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Role of Down-Regulated in Adenoma Anion Exchanger in HCO_3^- Secretion across Murine Duodenum

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Abstract

Background & Aims—The current model of duodenal HCO_3^- secretion proposes that basal secretion results from Cl^-/HCO_3^- exchange whereas cAMP-stimulated secretion depends on a cystic fibrosis transmembrane conductance regulator channel (Cftr)-mediated HCO_3^- conductance. However, discrepancies in applying the model suggest that Cl^-/HCO_3^- exchange also contributes to cAMP-stimulated secretion. Of two candidate Cl^-/HCO_3^- exchangers, studies of putative anion transporter-1 (Pat-1) knockout (KO) mice find little contribution of Pat-1 to basal or cAMP-stimulated secretion. Therefore, the role of down-regulated in adenoma (Dra) in duodenal HCO_3^- secretion was investigated using DraKO mice.

Methods—Duodenal HCO_3^- secretion was measured by pH stat in Ussing chambers. Apical membrane Cl^-/HCO_3^- exchange was measured by microfluorometry of intracellular pH (pH_i) in intact villous epithelium. Dra expression was assessed by immunofluorescence.

Results—Basal HCO_3^- secretion was reduced ~55-60% in the DraKO duodenum. cAMP-stimulated HCO_3^- secretion was reduced ~50% but short-circuit current (I_{sc}) was unchanged, indicating normal Cftr activity. Microfluorimetry of villi demonstrated that Dra is the dominant Cl^-/HCO_3^- exchanger in the lower villous epithelium. Dra expression increased from villous tip to crypt. DraKO and WT villi also demonstrated regulation of apical Na^+/H^+ exchange by Cftr-dependent cell shrinkage during luminal Cl^- substitution.

Conclusions—In murine duodenum, Dra Cl^-/HCO_3^- exchange is concentrated in the lower crypt-villus axis where it is subject to Cftr regulation. Dra activity contributes most basal HCO_3^- secretion and ~50% of cAMP-stimulated HCO_3^- secretion. Dra Cl^-/HCO_3^- exchange should be considered in efforts to normalize HCO_3^- secretion in duodenal disorders such as ulcer disease and cystic fibrosis.

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Introduction

Duodenal mucosal HCO_3^- secretion, important in the formation of an alkaline mucus barrier against gastric acid, involves contributions by apical membrane Cl^-/HCO_3^- exchange activity and requires Cftr activity for cyclic-nucleotide stimulated secretion[1,2]. The identity of the anion exchangers involved in duodenal HCO_3^- secretion has been a subject of investigation for several decades. Past immunolocalization studies have ruled out involvement of the SLC4 bicarbonate transporter SLC4A2 (anion exchanger isoform 2) which is expressed in intestinal epithelium but does not reside in the apical membrane compartment[3]. A recent addition to the family, i.e., SLC4a9 (anion exchanger 4, Ae4), has tentatively been localized to the apical membrane of murine duodenum but has very low levels of expression[4,5]. Rather, most evidence supports the involvement of the SLC26A family of multifunctional anion exchangers in duodenal Cl^-/HCO_3^- exchange. Of the 10 family members, two members have been localized to the apical membrane of intestinal epithelia in murine models. Dra (Slc26a3) exhibits high rates of Cl^-/HCO_3^- exchange and loss-of-function mutations are responsible for the human genetic disease congenital Cl^- losing diarrhea (CLD)[6-9]. Pat-1 (Slc26a6) is a robust Cl^-/HCO_3^- exchanger but also exchanges sulfate, oxalate and formate at lower rates[10-12]. Recent studies of murine duodenum indicate that Pat-1 is the dominant Cl^-/HCO_3^- exchcanger in the upper villous epithelium[5].

Although well recognized that duodenal HCO_3^- secretion involves the concerted activities of Cftr and anion exchanger(s), the relative contribution of each pathway to net HCO_3^- secretion under basal and cyclic nucleotide-stimulated conditions has been difficult to establish. Cftr has significant HCO_3^- permeability relative to Cl^- (~ 1:5, respectively[13]), increases Cl^-/HCO_3^- exchange activity by providing a Cl^- "leak" to recycle Cl^-[14,15], and, when activated, alters cell volume and membrane potential[16,17]. Changes in cell volume regulate the activity of the apical membrane Na^+/H^+ exchanger Nhe3[17] whereas changes in membrane potential may directly alter Cl^-/HCO_3^- exchange based on recently proposed electrogenic stoichiometries for PAT-1 ($1Cl^-$:2 HCO_3^-) and DRA ($2Cl^-$:$1HCO_3^-$)[18]. Particularly during stimulated secretion, these factors confound attempts to partition duodenal HCO_3^- secretion between a Cftr HCO_3^- conductance and Cl^-/HCO_3^- exchange.

In both human and murine duodenum, most studies agree that basal rates of HCO_3^- secretion depend primarily on Cl^-/HCO_3^- exchange activity. Inhibition of Cl^-/HCO_3^- exchange by removal of luminal Cl^- abolishes basal HCO_3^- secretion and, although reduced, a finite rate of basal secretion is retained in the absence of Cftr activity[2,19-21]. In contrast, the role of Cl^-/HCO_3^- exchange during cAMP-stimulated HCO_3^- secretion is less clear. Although a contribution of Cl^-/HCO_3^- exchange has been postulated[2], studies in which apical membrane Cl^-/HCO_3^- exchange is inhibited by luminal Cl^- removal have concluded that most cAMP stimulated secretion involves a Cftr-mediated HCO_3^- conductance[2,21]. This is further complicated by recent studies showing that the HCO_3^- permeability of recombinant CFTR may be increased in the absence of extracellular Cl^-[22]. Further, two studies of murine duodenal HCO_3^- secretion have yield indirect evidence of a significant contribution of Cl^-/HCO_3^- exchange during cAMP stimulation when physiological concentrations of Cl^- are present in the luminal bath. In the first study[2], inhibition of carbonic anhydrase activity reduced the rate of HCO_3^- secretion by 50% without altering the transepithelial I_{sc}, an index of Cftr activity. In a second study[23], measurements of serosal-to-mucosal HCO_3^- and Cl^- flux found that only 50% of cAMP-stimulated HCO_3^- secretion was associated with the I_{sc} during loss of $Na^+/K^+/2Cl^-$ cotransporter Nkcc1 activity. In efforts to identify the relevant Cl^-/HCO_3^- exchangers contributing to HCO_3^- secretion, recent investigations of Pat-1KO duodenum show that Pat-1 only provides ~20% of basal HCO_3^- secretion and no contribution to cAMP stimulated secretion[24]. Thus, it is imperative that the role of the other major Cl^-/HCO_3^- exchanger, Dra, be investigated for its contribution to duodenal HCO_3^- secretion.

Identifying the role of apical membrane Cl^-/HCO_3^- exchangers is fundamental to creating an accurate model of duodenal HCO_3^- secretion. The model depends upon studies of native intestinal mucosa, but specific inhibitors or stimulants that effectively discriminate between the exchanger isoforms are not available or exert additional effects on other anion transport proteins. Therefore, in the present study, we performed *ex vivo* duodenal studies using mice with gene-targeted deletion of Dra to assess its contribution to transepithelial HCO_3^- secretion.

