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Mechanism of intestinal absorption. Effect of clonidine on rabbit ileal villus and crypt cells.

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Research Article

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Mechanism of Intestinal Absorption

Effect of Clonidine on Rabbit Ileal Villus and Crypt Cells

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Abstract

In intact tissues studies, intestinal absorptogogues stimulate NaCl absorption that occurs via the dual operation of Na:H and Cl:HCO_3 exchanges on the brush border membrane (BBM) of villus cells. To determine the cellular mechanism of action of an intestinal absorptogogue, the effect of clonidine was determined on Na:H and Cl:HCO_3 exchange in rabbit ileal villus and crypt cells. Using 2,7-bis(carboxyethyl)-5,6-carboxy-fluorescein we have previously shown that recovery from an acid load occurs via Na:H exchange, whereas recovery from an alkaline load occurs via Cl:HCO_3 exchange in both cells.

In villus cells, the rate of recovery from a propionate-induced alkaline load was not altered by clonidine. However, clonidine stimulated recovery from an acid load induced by NH_4Cl, Na removal, or amiloride. These data suggest that clonidine stimulates Na:H exchange in villus cells.

In crypt cells, the rate of recovery from a propionate-induced alkaline load was also not altered by clonidine. However, in crypt cells, unlike the villus cells, clonidine inhibited recovery from an acid load induced by NH_4Cl, Na removal, or amiloride. These data suggest that clonidine inhibits Na:H exchange in crypt cells.

Stimulation of Na:H exchange on the BBM of villus cells would be expected to stimulate coupled NaCl absorption (which occurs by coupling of Na:H and Cl:HCO_3 exchange). Inhibition of Na:H in crypt cells, known to be present only on the basolateral membrane, will acidify the cell and may inhibit Cl:HCO_3 exchange on the BBM, resulting in the inhibition of HCO_3 secretion. (*J. Clin. Invest.* 1995. 95:2187–2194.) Key words: intestinal absorption and secretion • regulation of secretion • bicarbonate secretion • intestinal absorptogogues • intestinal electrolyte transport

Introduction

In the ileum coupled NaCl absorption occurs via the dual operation of Na:H and Cl:HCO_3 exchange on the brush border membrane (BBM)[1] of villus cells. Inhibition of either of these transporters will result in the inhibition of coupled NaCl absorption. In contrast, there is only a Cl:HCO_3 exchanger on the BBM of crypt cells, and thus, this cell is not capable of coupled NaCl absorption. However, there is a Na:H exchanger on the basolateral membrane (BLM) of crypt cells and stimulation of this will alkalinize the cell and may stimulate the BBM Cl:HCO_3 exchange resulting in HCO_3 secretion (1, 2).

We have previously demonstrated that ileal villus and crypt cells respond differently to a given secretogogue. We demonstrated that serotonin inhibited Cl:HCO_3 exchange on the BBM of villus cells, whereas it stimulated Na:H exchange on the BLM of crypt cells (3). We have also demonstrated that these two cell types respond differently to agonists that mediate their effects via different intracellular second messengers (3, 4). In these studies we demonstrated that cAMP and Ca inhibit NaCl absorption in villus cells by different mechanisms: cAMP inhibits Na:H exchange, whereas calcium inhibits Cl:HCO_3 exchange on the BBM of villus cells. Although both cAMP and calcium inhibit different transporters on the BBM of villus cells, in crypt cells both cAMP and calcium stimulate Na:H exchange that is found only on the BLM. This stimulation, as previously mentioned, may stimulate bicarbonate secretion by the crypt cells (3, 4).

Given these observations with secretagogues, the aim of this study is to determine the mechanism of action of an absorptogogue on these cells. Clonidine, an $\alpha 2$ agonist, known to stimulate NaCl absorption and inhibit bicarbonate secretion in intact tissue studies from the rabbit ileum was used as the absorptogogue (5–8).

