t = 10 or 60 min, the mucosa was sectioned with a cryostat, as described below.

**Intravenous fluorophore injection**

To simulate the diffusion of solutes between the systemic circulation and epithelial cells *in situ*, we injected fluorophore intravenously (*iv*) prior to examination of the mouse duodenum *in vivo*. The epithelial cells were first loaded with 5(6)-chloromethyl SNARF*®*-1 acetate (20 µM), as described above, in order to provide contrasting fluorescence. Then, under isoflurane anesthesia, the anesthetized mouse was placed prone on a microscope stage with the duodenal mucosa placed over the aperture of a perfusion chamber (RC22, Warner Instrument) as previously reported (26). The tail vein was cannulated with 30-G metal cannula connected to a PE-10 tube filled with pre-warmed saline for *iv* injection. The chamber was warmed at 37 °C by automatic temperature controller and perfused with pH 7.4 Krebs buffer at a rate of 200 µl/min using an infusion pump. After a 10-min stabilization, the upper villous was imaged using SNARF fluorescence, and the time was set as t = 0. At t = 5 sec, CF (1 mg/kg in 100 µl saline) was bolus *iv* injected into the tail venous cannula. A green fluorescent (495 nm) image was captured every 1 sec for 60 sec and every 1 min thereafter. At t = 10 min (10-min after *iv* injection), SNARF images and CF images were captured and the observed portion of duodenum was removed for preparation of the cryostat sections, as described below.

**Frozen sections**

We made cryostat sections in order to confirm the distribution of the fluorescent dye in the stripped duodenal mucosa either after perfusion of fluorescent compounds or after *iv* injection of fluorescent dye. Immediately following the experiments described above, the stripped mucosa or the observed portion of duodenum obtained from the *in vivo* study was mounted in O.C.T. compound (Miles, Inc., Elkhart, IN, USA) at −20°C and sectioned with a cryostat (Leica Microsystems, Wetzlar, Germany) at 8 µm thickness. After air-drying, the sections were coverslipped with glycerol containing 2.5 % w/v DABCO to retard fading. The sections were immediately observed with the Zeiss epifluorescent microscope; images were captured and recorded using a charge-coupled device color video camera (Hamamatsu Photonics, Hamamatsu, Japan) with imaging software, Simple PCI® (Compix Inc. Imaging Systems, Cranberry Township, PA).

**Statistics**

All data from six mice in each group are expressed as means ± SEM. Comparisons between groups were made by one-way ANOVA followed by Fischer’s least significant difference test. *P* values of < 0.05 were taken as significant.

**RESULTS**

**Stability of upper villous cell pH**

Since the villous structure stability is time-limited, we first determined the pH* sub i* stability in the upper villous cells in the stripped duodenal mucosa in our
perfusion system. Following a 10-min stabilization, $pH_i$ was stable for ~ 13 min during perfusion of pH 7.4 Krebs’ buffer in the mucosal solution and pH 7.4 gassed HEPES bicarbonate Ringer solution in the serosal solution (Fig. 2). $pH_i$ gradually declined during the 20-min measurement, presumably corresponding to accumulating villous cell damage as described previously (6, 7). To measure the accessibility of small solutes perfused in the serosal solution to the upper villous epithelial cell plasma membrane, the serosal solution pH was changed from pH 7.4 to pH 7.0 or 6.4. Since the serosal perfusate was CO$_2$/HCO$_3^-$-HEPES Ringer solution, [CO$_2$] in the serosal solution was 1.2, 2.8 and 8.3 mM, and $PCO_2$ 37, 87 and 260 Torr in pH 7.4, 7.0 and 6.4 solution, respectively. Compared with the stable $pH_i$ observed with perfusion of the pH 7.4 serosal perfusate, a 2-min perfusion of the pH 7.0 solution had no effect ($pH_i = 7.25 \pm 0.03$ in pH 7.0 group vs. $7.29 \pm 0.04$ in pH 7.4 group at $t = 4$, $p > 0.05$), whereas perfusion of the pH 6.4 solution rapidly acidified the upper villous cells, followed by a slow recovery of $pH_i$ to baseline after removal of the acid stress, followed by an irreversible decline of $pH_i$ after ~ 15 min (Fig. 2). These findings suggest that CO$_2$ or H$^+$ in the serosal perfusate rapidly enters the cytoplasm of the upper villous cells, but is slowly washed out, with $pH_i$ stability limited to ~ 15 min.