Methods

Animals

Mice with the gene-targeted disruptions of murine *Slc26a3* (*Dra*)[25], *Slc26a6* (*Pat-1*)[24], *Abcc7* (*Cftr*)[26] or *Slc4a9* (*Ae4*)[5] on a mixed genetic background were used. All comparisons of homozygous knockout mice were made with gender- and age-matched (+/+) siblings (WT). Mutant mice were identified using PCR-based analysis of tail-snip DNA[27]. Mice were maintained on standard laboratory chow (Formulab 5008 Rodent Chow; Purina) and tap water until the day before an experiment. The drinking water of DraKO (and WT littermate) mice routinely contained 50% Pedialyte® to prevent dehydration secondary to diarrhea[25]. Mice (2-4 months) were fasted overnight before experimentation but were provided water *ad libitum*. The mice were singly housed in a temperature (22-26°C) and light (12:12-h light-dark)-controlled room in the AAALAC-accredited animal facility at the Dalton Cardiovascular Research Center. All experiments involving animals were approved by the University of Missouri Animal Care and Use Committee.

pH stat

The method for pH stat measurement of transepithelial HCO_3^- secretion in murine duodenum has been previously described[23]. Excised proximal duodenum was stripped of external muscle layers and mounted on Ussing chambers (0.25 cm^2 aperature). Neural activity and prostaglandin generation were blocked using tetrodotoxin (0.1 μM, serosal) and indomethacin (1 μM, bilateral). Spontaneous potential difference was voltage-clamped to 0 mV allowing measurement of the transepithelial I_{sc} (μeq/cm^2 tissue surface area·hr) and conductance (G_t, mS/cm^2). All experiments were carried out under short-circuited conditions with the serosal bath serving as ground. The mucosal surface was bathed with unbuffered NaCl solution and vigorously gassed with 100% O_2. The serosal-to-mucosal bicarbonate flux (J_{sm}^{HCO3}) was measured by clamping luminal bath pH at 7.4 using 5 mM HCl administered by an automatic titrator (Radiometer Analytical, Lyon, France). In some experiments, Cl^- content of the luminal solution was replaced equimolar using $gluconate^-$ and $isethionate^-$. The serosal bath contained Krebs bicarbonate Ringers solution (KBR) containing 10 mM glucose (pH 7.4; gassed with 95% O_2:5% CO_2; 37°C). Subsequent experiments consisted of two 30 min flux periods: an untreated period (Basal), followed by a treatment period (cAMP) beginning 15 min after bilateral 10 μM forskolin addition.

Intracellular pH (pH_i) measurement

The method used for imaging villous epithelial cells in intact murine duodenal mucosa has been previously described[5,15]. Briefly, muscle-stripped duodenum was mounted in a horizontal perfusion chamber where luminal and serosal surfaces of the tissue were independently bathed. Villi immobilized under a fine mesh were selected for observation using an Olympus BX50WI microscope with water immersion objective (Olympus, Melville, NY). For pH_i measurements of the lower villous epithelium, the mucosa was placed over a short piece of 1.2 mm glass tubing to enable visualization and rapid solution changes. The mucosa was incubated on the luminal side with 16 μmol/L of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 10 minutes before superfusion. Intracellular pH was measured by the dual excitation wavelength technique (440-and 495-nm),

and 10 villous epithelial cells were imaged at 535-nm emission. The 440/495-nm ratios were converted to pH_i using a standard curve generated by the K^+/nigericin technique[28]. For Cl^-/HCO_3^- exchange measurements, the luminal superfusate was a 55 mM Cl^- KBR solution whereas the serosal superfusate was Cl^--Free KBR (Cl^- replaced with isethionate$^-$) containing 1 μM 5-(N-ethyl-n-isopropyl)-amiloride (EIPA) to block the activity of the basolateral membrane Na^+/H^+ exchanger NHE1 (gassed with 95% O_2: 5% CO_2; 37°C;pH 7.4). Cl^-/HCO_3^- exchange activity was estimated from the $\Delta pH_i/\Delta t$ during removal and replacement of luminal Cl^-, taking into account the total buffering capacity of the system. For Na^+/H^+ exchange measurement, the luminal perfusate was 55 mM Cl^- KBR except NaTES replaced $NaHCO_3$ on an equimolar basis (gassed with 100% O_2). The serosal superfusate was identical except N-methyl-D-gluconate ($NMDG^+$) replaced Na^+ on an equimolar basis. Na^+/H^+ exchange activity was estimated from the $\Delta pH_i/\Delta t$ during removal and replacement of luminal Na^+[29].

Immunofluorescence

Dra protein expression in 4 μm frozen sections of duodenum was estimated by immunofluorescence, as previously described[30].

Villous epithelial cell height

Villous epithelial cell height was used as an index of cell volume[17]. Fresh sections (1-2 mm) of duodenum were immobilized under a nylon mesh in KBR solution at room temperature. Cross-sectional views of epithelial cell height in the lower one-third villous axis were selected under bright field microscopy (Olympus BX50WI microscope) and images were acquired with a Sensicam CCD camera (Cooke, Auburn Hills, MI). After a control period, KBR was replaced with Cl^--Free KBR for 5 min before reacquisition of images at the same locus. Morphometry of cell height before and after images was performed using ImagePro Plus (Media Cybernetics, Carlsbad, CA).

Materials

The fluorescent dye BCECF-AM was obtained from Invitrogen (Carlsbad, CA). Tetrodotoxin and forskolin were obtained from Biomol International L.P. (Plymouth Meeting, PA). Other materials were obtained from either Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Statistics

All values are reported as mean±SEM. Data between two treatment groups were compared using a 2-tailed unpaired Student *t*-test assuming equal variances between groups. Data from multiple treatment groups were compared using a one-way ANOVA with a post hoc Tukey's *t*-test. Probability $p<0.05$ was considered statistically significant.

Results

Dra contributes to basal HCO_3^- secretion across the duodenum

Recent investigations of Pat-1KO duodenum show a minor contribution (~20%) of Pat-1 to net HCO_3^- secretion under basal conditions[24]. Therefore, the contribution of Dra to basal and cAMP-stimulated HCO_3^- secretion was measured in comparisons of DraKO and WT duodena. As shown in Figure 1 (top panel, left), basal HCO_3^- secretion was greatly reduced (~80%) in the DraKO as compared to WT duodenum. The residual HCO_3^- secretion was largely dependent on Cl^-/HCO_3^- exchange because removal of luminal Cl^- completely eliminated net HCO_3^- secretion in the DraKO duodenum (Figure 2, top panel, left). Since proton secretion via Na^+/H^+ exchange activity can mask a portion of HCO_3^- secretion in murine duodenum[19], it is possible that the contribution of Dra to basal HCO_3^- secretion was

overestimated due to "autotitration" by concurrent Na^+/H^+ exchange activity. Therefore, basal HCO_3^- secretion across DraKO duodenum was measured after addition of 100 μM EIPA to the luminal bath to inhibit Nhe2 and Nhe3[31]. EIPA significantly increased basal HCO_3^- secretion in the DraKO duodenum (DMSO control = 0.4±0.1 vs. EIPA =1.0±0.2 μeq/cm^2·h, n = 4–7, $p<0.05$), indicating that Dra activity contributes ~55-60% to basal HCO_3^- secretion after inhibition of concurrent H^+ secretion. An unexpected observation was a small but consistent increase in the basal I_{sc} of the DraKO (Figure 1, lower panel, left). Although the ionic basis of the DraKO I_{sc} was not investigated, a favorable Cl^- concentration gradient resulting from luminal Cl^- removal increased the basal I_{sc} in both the DraKO and WT to the same magnitude (Figure 2, lower panel, left). It is important to note that the lack of net HCO_3^- secretion under this condition suggests that the DraKO I_{sc} is not a HCO_3^- secretory current.