Methods

Cell isolation. Villus and crypt cells were separated from rabbit ileum by a calcium-chelation technique previously reported from our laboratory (9) and maintained in short-term culture. Using this method six fractions of cells were sequentially collected. Fraction 1 was used as villus cells and fraction 6 as crypt cells. Several criteria were used to ensure good crypt-villus cell separation, including enzyme markers (alkaline phosphatase for villus and thymidine kinase for crypt), morphology (a well-developed brush border on villus but not crypt cells), transporter specificity (i.e., presence of Na-glucose cotransport in villus but not crypt cells), higher baseline intracellular pH in crypt as compared with villus cells, and higher rates of protein synthesis in crypt as compared with villus cells. The cells were maintained in short-term culture for up to 6–8 h. Viability was assessed by trypan blue exclusion, linear incorporation of leucine into protein, Na-stimulated glucose uptake (villus cells), and preserved cell structure by electron microscopy (9). The presence of Na:H and Cl:HCO_3 exchange and their role in the regulation of intracellular pH has been demonstrated in both cell types, whereas Na-HCO_3 cotransport could not be demonstrated in either cell type (9).

Intracellular pH measurements. Intracellular pH was measured using 2,7-bis(carboxyethyl)-5,6-carboxy-fluorescein (BCECF) as previously described (9). Briefly, the cells were loaded with 10 μM of the

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1. *Abbreviations used in this paper:* BBM, brush border membrane; BCECF, 2,7-bis(carboxyethyl)-5,6-carboxy-fluorescein; BLM, basolateral membrane.

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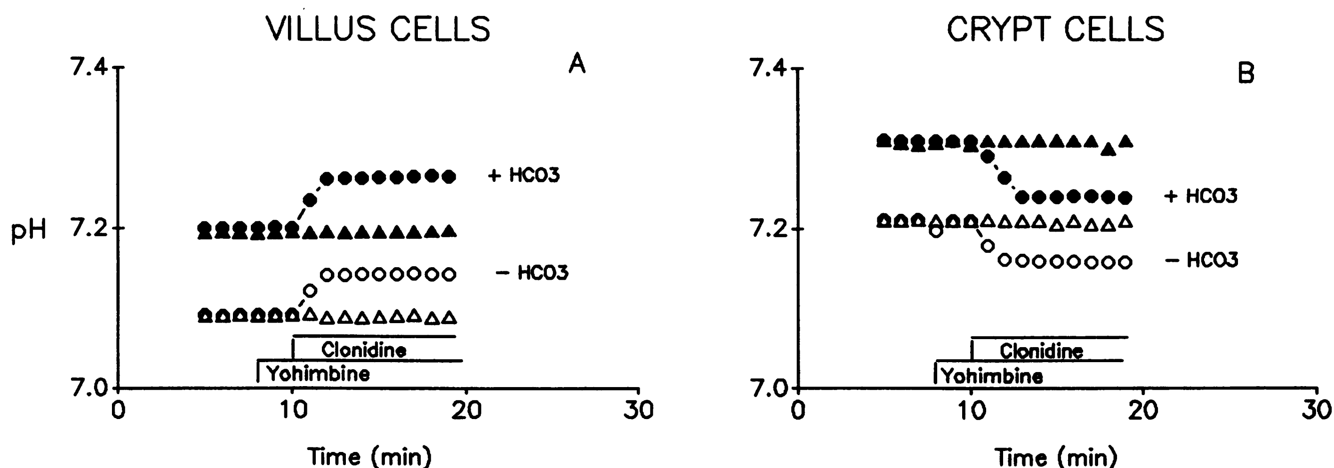

Figure 1. Effect of clonidine on intracellular pH in villus (*A*) and crypt (*B*) cells. Cells were perfused with either HCO_3-containing (●, ▲) or HCO_3-free (○, △) solutions. Cells were also perfused with 10 μM clonidine (●, ○) or 10 μM yohimbine followed by clonidine (▲, △) during the period shown.