![Fig. 2. Basal Intracellular pH ($pH_i$) of the upper villous cells; effect of serosal acidification on $pH_i$. Upper villous cell $pH_i$ was stable during serosal perfusion of pH 7.4 HEPES bicarbonate Ringer solution until ~ 13 min after $pH_i$ measurement started, followed by the gradual decline of $pH_i$. Serosal pH 6.4 HEPES bicarbonate Ringer solution rapidly acidified the upper villous cells, followed by slow $pH_i$ recovery to the baseline, after which $pH_i$ declined. Data are expressed as means ± SEM (n=6). *$p < 0.05$ vs. pH 7.4 group.](image-url)
Effect of perfusion of small molecule inhibitors on CO₂-induced acidification

Upper villous cell pHᵢ in stripped duodenal mucosa was stable during luminal perfusion with pH 7.4 Krebs solution (basal period) or with perfusion of pH 6.4 saline ([CO₂] ~ 0) during the challenge period, used as a control for the high CO₂ solution, and during perfusion of the pH 7.4 solution during the recovery period (Fig. 3). Perfusion of the high CO₂ solution (pH 6.4, P_{CO₂} = 260 Torr) rapidly acidified the cells, followed by a plateau during CO₂ exposure, followed by recovery after CO₂ removal (Fig. 3).

To selectively inhibit the basolateral ion transporter in the villous cells, we examined the effect of the selective NHE-1 inhibitor ZP on CO₂-induced acidification. Since ZP inhibits only NHE-1 in the nM - µM range and NHE-3 in the mM range (32), 10 µM ZP was chosen so as to be in the NHE-1 selective range. Abundant data, obtained with a variety of techniques, strongly support the localization of NHE-1 to the intestinal epithelial cell basolateral membrane (33, 34) and NHE-1 plays a role in pHᵢ regulation in duodenal villous cells (25). Thus, any effect of ZP under these conditions would be predicted to be predominantly due to interaction of the compound with a transporter expressed in the epithelial cell basolateral membrane. Luminal ZP perfused with the mucosal high CO₂ solution augmented cellular acidification, whereas ZP perfused in the serosal solution during the challenge period had no effect on CO₂-induced acidification of the upper villous cells (Fig. 3). These results are consistent with luminally perfused ZP inhibiting basolaterally located NHE-1, enhancing acidification, and with ZP perfused in the serosal solution failing to reach the basolateral membrane of the upper villous cells at adequate inhibitory concentrations within the observation period.

Furthermore, we examined the effect of another NHE-1 non-selective inhibitor DMA (0.1 mM) or a NBC inhibitor DIDS (1 mM) in the serosal solution during the mucosal CO₂ challenge period. NBC is localized on the epithelial basolateral membrane in the duodenum (35 - 37). Unexpectedly, perfusion of DMA or DIDS in the serosal solution had no effect on CO₂-induced acidification (Fig. 4). These data suggest that either NHE-1 or NBC does not contribute to CO₂-induced epithelial pHᵢ regulation or that either DMA or DIDS fails to reach its molecular targets in the basolateral membrane of the upper villous cells when perfused in the serosal solution in this short-term experiment.

In preliminary studies, we used interventions described in recent publications describing a similar technique, in order to determine if they would increase the longevity of the stability and viability of the preparation. Interventions included using an O₂-bubbled mucosal perfusate, pretreating with indomethacin (1 µM) to inhibit surgical stress-induced prostaglandin synthesis, with TTX (10 µM) to eliminate the neural reflex, and loading the villous cells with BCECF after stripping, mounting, and stabilization, as described (19, 20). Nevertheless, the results in terms of pHᵢ stability, the pHᵢ response to the luminal CO₂, and the effect of serosal inhibitors, were similar, to results obtained in the absence of these interventions.
These data suggest that our methodological modifications, with the exception of the small volume perfusion chamber, are not the reason why pH\textsubscript{i} stability was short, pH\textsubscript{i} recovery was slow and the serosal inhibitors had no effect on pH\textsubscript{i}.