Dra contributes to cAMP-stimulated HCO_3^- secretion across the duodenum

To investigate whether Dra contributes to stimulated HCO_3^- secretion, HCO_3^- secretory rates were measured in DraKO and WT duodena treated with forskolin to increase intracellular cAMP. As shown in Figure 1 (upper panel, right, and inset), cAMP stimulation of HCO_3^- secretion was reduced in the DraKO as compared to WT duodenum. The effect of cAMP on J_{sm}^{HCO3} in WT did not result from direct stimulation of Dra activity because cAMP stimulation of Pat-1/Cftr double KO duodena (used to isolate Dra activity) did not increase J_{sm}^{HCO3} (Basal J_{sm}^{HCO3} = 0.2±0.1 vs. Stimulated J_{sm}^{HCO3} = 0.3±0.1, n=6, *ns*). Although cAMP-stimulated HCO_3^- secretion was reduced in the DraKO, the cAMP-stimulated I_{sc} was similar between the DraKO and WT (Figure 1, lower panel, right, and inset), indicating that Cftr activity, responsible for most I_{sc} stimulation by forskolin[2], was not different between DraKO and WT duodenum. The contribution of Dra, as shown above, contrasts with the existing model where it is proposed that cAMP-stimulated HCO_3^- secretion is largely carried by a Cftr-mediated HCO_3^- conductance[2,21]. Since the earlier model was largely based on studies showing normal cAMP-stimulated HCO_3^- secretion in the absence of luminal Cl^- (to inhibit Cl^-/HCO_3^- exchange), we attempted to reconcile the present findings by measuring cAMP-stimulated HCO_3^- secretion during removal of luminal Cl^-. Interestingly, as shown in Figure 2 (upper panel, right, and inset), cAMP-stimulated HCO_3^- secretion was no longer reduced in the DraKO in the absence of luminal Cl^-. Further, there was no difference in the cAMP-stimulated I_{sc} between the DraKO and WT duodenum with the favorable Cl^- gradient. Finally, to evaluate whether changes in the expression of other relevant transporters might be altered in DraKO mice, duodenal mRNA expression of Pat-1, Nhe3 and Cftr was measured in Dra WT and Dra KO gender-matched littermates but no differences in expression of these transporters was detected (Supplemental Figure 1).

Dra is the major Cl^-/HCO_3^- exchanger in the lower villous epithelium of the duodenum

Previous measurements of Cl^-/HCO_3^- exchange activity in the *upper* duodenal villous epithelium of knockout mice show that 70% is attributable to Pat-1, 30% to Dra and <5% to Ae4[5]. As shown in Figure 3A, the rates of Cl^-/HCO_3^- exchange in the upper villous epithelium as estimated by the Cl^--dependent $\Delta pH_i/\Delta t$ during Cl^- removal and re-addition are only moderately reduced in the DraKO as compared to WT. Despite the dominance of Pat-1 activity in the upper villus, Pat-1 Cl^-/HCO_3^- exchange contributes <20% of basal HCO_3^- secretion in mouse duodenum[37]. In contrast, the pH stat studies (Figures 1 and 2) indicate a major role of Dra in duodenal HCO_3^- secretion. Since Dra mRNA expression is reportedly greatest in the lower villus and crypt region of the duodenum[32], the contribution of different apical membrane Cl^-/HCO_3^- exchangers to Cl^--dependent $\Delta pH_i/\Delta t$ in the lower villous epithelium was measured in Pat-1KO, Ae4KO and DraKO duodena. As shown in Figure 3C, Cl^-/HCO_3^- exchange in the lower villus, unlike the upper villus[5], was not reduced in the Pat-1KO duodenum (left). Similarly, Cl^-/HCO_3^- exchange was not reduced in the Ae4KO lower villous epithelium (middle). In contrast, as shown in Figures 3B and 3C (right), Cl^-/HCO_3^- exchange was

completely eliminated in the DraKO lower villus epithelium. Interestingly, removal of luminal Cl^- resulted in significant *acidification* of the epithelium whereas Cl^- return induced *alkalinization* of the DraKO lower villous epithelium. Dra protein expression along the villous axis was also evaluated and, consistent with the functional studies, showed an increasing gradient from villous tip to crypt in the murine duodenal epithelium (Figure 4). Likewise, Pat-1 mRNA expression mirrors its functional activity with a decreasing gradient from upper villous to crypt epithelium (Supplemental Figure 2).

Abnormal pH_i regulation in the DraKO villous epithelium during luminal Cl^- substitution results from CFTR-dependent cell shrinkage and inhibition of Na^+/H^+ exchange

The novel observation that the DraKO lower villous epithelium acidifies and re-alkalinizes during luminal Cl^- removal and replacement, respectively, was further investigated. High levels of Cftr are expressed in the lower villus of the duodenum[33]. It was reasoned that removal of luminal Cl^- (in the absence of basolateral Cl^-) may induce Cftr-dependent cell volume reduction [17,34], a potent inhibitor of the apical membrane Na^+/H^+ exchanger Nhe3[35], and thereby acidify the epithelium. In other species including human, Nhe3 is expressed at greatest levels in villous epithelium but with extension into crypt epithelium[36]. A similar pattern of Nhe3 mRNA expression is present in murine duodenum (Supplemental Figure 2). To examine Cftr dependence of the acidifying effect of Cl^- removal, the Cl^--dependent $\Delta pH_i/\Delta t$ of the lower villous epithelial was measured in double Dra/Cftr KO mice. As shown by the experiment in Figure 5A and 5B, removal and replacement of luminal Cl^- did not alter pH_i in the lower villous epithelium of the Dra/Cftr double KO. Viability of the Dra/Cftr double KO preparations was verified by demonstration of apical membrane Na^+/H^+ exchange activity in the lower villus (Figure 5A). The role of Nhe3, the major apical membrane Na^+/H^+ exchanger in murine intestine, was investigated by pre-treating the mucosa with an inhibitory concentration of EIPA (100 μM, luminal) before luminal Cl^- removal[31]. As shown in Figure 5B, EIPA treatment prevented pH_i acidification during luminal Cl^- removal in the DraKO duodenum. Thus, the activities of Cftr and Nhe3 are required for pH_i acidification during luminal Cl^- removal in the DraKO lower villus. To investigate whether Cl^- removal under these conditions induces lower villous cell shrinkage, we measured changes in epithelial cell height (an index of villous cell shrinkage[17]) in the lower villus after removal of bath Cl^-. As shown Figure 6A and 6B, removal of superfusate Cl^- from both WT and DraKO duodenal mucosa caused significant decreases in epithelial cell height within 5 min. The decrease in cell height was slightly though significantly greater in the DraKO as compared to WT villous epithelium (DraKO = -16.2 ±2.8% vs. WT = -9.6±1.6%, $p < 0.05$), perhaps indicating that the greater basal I_{sc} in the DraKO is secondary to an increased Cl^- conductance. The magnitude of cell height reduction in the DraKO epithelium under these conditions is ~65% of the decrease in villous cell height that results from exposure to strongly hypertonic medium (650 mOsm/l)[17]. Villous cell shrinkage induced by Cl^- removal was rapidly reversed (113±6% within 3–10 min; n=6) when the mucosa was subsequently exposed to superfusate Cl^-.