acetoxy methylester of BCECF from a 10-mM stock in DMSO for 10 min at 37°C. A coverslip coated with subconfluent monolayer of cells was mounted in a thermostatically controlled cuvette (37°C) in a Perkin-Elmer (Norwalk, CT) LS-5 spectrofluorometer with constant perfusion to wash away any leaked dye. The dye was alternatively excited at 450 and 500 nm, and the fluorescence emission was measured at 530 nm. The BCECF fluorescence excitation ratio was calibrated using the high-K/nigericin technique (10). All experiments were performed in CO_2/HCO_3 or Na-Hepes (HCO_3 free) solutions. The standard CO_2/HCO_3 solution contained (in mM) NaCl 115, $NaHCO_3$ 25, K_2HPO_4 2.4, KH_2PO_4 0.4, $MgCl_2$ 1.25, $CaCl_2$ 1.25, and gassed with 5% CO_2, 95% O_2 (pH 7.4 at 37°C). The Na-Hepes solution contained (in mM) NaCl 130, KCl 4.5, KH_2PO_4 1.2, $MgSO_4$ 1, $CaCl_2$ 1.25, Hepes 20, and gassed with 100% O_2 (pH 7.4 at 37°C).

BCECF-AM was purchased from Molecular Probes (Eugene, OR). Amiloride, 4,4′-diisothiocyanostilbene-2-2′ disulfonic acid, clonidine, and yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO).

Data presentation. Cell viability was assessed after each experiment by trypan blue dye exclusion. Only those experiments in which dye exclusion was > 85% were evaluated. Any given experiment and its control were performed on cells isolated from a single rabbit. Each villus and crypt cell comparison experiment was performed from the same ileum. Where representative examples are shown, experiments were performed on three to seven separate cell preparations from different animals. Paired Student's *t* test was used for statistical analysis. Mean±SEM is shown on Figs. 2, *C* and *D*, through 5, *C* and *D*, and SEM is inclusive when not obvious by the presence of error bars. dpH/d*t* is calculated as change in pH from pH nadir or peak for ≥ 1.5 min and expressed as change in pH per minute.

Results

Fig. 1 demonstrates the effect of clonidine on resting intercellular pH in villus and crypt cells. After achieving a steady state pH, the cells were exposed to clonidine in the presence and absence of HCO_3. Clonidine (10 μM) causes an immediate alkalinization of villus cells irrespective of the presence of HCO_3 (Fig. 1 *A*). Clonidine increased baseline pH from 7.21±0.01 to 7.28±0.01 (n = 4, $P < 0.01$) in villus cells in the presence of HCO_3 and from 7.10±0.01 to 7.16±0.01 (n = 4, $P < 0.01$) in the absence of HCO_3. In contrast to the villus cells, clonidine causes an immediate acidification of crypt cells both in the presence and absence of HCO_3 (Fig. 1 *B*). Clonidine decreased intracellular pH from 7.30±0.01 to 7.25±0.01 (n = 4, $P < 0.005$) in the presence of HCO_3 and from 7.20±0.01 to 7.14±0.01 (n = 4, $P < 0.005$) in the absence of HCO_3.

The effects of clonidine are blocked in both villus and crypt cells (Fig. 1) when the cells are first exposed to 10 μM yohimbine, and α2 antagonist, which by itself has no effect on these cells.

Because the effect of clonidine is HCO_3 independent in both cell types, it is unlikely that the effect of clonidine is on Cl:HCO_3 exchange in either cell type. To confirm that clonidine does not affect Cl:HCO_3 exchange in either cell type, the effect of clonidine on recovery from an alkaline load, known to occur via Cl:HCO_3 exchanger in both cell types (9), was studied. A propionate pulse is used to alkalinize both the villus and crypt cells (Fig. 2). In both the villus (Fig. 2 *A*) and crypt (Fig. 2 *B*) cells recovery from this alkaline load, known to occur via Cl:HCO_3 exchange, was not altered by clonidine. The initial rate of recovery (dpH/d*t*) is not affected by clonidine in villus (Fig. 2 *C*; 0.12±0.01 dpH/min in control and 0.13±0.01 in clonidine, n = 3, P = NS) or crypt cells (Fig. 2 *D*; 0.06±0.01 dpH/min in control and 0.06±0.01 in clonidine, n = 3, P = NS). These data confirm that clonidine does not affect Cl:HCO_3 exchange in either cell type.