**Effect of the serosal 5-HT on villous tip cell pH\textsubscript{i}**

To examine the effect of mucosal neural activation on the villous tip cell pH\textsubscript{i}, 5-HT (0.1 mM) was perfused in the serosal solution with or without TTX (10 µM) treatment. Compared to the stable pH\textsubscript{i} in the control group, serosal 5-HT rapidly and progressively acidified the upper villous cells (*Fig. 5*). 5-HT-induced acidification was abolished by TTX treatment, suggesting that the effect of 5-HT is neuronally mediated, presumably by activation of the submucosal plexus, releasing acetylcholine in the villi (3; 28).

**Diffusion of fluorescent compounds from the serosal perfusate**

In order to further determine the diffusion properties of small molecules in the stripped preparation, we perfused the mucosa with several fluorescent
compounds with physical properties similar to commonly used receptor ligands and transport inhibitors. We additionally injected these compounds iv in vivo prior to obtaining the tissue in order to measure the diffusion of small compounds from the circulation into the mucosa.

Initial studies were directed at the measurement of the diffusion of fluorescent compounds perfused in the serosal or mucosal solutions. First, we examined a cell-impermeant green fluorescent compound, CF (MW 376.32). When perfused over the serosal surface and examined form the apical side, CF (10 µM) was easily visualized during 10-min challenge and was present after 10-min of washout, but did not stain the villi, which appear as negatively-stained structures overlying the bright fluorescence from the crypts. (Fig. 6A). To label the epithelial cells, we incubated a compound known to be taken up by epithelial cells, the intracellularly trapped fluorescent pH reporter SNARF-1 acetate (20 µM; 20 min) over the apical surface of the duodenum, which yielded a dye distribution consistent with loading of the epithelial cells (Fig. 6B). Fig. 6C depicts a merged image, in which the red, SNARF-stained villous epithelial cells are distinct from the green, CF-stained crypt fluorescence. CF distribution in the stripped mucosa was confirmed by cryostat sections corresponding to the same conditions depicted in 6A-6C. Examination of

Fig. 4. Effect of dimethylamiloride (DMA) or DIDS on CO₂-induced acidification. Serosally perfused DMA (0.1 mM) or DIDS (1 mM) had no effect on CO₂-induced acidification and recovery. Data are expressed as means ± SEM (n=6).
the sections confirms that the stripping procedure removed the serosa, muscle layer and part of the submucosa, leaving intact the muscularis mucosae and crypt cells (Figs. 6D, E). Furthermore, the green fluorescence was observed only in the muscularis mucosa and around the crypt cells, with no fluorescence was observed in

![Image](image.png)

**Fig. 5.** Effect of serosal 5-hydroxytryptamine (5-HT) on intracellular pH (pH<sub>i</sub>). Serosal 5-HT (0.1 mM) rapidly acidified the upper villous cells. TTX (10 µM) treatment in the serosal perfusion abolished the effect of 5-HT. Data are expressed as means ± SEM (n=6). *p < 0.05 vs. vehicle group, †p < 0.05 vs. 5-HT group.

**Fig. 6.** Distribution of fluorescent dye in mouse duodenal villi. A-F: Perfusion of 5(6)-carboxyfluorescein (CF, 10 µM) in the serosal solution in the stripped duodenal mucosa. After washout, the green CF fluorescence remained in the crypt mucosa, but did not stain the villous core (A). In contrast, SNARF®-1 red fluorescence was localized in the villous cells (B); C is merged SNARF-CF images. D-F: Corresponding frozen sections revealed the limited localization of CF on the muscularis mucosa (MM) and in the crypt area (c), but not in the villi (v) (D, E), with SNARF localized to the epithelial cells (F). G-K: Localization of iv injected CF (1mg/kg) in vivo. CF was quickly distributed to the villous core, but not the villous epithelial cells (G), as compared with SNARF®-1-positive villous epithelial cells (H); I is merged SNARF-CF images. J-K: Corresponding frozen sections demonstrate the interstitial distribution of iv CF in the villous core (v), crypt (c) and muscularis propria (PM). Bars = 100 µm.
the villous core or in the villous cells. Figs. 6E and F are the same sections in which the green CF fluorescence was present only in the muscularis mucosa and in the vicinity of the crypt cells, but not in the villous core or in the villous cells (Fig. 6E) with the SNARF red fluorescence limited to the cytoplasm of the upper villous cells (Fig. 6F). These images correspond to the image obtained in vitro (Fig. 6A-C), suggesting that the lack of villous CF staining was not due to nonspecific bleaching or dye leaching, or due to the lack of stainable villous cells.