DIscussion

Previous evaluations of duodenal HCO_3^- secretion in humans and mice have shown that Cl^-/HCO_3^- exchange provides the major portion of basal HCO_3^- secretion[2,20,21]. In addition, most studies show that Cftr provides a small though measurable contribution which may involve a Cl^- recycling function and/or direct interaction that facilitates Slc26a Cl^-/HCO_3^- exchange[2, 15,19-21]. Since recent studies of Pat-1KO duodenum indicate that Pat-1 contributes only 20% of basal HCO_3^- secretion, it is significant that the present study found that the major fraction of basal HCO_3^- secretion is dependent on Dra activity. When proton efflux to the luminal surface (from "uncoupled" Na^+/H^+ exchange) was pharmacologically inhibited, Dra contributed 55-60% of basal HCO_3^- secretion. Thus, combined activities of Dra and Pat-1

account for 75-80% of the basal HCO_3^- secretion across murine duodenum. The remaining fraction of basal HCO_3^- secretion (~20%) is likely divided between a HCO_3^- conductance mediated by Cftr[13] and paracellular movement of HCO_3^- down the serosal-to-mucosal HCO_3^- concentration gradient in pH stat (see model-Figure 7).

An important observation was that Dra also contributes a significant fraction (~50%) to cAMP-stimulated HCO_3^- secretion across the murine duodenum. Previous studies of duodenal HCO_3^- secretion in mice have concluded that at least 80% of cAMP-stimulated HCO_3^- secretion results from a Cftr-mediated HCO_3^- conductance[2,21]. This model was based on studies showing near-normal HCO_3^- secretory responses to cAMP during removal of luminal Cl^- to inhibit Cl^-/HCO_3^- exchange activity. However, exceptions to the model of a dominant Cftr-mediated HCO_3^- conductance during stimulated secretion have been provided by studies using inhibitors of carbonic anhydrase or Nkcc1[2,23]. These studies estimated that 50% of cAMP-stimulated HCO_3^- secretion across murine duodenum is electroneutral when *physiological* concentrations of luminal Cl^- are present. The present findings support this estimate in that the cAMP-stimulated HCO_3^- secretion was reduced ~50% in the DraKO duodenum without altering the magnitude of the cAMP-stimulated I_{sc} (an index of Cftr activity). Consistent with studies of human DRA[9], direct cAMP stimulation of Dra was not found in experiments isolating Dra by using Pat-1/Cftr double KO duodenum. Rather, Cftr is required to enhance Dra activity either by provision of Cl^- "recycling"[15] and/or though direct intermolecular interactions[37]. Since Pat-1 does not contribute to cAMP-stimulated HCO_3^- secretion[24], it is likely that the remaining 50% of stimulated secretion in physiological Cl^- solutions is due to a Cftr-mediated HCO_3^- conductance. This fraction of HCO_3^- secretion represents ~13% of the stimulated I_{sc}, thus approximating the HCO_3^- to Cl^- permeability ratio of CFTR (1:5)[13] (see model-Figure 7).

To reconcile the present findings with the "HCO_3^- conductance" model, cAMP stimulated HCO_3^- secretion across DraKO duodenum was measured in the absence of luminal Cl^-. Surprisingly, nearly equal rates of HCO_3^- secretion were present in WT and DraKO duodena. The equality of HCO_3^- secretory responses resulted from both a decrease (-20%) in WT secretion and an increase (+30%) in DraKO secretion in the absence of luminal Cl^-. Increased stimulated HCO_3^- secretion during luminal Cl^- removal may indicate increased HCO_3^- permeability of Cftr in the absence of extracellular Cl^- [22]. Alternatively, increased HCO_3^- secretion may result from changes in paracellular HCO_3^- permeability during removal of luminal Cl^-. Physiologically, it is unlikely that an environment devoid of luminal Cl^- would be encountered in the duodenum where 24-hr measurements in humans indicate that Cl^- concentration is maintained above 40 mM[38]. In contrast, enhancement of the HCO_3^- conductance of Cftr in the absence of luminal Cl^- may be more important in epithelia such as the pancreatic duct where low Cl^- concentrations occur during stimulated secretion[39].

The functional activity of Dra along the villus axis in the murine duodenum mirrors its expression pattern where greatest levels of Dra protein and mRNA[32] are found in the lower half of the crypt-villus axis. Enrichment of Dra activity in the lower villous/crypts puts the exchanger in the location of greatest Cftr activity[36], which should facilitate the mechanism of duodenal HCO_3^- secretion outlined above. This is apparently an efficient process for transepithelial HCO_3^- secretion because expression levels of Dra in the duodenum are several-fold less as compared to large intestine[40]. In the *upper* villous epithelium, Dra activity is less (30%) and high levels of Pat-1 expression dominate Cl^-/HCO_3^- exchange activity (70%)[5]. Further, it is likely that Dra in the upper villous epithelium is coupled with Nhe3 for NaCl absorption and may not contribute to net HCO_3^- secretion. As shown in this and previous studies[36,41], highest levels of Nhe3 expression are found in the villous epithelium. Coupling with Nhe3 may also account for the small contribution of Pat-1 to net HCO_3^- secretion;

however, Pat-1 has other transport functions including oxalate secretion, sulfate uptake and regulation of intracellular pH[5,12].

The evidence that Pat-1 and Dra are largely segregated between upper and lower villus is contrary to speculation that these two anion exchangers have opposite electrogenic Cl^-:HCO_3^- stoichiometries that operate in parallel to yield electroneutral HCO_3^- secretion and Cl^- absorption[42]. Although some overlap of Pat-1 and Dra activities exists in the upper villus[5], genetic ablation of Pat-1 does not affect the duodenal I_{sc}[43], which is inconsistent with electrogenic Cl^-/HCO_3^- exchanger activity. Recent studies in expression systems also do not support electrogenic Pat-1 Cl^-/HCO_3^- exchange[44]. Interestingly, a small increase in the basal I_{sc} was present in the DraKO duodenum but will require further examination to determine its ionic basis. Compensation of one anion exchanger by the other also is not apparent in the KO mice. In Pat-1KO duodenum, Dra activity does not increase in upper villous epithelium[5] whereas, in the present study, Pat-1 did not compensate for loss of Cl^-/HCO_3^- exchange in the DraKO lower villous epithelium. Additionally, no changes in mRNA expression of Pat-1, Nhe3 or Cftr were found in DraKO duodenum.

In the lower villous epithelium, Dra is exposed to the cellular effects of Cftr activity as demonstrated by the effect of luminal Cl^- removal on cell shrinkage and Nhe3 function. The unexpected observation of cell acidification during luminal Cl^- substitution in the DraKO lower villus led to the hypothesis that Cl^- removal may induce Cftr-dependent cell shrinkage and Nhe3 inhibition. This hypothesis was based on past studies showing that acute cAMP activation of Cftr results in villous cell shrinkage and inhibition of Nhe3[17]. Findings in the present investigation were consistent in that Cl^--dependent acidification did not occur in the absence of Cftr or Nhe3 activity. Subsequent studies demonstrated reduced cell volume in WT and DraKO villous epithelium during Cl^- removal, a phenomenon not unlike that demonstrated in other epithelia expressing active Cl^- channels[45]. Thus, Cftr extends a subtle but potent effect on Nhe3 and pH_i through its regulation of epithelial cell volume.

In conclusion, investigation of the DraKO intestine defines a central role for Dra Cl^-/HCO_3^- exchange activity in duodenal HCO_3^- secretion. Dra is responsible for ~55-60% of basal HCO_3^- secretion and ~50% of cAMP-stimulated HCO_3^- secretion when physiological Cl^- concentrations are present in the luminal solution. This finding resolves past inconsistencies with a model proposing that cAMP-stimulated HCO_3^- secretion largely results from a Cftr-mediated HCO_3^- conductance[2,21]. In the revised model, a Cftr-mediated HCO_3^- conductance is responsible for the remaining ~50% of cAMP-stimulated HCO_3^- secretion. Dra activity is largely concentrated in the lower villous and probably crypt epithelium where its function is influenced by Cftr. In light of its prominent role in intestinal HCO_3^- secretion, future investigations should consider Dra activity in pathophysiological models and potential therapies of duodenal disorders such as ulcer disease and intestinal manifestations of cystic fibrosis.