The effect of clonidine on Na:H exchange in villus and crypt cells was next studied. This was accomplished by determining whether the effect is Na dependent, amiloride sensitive, and by looking at recovery from an acid load that is known to occur via Na:H exchange in both cells (9). The acid load was induced by three different methods: NH_4Cl pulse, Na removal, and addition of amiloride. Fig. 3 demonstrates the effect of clonidine on recovery from an acid load induced by NH_4Cl. After establishing a steady state pH, cells were briefly pulsed with NH_4Cl, which results in acidification of the villus and crypt cells (Fig. 3). Recovery from this acid load, known to occur via Na:H exchange in both cells, is accelerated in villus cells (Fig. 3 *A*) but slowed in crypt cells (Fig. 3 *B*) by clonidine. In the presence of clonidine, the rate of recovery (dpH/d*t*) from the acid load

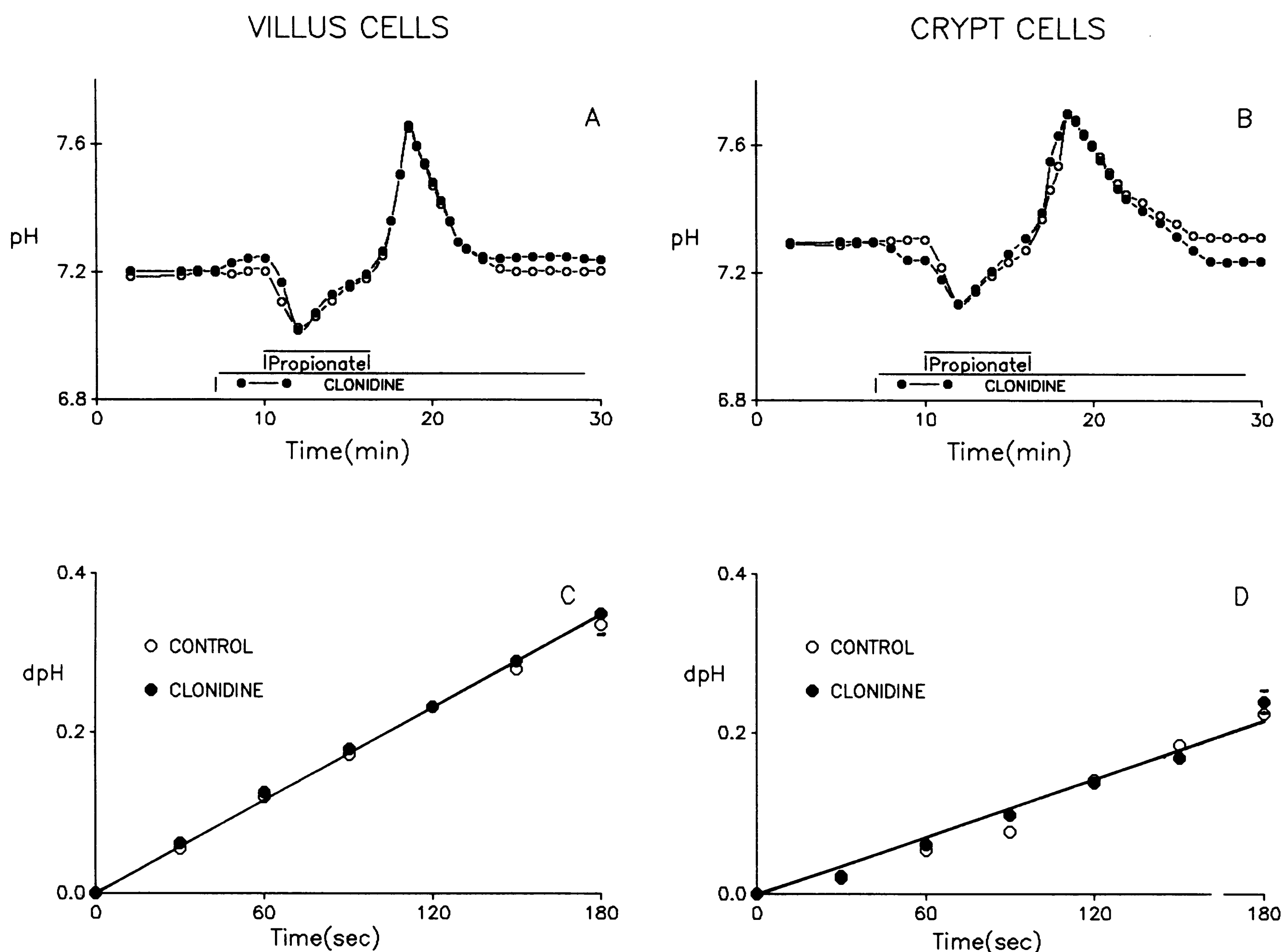

Figure 2. Effect of clonidine on recovery from an alkaline load in villus (*A*) and crypt (*B*) cells. The cells are first perfused with the standard CO_2/HCO_3 solution and then with 50 mM Na-propionate replacing 50 mM of NaCl, in the presence (●) or absence (○) of 10 μM clonidine during the time period shown. The rate of recovery, dpH/d*t*, from an alkaline load is unaffected by clonidine in villus (*C*; 0.12±0.01 dpH/min in control and 0.13±0.01 in clonidine, $n = 3$, P = NS) or crypt cells (*D*; 0.06±0.01 dpH/min in control and 0.06±0.01 in clonidine, $n = 3$, P = NS). *A* and *B* are representative experiments. In *C* and *D*, mean±SEM is shown, and where indicated (*), the dpH/d*t* at any given time is also significantly different from that of control.