To simulate the path of systemically administered compounds through the mucosa, we injected CF iv (1 mg/kg) during direct microscopic observation in vivo. In most tissues, iv injected CF rapidly leaks from capillaries and is distributed in the interstitial fluid (38, 39). CF quickly appeared in the villous core vasculature and was distributed in the core interstitium within ~ 3 sec after iv injection, where it remained during the experiment. Fig. 6G depicts the positive staining of the villous core, without staining of the epithelial cells, compared to SNARF staining of the upper villous cells (Fig. 6H, I) 10-min after iv injection. Positive staining of the villous core, crypt and muscularis propria was confirmed by corresponding frozen sections (Fig. 6J, K), in contrast to the staining pattern of CF perfused over the serosal surface of the stripped mucosa (Fig. 6D, E).

Since the diffusion of chemical compounds into the tissue is time- and concentration-dependent, we examined the effects of perfusion time and concentration of CF on the diffusion of the fluorescent signal into and across the stripped duodenal mucosa. CF movement from the serosal to the mucosal solutions gradually and concentration-dependently increased until ~ 40 min, reaching a

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Fig. 7. Diffusion of a cell-impermeant dye, carboxyfluorescein (CF) from the serosal solution into the mucosal solution. A: CF permeation from the serosal to mucosal solution (s-to-m) or from the mucosal to serosal solution (m-to-s). CF permeation (s-to-m) gradually and dose-dependently increased, reaching a plateau after ~ 40-min of perfusion. CF permeation in 100 µM (s-to-m) group was only twice that of the 10 µM (s-to-m) group, whereas m-to-s permeation with 10 µM CF mucosal perfusion was similar to the rate of s-to-m permeation with 100 µM CF serosal perfusion. B: Villous fluorescent intensity measured in the stripped preparation during serosal perfusion with CF. Intensity quickly reached a plateau at 5 min, with no further increase. The intensity in the 100 µM (s-to-m) group was about ten times as high as that in 10 µM (s-to-m) group. C: In vitro images of villi in the stripped mucosa perfused with the serosal CF, used for the measurements depicted in A and B. CF concentrations and times used: 10 µM (a, b), 100 µM (c, d) at 10 min (a, c) and 60 min (b, d). The villi were negatively stained during serosal perfusion with CF at 10 min (a, c), but were less visible at 60 min (b, d). Bars = 100 µm. D: Corresponding frozen sections of the stripped mucosa perfused with serosal CF 10 µM (a, b) and 100 µM (c, d) at 10 min (a, c) and 60 min (b, d). The luminal surface of the villi is denoted by the white line. CF 10 or 100 µM only stained the muscularis mucosa (MM) and crypts at 10 min (a, c), but CF appeared in the villi at 60 min (b, d). Note that the villous height was much shorter at 60 min than that at 10 min. Bars = 100 µm. E: Mucosal perfusion of CF 10 µM over the stripped mucosa. The villi were negatively stained at 5 min (a) and were stained with CF at 10 min (b), more visible after a 10-min washout (c). Note that villous cells remained negative, whereas the villous core was CF-positive after washout (c). Corresponding frozen sections revealed CF staining in the villous (v) cores (d). Bars = 100 µm.
plateau by 60 min (Fig. 7A). Despite a 10-fold concentration difference, the plateau rate of CF movement in the 100 μM group was only twice as high as that in the 10 μM group in the serosa-to-mucosa (s-to-m) direction. Furthermore, CF leakage from the mucosa-to-serosa (m-to-s) direction at 10 μM was similar to that of 100 μM s-to-m direction. These data suggest that the s-to-m diffusion constant is unlikely consistent with simple diffusion, and that m-to-s diffusion (absorption) is much higher (~10 times greater) than s-to-m diffusion (secretion) in the mouse duodenal mucosa. In contrast, the fluorescent intensity measured in vitro over the villi during serosal CF perfusion rapidly reached a plateau at 5 min and remained at the same intensity, with fluorescent intensity during serosal perfusion of 100 μM CF ~ 10-fold