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Abbreviations

Ae4 anion exchanger 4

Cftr cystic fibrosis transmembrane conductance regulator

EIPA 5-(N-ethyl-n-isopropyl)-amiloride

dKO double knockout

Dra down-regulated in adenoma

G_t transepithelial conductance

IBMX isobutylmethyl xanthine I_{sc}, short-circuit current

J ion flux

KO knockout

Nhe3 Na^+/H^+ exchanger isoform 3

Pat-1 putative anion transporter-1

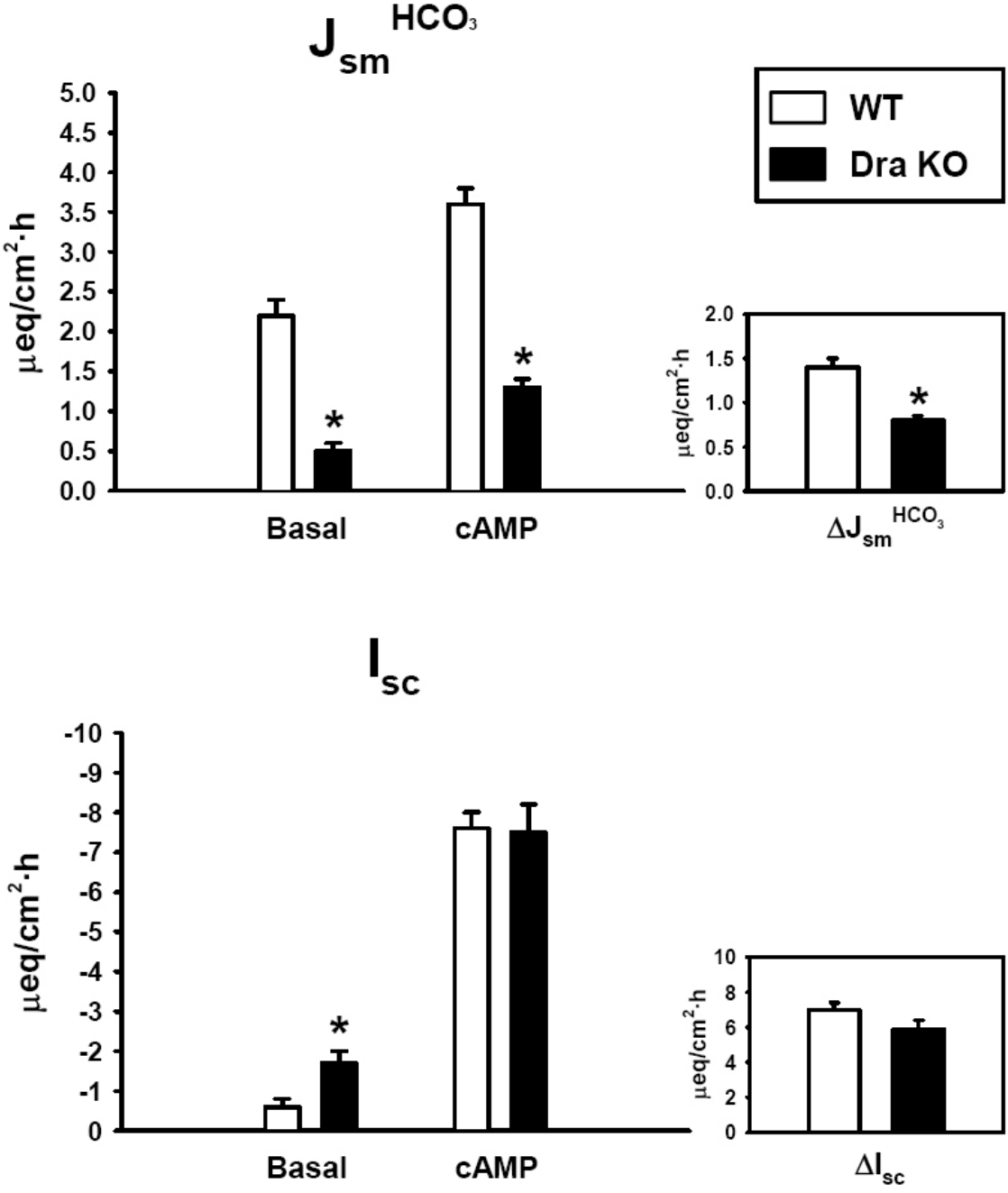

Figure 1.

Basal and cAMP-stimulated HCO_3^- secretions are reduced in Dra knockout (DraKO) duodenum. *Upper*: Transepithelial J_{sm}^{HCO3} in DraKO (black bars) and WT (white bars) during 30-min pH stat flux periods. Inset, change in J_{sm}^{HCO3} from basal to cAMP-stimulated flux periods. *Lower*: Basal and cAMP-stimulated I_{sc} across WT and DraKO duodena during 30-min pH stat flux periods. Inset, change in I_{sc} from basal to cAMP-stimulated flux periods. Transepithelial conductances under basal conditions were WT=50.2±1.9 and DraKO=44.0±2.6 mS/cm^2. (n=11 mice) *p<0.05 vs. WT.