higher than villi perfused with 10 µM CF (Fig. 7B). These studies suggest that CF intensity measured in vitro corresponded to the fluorescence of CF in the serosal perfusate, and not of the villous cells or the villous core. Fig. 7C depicts images of stripped mucosa serosally perfused with CF. At 10 min, the villi were negatively stained with 10 or 100 µM CF (Fig. 7C a, c), with little change observed until ~ 30 min (data not shown). However, marked loss of negative villous staining was observed at 60 min at either concentration (Fig. 7 b, d). To further examine CF-related villous staining, we examined corresponding frozen sections (Fig. 7D). Serosal perfusion of 10 µM CF (Fig. 7D a) and 100 µM (c) at 10 min stained the muscularis mucosa and the crypts, but did not stain the villous core or the villous cells. 10 µM CF (Fig. 7D b) and 100 µM (d) stained the villi as well as crypts and muscularis mucosa at 60 min, although villous height at 60 min was shorter than that at 10 min representing likely severe damage. These data suggest that the lack of negative staining observed in vitro at 60 min (Fig. 7C) are consistent with shortened, damaged villi (Fig. 7D). In contrast, mucosal perfusion of CF (10 µM) rapidly stained the villous core within 10 min (Fig. 7E a, b). The in vitro image after washout of mucosal CF clearly shows positive villous core staining with negative staining of the villous epithelial cells (Fig. 7E c), confirmed by inspection of the corresponding frozen sections (Fig. 7E d). This pattern is also comparable to the villous core staining observed in vivo after iv CF injection (Fig. 6K), suggesting that luminal fluorescence rapidly diffuses into the villous core, consistent with the rapid CF permeation observed in the m-to-s direction (Fig. 7A). All of these data demonstrate that at least in the short-term, diffusion of fluorescent compounds into the structurally intact villi from the serosal perfusate was not observed, and that in the longer term, the fluorescent compound diffused into damaged villous structures and epithelial cells. Furthermore, the gradual increase in transmucosal CF permeation (Fig. 7A) with the negative CF staining in the villi but the positive staining in the crypts (Fig. 7D) up to ~ 30 min also suggests that s-to-m CF permeation represents transmucosal dye permeation across the crypt and not the villous region of the mucosa. Possible explanations as to why CF permeation reached a plateau by 60 min with CF staining in the shortened villi at 60 min is that the leaked CF from into the lumen could be back-diffused (absorbed) into the already damaged villi. Moreover, the rapid diffusion of the luminal fluorescence into the villous core, presumably due to the paracellular permeability as previously reported (40, 41), further supports our results that the luminal inhibitors quickly affect the basolateral membrane ion transporters of the upper villous cells. It is possible that edge damage, as previously reported (42), may explain the rapid transmucosal leakage of CF or the observed plateau phase of CF permeation. Nevertheless, the observed asymmetry of transmucosal dye diffusion does not support this possibility.

Since many commonly used inhibitors and receptor ligands have varying water solubility, we examined the diffusion of the hydrophilic fluorophore AO (MW 301.82) and the hydrophobic fluorophore HAF (MW 585.74) perfused from the serosal solution into stripped mucosa. Frozen sections of the stripped mucosa
confirmed that serosal perfusion of AO (Fig. 8A) and HAF (Fig. 8C) stained the crypt cells and nuclei in the crypt area, but did not stain the villous core or villous epithelial cells, suggesting that the lipid solubility of the fluorophore does not affect its staining pattern. In contrast, post-staining of serial frozen sections with AO (Fig. 8B) or HAF (Fig. 8D) revealed that the cell cytoplasm, nuclei or plasma membranes, were readily stained if the dyes had free access to these structures.

DISCUSSION

We demonstrated that in perfused mouse stripped duodenal mucosa, compounds in the mucosal perfusate readily and rapidly penetrated into the epithelial layer, and affected epithelial function, regardless of the epithelial polarity of the transporter
inhibited. Fluorescent compounds infused iv rapidly penetrated the villous core. In contrast, compounds in the serosal perfusate had limited time and concentration-dependent access to the villous epithelial cells, although they had rapid access to sub-epithelial neural elements. Furthermore, the villous cell viability reflected by pHi and the villous structural stability observed in vitro and in frozen sections were time-limited in chambered, stripped duodenal mucosa.

The stripped, chambered intestinal preparation has been used since the 1970s for the investigation of net epithelial transmucosal solute and electron (Isc) movement in the intestine. Advantages of the chambered preparation include ability to access the serosal membrane and ability to measure and correlate bidirectional tracer fluxes, voltage, resistance, and current. Although much data have been obtained with the use of the chambered system, these data should be interpreted in light of the architectural features of the mucosa. Frog skin, a relatively flat, uniform, and tight epithelium was chosen for initial studies of epithelial function in vitro (1). The small intestine is, by contrast, thrown into villi, with the average length in mice from crypt base to upper villous 400 µm. Therefore, a solute perfused in the serosal solution might traverse 100 µm prior to encountering the crypt epithelial cell basolateral membrane, whereas solutes must traverse a distance of 400-500 µm in order to reach the upper villous epithelial cells. Assuming simple diffusion, uniform diffusion constants, and identical initial concentrations, the time for a solute to reach a given concentration remote from its starting point is related to the (distance diffused)² (43). If the distance from the serosal perfusate to the crypt cell is 100 µm and to the upper villous cell is 500 µm, it will take 25 times longer, by simple diffusion, for the solute to achieve the same concentration at the villous cell than in the crypt cell. Over a long enough period, a steady-state will be achieved, that will minimize these differences, as we also demonstrated with our studies of transmucosal CF diffusion (39). Flat, tight epithelia such as esophagus, stomach, and colon are well-suited for in vitro study of intact tissue. The leaky, fragile, villous-structured duodenum thus presents many interpretational challenges in this regard.

Relatively few studies have addressed the comparative contributions of the mucosal vs. muscular layers towards the overall viability of the chambered preparation. Recent data suggests that the duodenum is the most fragile intestinal segment in this regard, with the villous tips and edges of the preparation being particularly at risk for damage (6, 7, 42, 44). Under direct observation, we noted marked epithelial damage within 30 min of mounting the tissue after the stripping, which is why we studied duodena within 30 min of harvest, with monitoring of villous cell function (pHₐ) and morphology. Our results showed that pHₐ was stable for ~ 15 min followed by pHₐ decrease and that the villous structure was intact for ~ 30 min followed by the villous damage and shortening by 60-min perfusion, although stable pHₐ and minimal villous cell damage is observed for ~ 60 min in the murine and rat duodenum in vivo (25, 26, 37). Most studies of chambered mucosa however are made using classic Ussing-type
chambers, which have a relatively large aperture and gas lifts in both hemichambers, which would likely oxygenate the tissue more effectively than we experienced with the optical chamber we employed, which relies on the perfusion of oxygenated solutions and has a relatively small aperture. Although the combination of a small aperture and continuous perfusion may place undue shear stress on the villi relative to that in conventional Ussing chambers, conventional Ussing chambers do not permit tissue microscopy. Surprisingly, in prior studies, duodenal transmucosal electrical conductance or the transmucosal diffusion of horseradish peroxidase is unaltered by muscular stripping, suggesting that the muscle layer offers little resistance to the permeation of large molecules (42), whereas forskolin-induced depolarization is present despite frank sloughing of the villous epithelial cells (7), which suggests particular care must be given to the interpretation of bulk transmucosal permeability and electrical measures in chambered intestinal preparations.