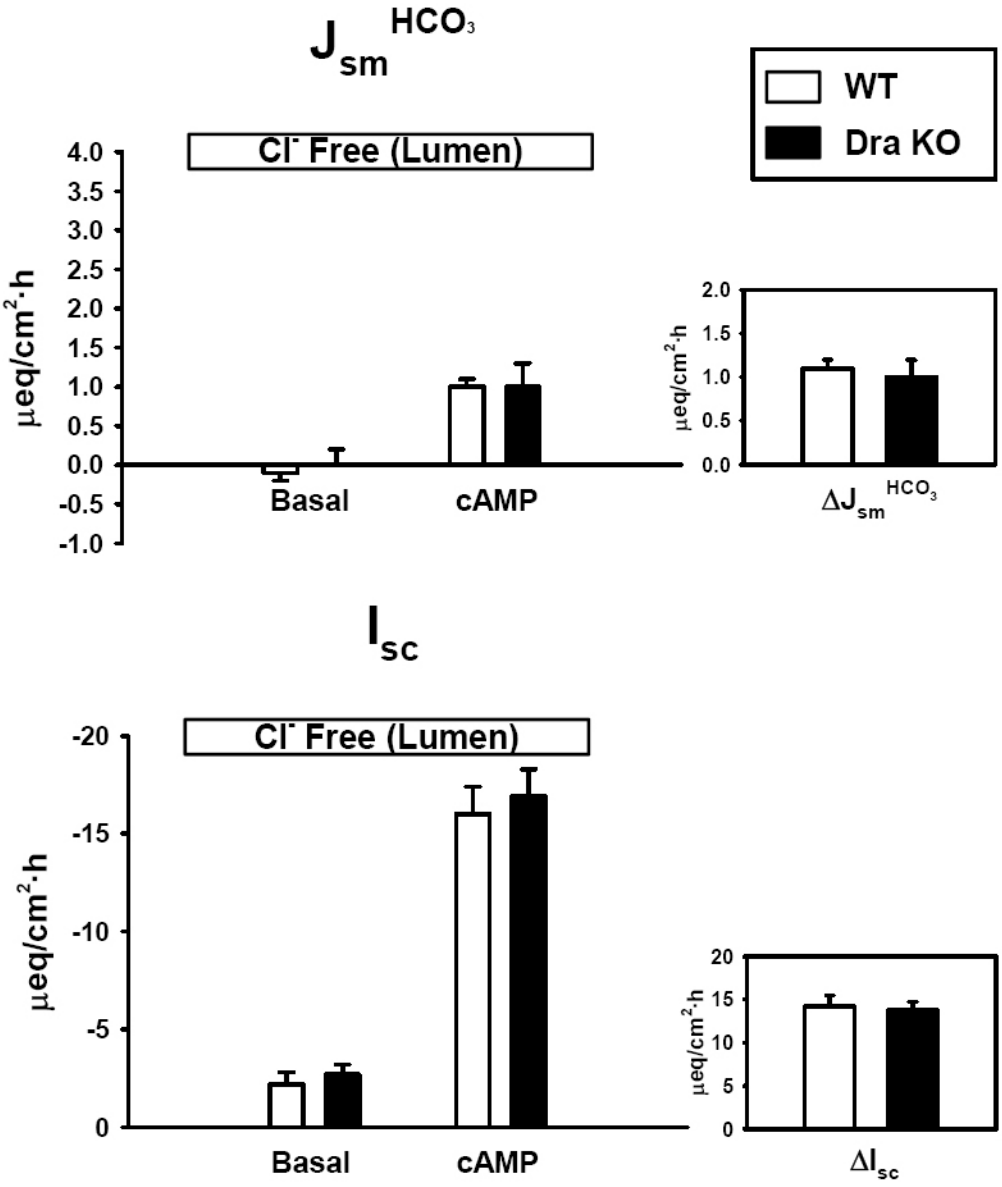

Figure 2.

Inhibition of Cl^-/HCO_3^- exchange by Cl^- removal eliminates differences in duodenal HCO_3^- secretion between WT (white bars) and DraKO (black bars) mice. *Upper*: Transepithelial J_{sm}^{HCO3} in the absence of luminal Cl^- during 30-min pH stat flux periods. Inset, change in J_{sm}^{HCO3} from basal to cAMP-stimulated flux periods. *Lower*: Basal and cAMP-stimulated I_{sc} across the WT and DraKO duodena in the absence of luminal Cl^-. Inset, change in I_{sc} from basal to cAMP-stimulated flux periods. Transepithelial conductance under basal conditions were WT=64.6±9.4 and DraKO=68.4±7.9 mS/cm^2. (n=5 mice).

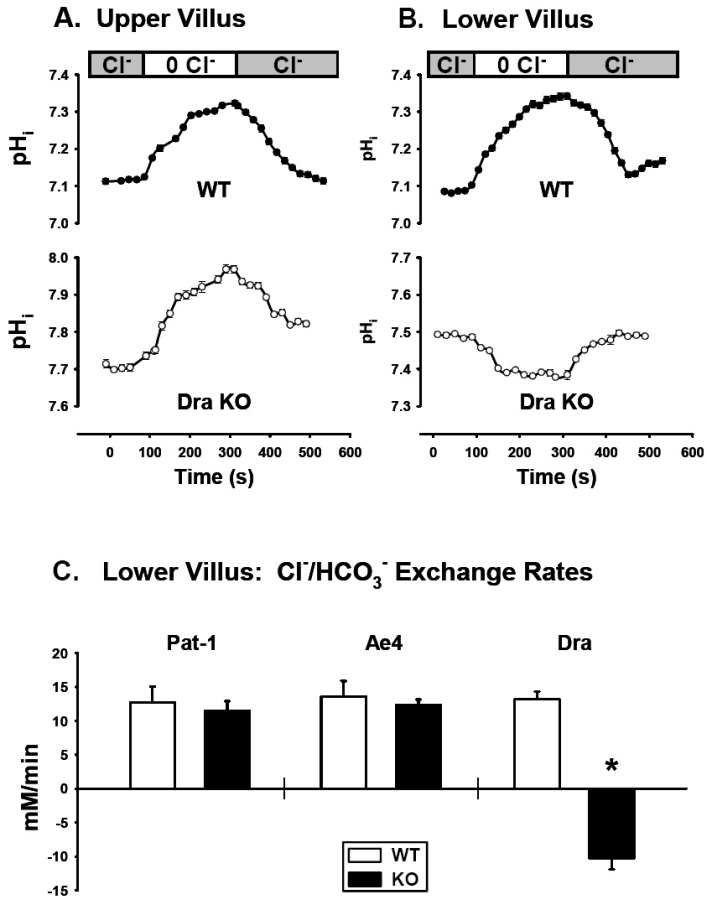

Figure 3.

Dra is the major Cl^-/HCO_3^- exchanger in the lower villous epithelium of murine duodenum. *(A)* Changes in cellular pH_i in the upper villous epithelium during removal and replacement of luminal Cl^- in WT and DraKO duodenum (representative of 8 mice). *(B)* Changes in pH_i in the lower villous epithelium during removal and replacement of luminal Cl^- in WT and DraKO duodenum (representative of 6 mice). *(C)* Rates of Cl^-/HCO_3^- exchange in the lower villous epithelium of Pat-1KO, Ae4KO, DraKO and WT littermates. **p*<0.05 vs. WT (n=6–10).