In order to study the polarity of inhibitor action, we studied the basolaterally-expressed proteins NHE-1 and NBC, which facilitate recovery from intracellular acidification. Inhibition of NHE-1 or NBC enhances acidification and delays recovery from low pH\textsubscript{i}, as previously reported (14, 25, 35, 45). Nevertheless we failed to demonstrate inhibition due to serosally perfused DMA, DIDS or ZP on luminal CO\textsubscript{2}-induced acidification in the upper villous cells. In contrast, serosal acidification rapidly acidified the upper villous cells, with slow recovery from the acidification, suggesting that CO\textsubscript{2}/H\textsuperscript{+} could reach the epithelial cells from the serosal solution only with a large concentration gradient across the mucosa, consistent with prior observations of alterations of villous epithelial pH\textsubscript{i} and membrane potential in response to serosal ion composition changes (9, 20). Since the accessibility of serosally perfused inhibitors to the basolateral membrane of the upper villous cells has not been directly studied in stripped intestinal mucosa, these results prompted the investigation of the diffusion characteristics of the serosally perfused fluorescent compounds.

We demonstrated that in our stripped preparation, the highly selective NHE-1 inhibitor, ZP lowers pH\textsubscript{i} when perfused apically, but has no effect when perfused serosally. Since NHE-1 is unequivocally localized to the epithelial basolateral membrane (33, 34, 46), ZP can best affect pH\textsubscript{i} if it is present at the epithelial cell basolateral membrane at inhibitory concentrations. Thus, within 10 min of perfusion, apically perfused ZP likely reached the basolateral membrane, whereas serosally perfused ZP did not. One explanation for this seeming anomaly is that the small intestine, having low resistance (~30 \(\Omega\text{\cdot cm}^2\)) is readily permeable to small organic molecules. Indeed, this permeability is the basis for the oral bioavailability of many pharmaceuticals, even though it is unknown whether this permeability is transcellular via drug transport proteins or if it is paracellular. The ready penetration of mucosal CF into the mucosa further supports this hypothesis. Another possibility is that ZP interacted with NHE-1 expressed cells near the epithelial cells, although it is unclear how this interaction would rapidly alter...
epithelial cell pH$_i$ regulation. The distance traversed, from the apical perfusate to the epithelial cell basolateral membrane is ~ 10 µm, which is greater than the distance between the subepithelial capillaries and the enterocyte basolateral membrane, but far less than the distance from the serosal surface of the stripped

![Fig. 9. Scheme of solute diffusion in intestinal epithelia. Distances (a-e) in the duodenal mucosa over which solute must diffuse to reach the upper villous cell basolateral membrane are shown. In mucosa stripped of muscularis propria (pm) and serosa (s), serosal solutes must traverse the distance between the serosal solution to the upper villous cell basolateral membrane (b), much further than the distance to the crypt cell basolateral membrane (a). In contrast, the luminal solute traverses a much shorter distance (d) by transcellular or paracellular diffusion. In vivo, the vascular network (red and blue) present in the villous core transports the solute to the epithelial basolateral membrane in the shortest distance (c). Furthermore, submucosal plexus neurons (sp) stimulated by the serosal solutes quickly conduct the signals to the upper villous, releasing mediators just beneath the villous cell basolateral membrane (e). In the murine duodenum these distances are approximately (in µm): $a = 100, b = 400-500, c = < 10, d = 10, e = < 10$. Arteriole; A (red), venule; V (blue), lymphatic vessels; Ly (yellow), muscularis mucosa; mm, submucosal layer; sm, myenteric plexus; mp]
preparation to the upper villous epithelial cell basolateral membrane (Fig. 9). As a consequence, compounds such as ZP that are predicted only to interact with epithelial basolateral membrane proteins are more likely to rapidly exert their effects when perfused mucosally than when perfused serosally in a stripped preparation. This has been previously observed with DIDS, which inhibits basolateral NBC with much greater avidity than apical membrane SLC26A anion exchangers (35, 47), but exerts its effect in the stripped system only when introduced from the apical side (19), probably due to the differential inhibition affinities to SLC26A or NBC, or the relative contribution of SLC26A or NBC to pH regulation. Nevertheless, transepithelial small molecule permeation is more frequently observed in leaky epithelia such as duodenum, and not with tight epithelia such as colon. In whole-tissue studies, it is not possible to distinguish effects of serosal compounds on the crypt vs. villous cells, clouding interpretation somewhat. In intact intestine, we have shown that luminal H⁺ is converted to CO₂ prior to traversing the enterocyte apical membrane. Cellular CO₂ is then reconverted to H⁺, where it traverses the enterocyte basolateral membrane, increasing H⁺ in the sub-epithelial interstitium, where it can then activate submucosal acid sensors on afferent nerves (21). Efferent responses to this activation include the stimulation of mucus and HCO₃⁻ secretion and augmentation of mucosal blood flow (48). One possibility is that the 5-HT pathway mediates the acid-induced HCO₃⁻ secretory response.