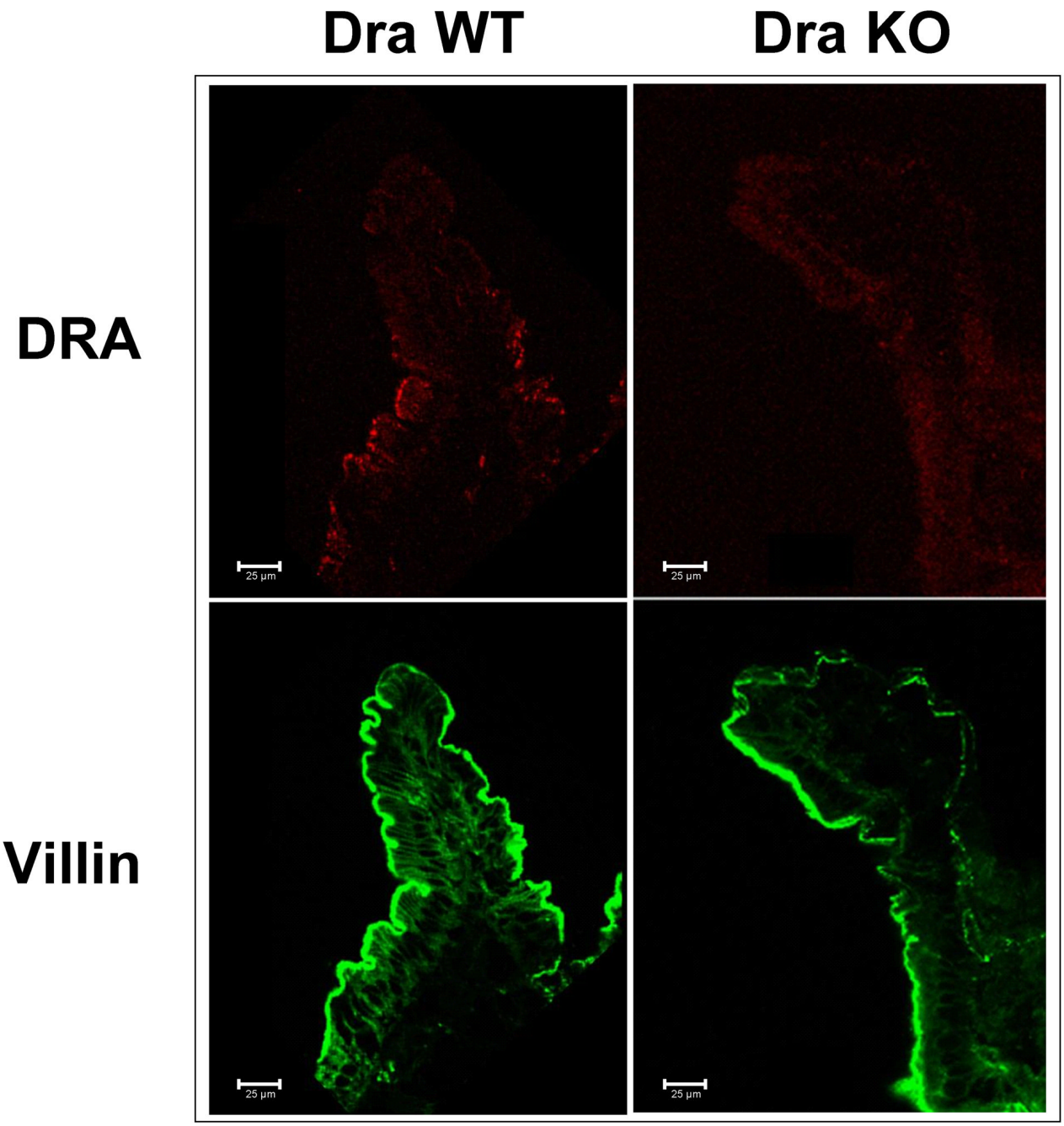

Figure 4.
Immunofluorescent localization of Dra (red) and villin (green) in duodenal epithelium of WT and Dra KO mice. WT shows increasing apical membrane immunolocalization of Dra from villous tip to villous base. Immunolocalization of Dra was not present in the Dra KO. Representative of 6 experiments.

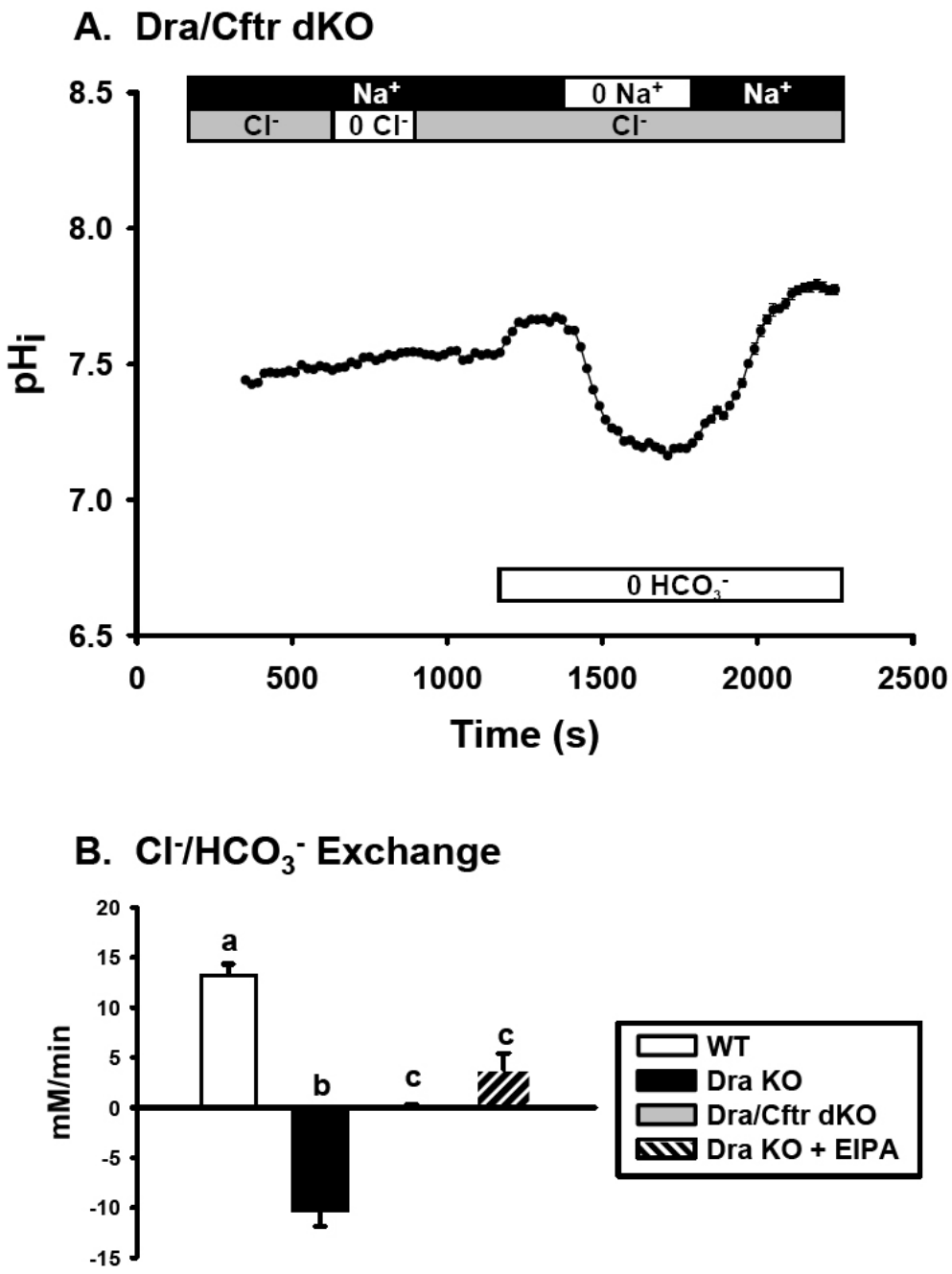

Figure 5.

Acidification in the DraKO lower villous epithelium during luminal Cl^- removal is Cftr-and Nhe-dependent. *(A)* Changes in pH_i in the Dra/Cftr double KO lower villous epithelium during Cl^- removal and replacement. Viability of the preparation was demonstrated by measurement of apical membrane Na^+/H^+ exchange (representative of 3 mice). *(B)* Comparison of Cl^-/HCO_3^- exchange rates between WT (white bar), DraKO (black bar), Dra/Cftr double KO (gray bar) and DraKO treated with 100 μM EIPA in the luminal bath (striped bar). Data for WT and untreated DraKO from data in Figure 3. [a,b,c]Means with the same letters are not significantly different (n=3–6 mice).