Three fluorescent dyes, cell-impermeant CF, hydrophilic, nucleophilic AO, and hydrophobic HAF, were used to investigate how a compound’s physical properties affect its diffusion properties. When perfused in the serosal solution, all dyes failed to stain the villous core and villous cells, as judged by in situ fluorescent imaging in the stripped mucosa and in the corresponding frozen sections, but appeared in the crypt area and in the muscularis mucosa. CF penetrated the mucosa, reaching steady-state in the apical solution in ~40 min. These results suggest that the dye perfused in the serosal solution only slowly reaches the upper villous cells, supporting our findings that the serosally perfused inhibitors had no measurable effect on villous epithelial cell function within 10 min after application. In contrast, iv injected CF rapidly distributed in the villous core, consistent with rapid dye delivery and homogenous distribution into the interstitial space via blood flow, and mucosally perfused CF readily penetrated the mucosa within 10 min, probably due to paracellular diffusion, then stained the villous core, supporting our findings that the luminaally perfused inhibitors rapidly affect the function of the basolateral membrane ion transporters of the upper villous cells.

Many studies of stripped mucosa document rapid changes of Iₛₑ, accompanied by slower changes of absorption and secretion in response to serosal perfusion of bioactive compounds (49; 50). The rapid release of acetylcholine by basolateral 5-HT, inhibited by the neurotoxin TTX supports its mediation by a cholinergic neural pathway (28). Our studies, in which serosal 5-HT rapidly decreased pHᵢ, inhibited by TTX, are consistent with neural mediation of the effects of serosally perfused 5-
HT, suggesting that acetylcholine released in the upper villus by serosally applied 5-HT via submucosal plexus nerves stimulates HCO$_3^-$ secretion from the upper villous cells, acidifying the villous cells. It is unknown, however, if the response to TTX reflects a direct effect of Ach on the enterocyte basolateral membrane or an indirect effect. Prior studies of intestine in vitro indicated that enterocyte M3 receptors mediate intestinal anion secretion, whereas M1 receptors are sited on submucosal neurons (51; 52). In duodenum, carbachol-stimulated HCO$_3^-$ secretion is associated with elevation of cytosolic Ca$^{++}$ concentration (5). Therefore, it is likely that the 5-HT-induced epithelial acidification resulted from Ach secretion from intra-epithelial efferent nerves activating epithelial M3 receptors, increasing HCO$_3^-$ secretion. In general, rapid responses to low concentrations of serosally perfused compounds are inhibited by TTX, in contrast to the slow responses to high concentrations of serosally perfused compounds whose effects are TTX-insensitive, consistent with much greater access of the serosal perfusate to intact neural elements than to the villous cells in the preparation. The TTX sensitivity of the acidification combined with the known lack of effect of luminal serotonin on duodenal HCO$_3^-$ secretion (53) strongly suggests that the 5-HT effect is neurally-mediated and not a direct effect on the enterocytes, although more recent studies suggest possible expression of 5-HT receptors on enterocytes (4).

In summary, the villous structure, the leaky tight junctions, and limited in vitro viability (in our perfusion chamber) of the proximal intestine confound studies conducted in stripped preparation in that solutes perfused in the apical solution can readily penetrate the mucosa, whereas those perfused in the serosal bath have only limited, time-dependent access to the villous epithelium. The 400-500 μm distance between the serosal surface and the basolateral membrane of the upper villous epithelial cells presents a formidable and unphysiological diffusion barrier, even in the absence of other cellular barriers, such as restrictive intercellular junctions. Finally, the presence of submucosal plexus neurons with villous mucosal projections of secretomotor fibers, combined with electrophysiological studies, strongly supports neural mediation of the rapid epithelial responses to low concentrations of bioactive compounds introduced into the serosal perfusate.

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