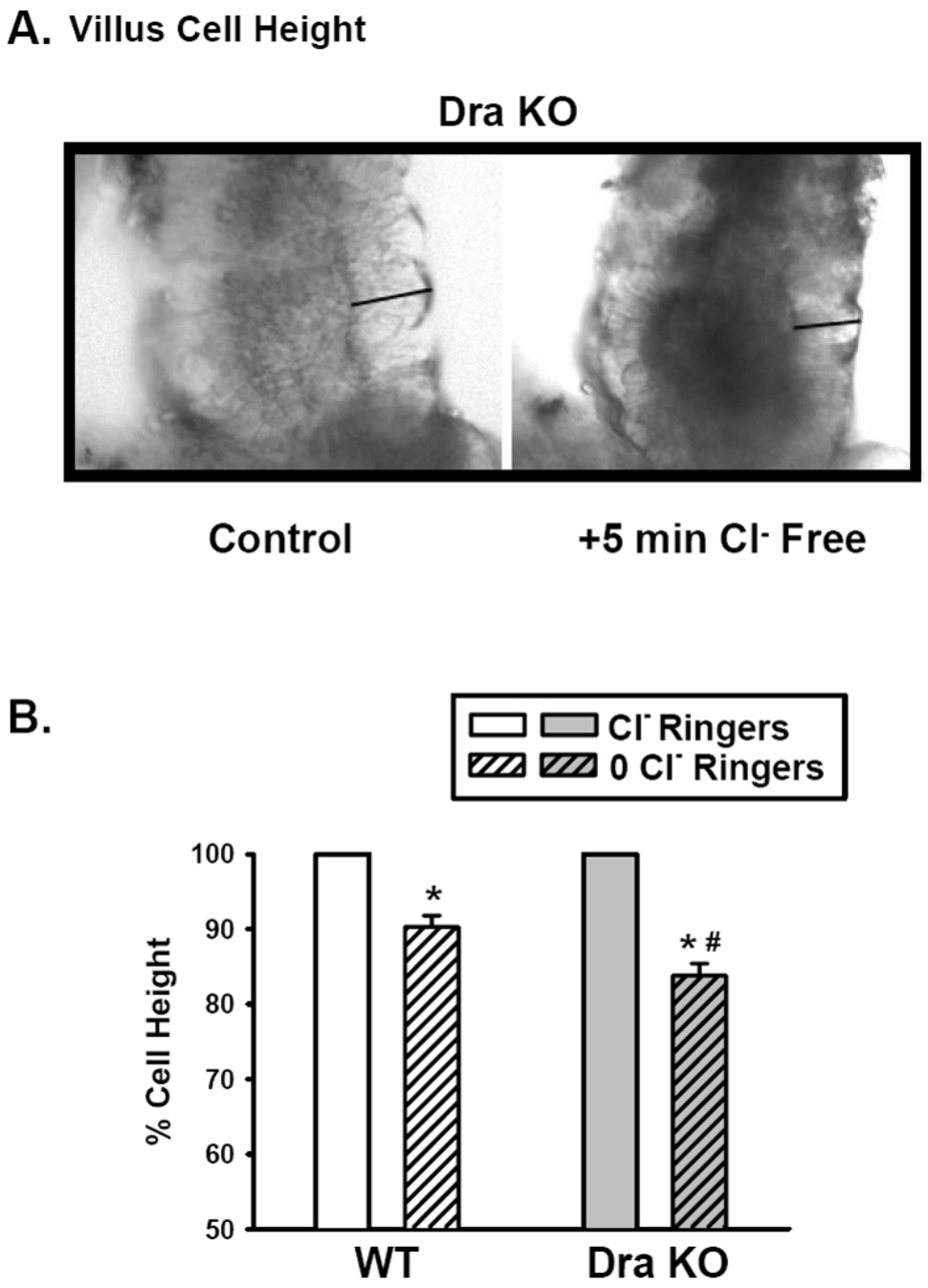

Figure 6.

Epithelial cell shrinkage in the lower villus during Cl^- removal in WT and DraKO duodenum. *(A)* Bright field images showing changes in epithelial cell height (an index of epithelial cell volume[17]) before and 5 min after removal of bath Cl^- in a single DraKO villus. Magnification: 100X. (B) Comparison of changes in epithelial cell height in WT and DraKO villi before and 5 min after removal of bath Cl^-. *p<0.05 vs. Cl^- Ringers; #p<0.05 vs. WT in 0 Cl^- Ringers (n=3-4 mice).

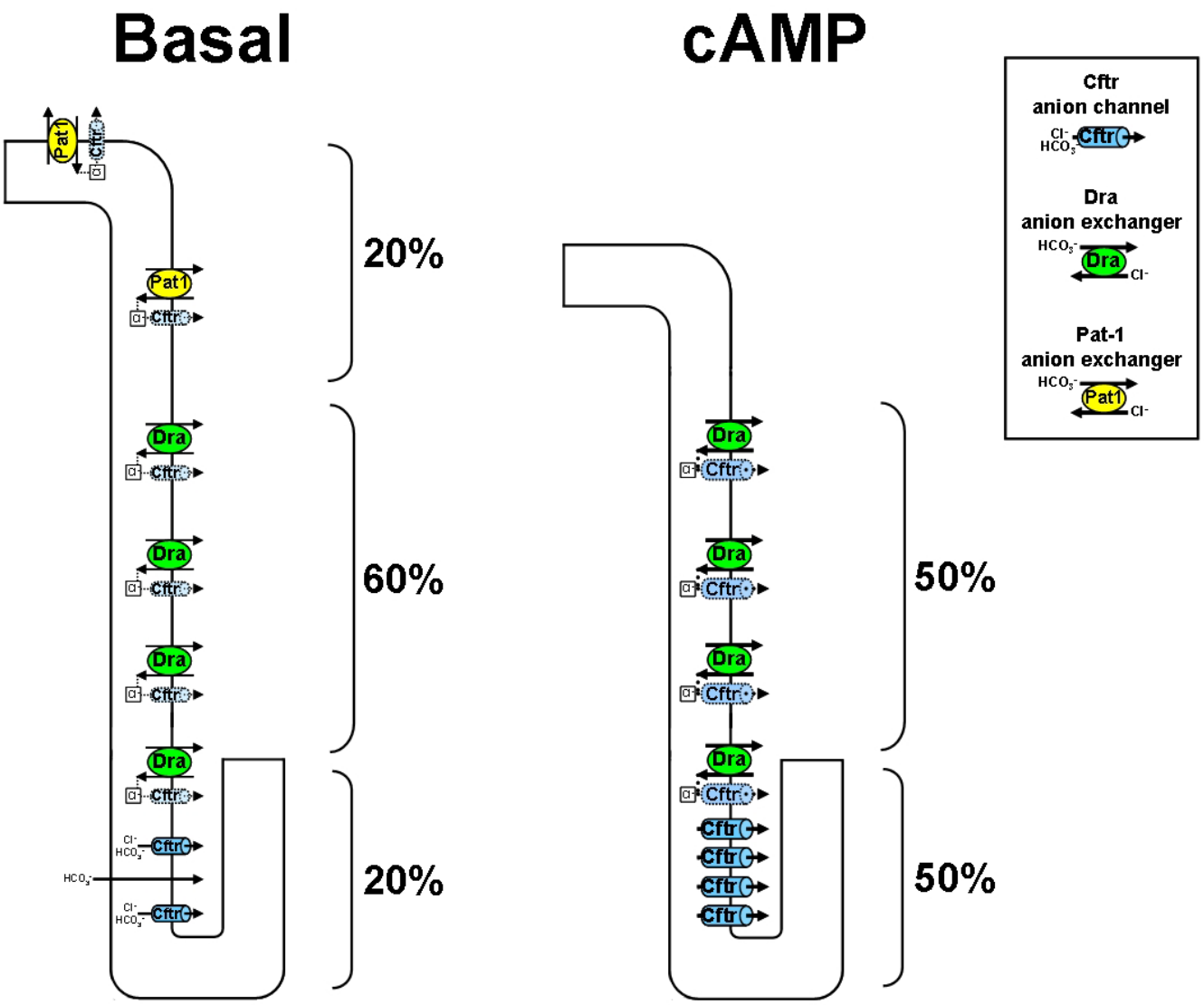

Figure 7.
Model for duodenal HCO_3^- secretion under basal (left panel) and cAMP-stimulated conditions (right panel). Villus contraction during cAMP stimulation is also depicted.