was faster in villus cells (Fig. 3 *C*, 0.10±0.01 dpH/min in control and 0.21±0.01 in clonidine, $n = 4$, $P < 0.005$). In contrast, in the crypt cells, the rate of recovery from the acid load was slower in the presence of clonidine (Fig. 3 *D*; 0.08±0.01 dpH/d*t* in control and 0.04±0.01 in clonidine, $n = 4$, $P < 0.001$). These data suggest that clonidine stimulates Na:H exchange in villus cells, whereas it inhibits Na:H exchange in crypt cells.

To confirm these observations, the effect of clonidine on recovery from an acid load induced by Na removal was next determined (Fig. 4). Removal of Na acidifies both cells (Fig. 4, *A* and *B*) by inhibiting Na:H exchange (9). These maneuvers prevented the alkalinization in villus cells and further acidification in crypt cells by clonidine (Fig. 4, *A* and *B*) previously seen in baseline conditions (Fig. 1). Thus, these data suggest that the effect of clonidine is Na dependent in both cell types. When Na is re-presented to the villus cells, the rate of recovery from this acid load was faster in the presence of clonidine (Fig. 4 *C*; 0.12±0.01 dpH/min in control and 0.19±0.01 in clonidine, $n = 5$, $P < 0.001$). In contrast, in the crypt cells when Na is re-presented, the rate of recovery from the acid load was slower in the presence of clonidine (Fig. 4 *D*, 0.06±0.01 dpH/min in control and 0.03±0.01 in clonidine, $n = 5$, $P < 0.001$).

To further confirm the observations that clonidine stimulates Na:H exchange in villus cells while inhibiting Na:H exchange in crypt cells, the effect of clonidine on recovery from an acid load induced by amiloride was determined (Fig. 5). Addition of amiloride acidifies both cells (Fig. 5, *A* and *B*) by inhibiting Na:H exchange (9). These maneuvers also prevented the alkalinization in villus cells and further acidification in crypt cells by clonidine (Fig. 5, *A* and *B*) previously seen in baseline conditions (Fig. 1). Thus, these data suggest that the effect of clonidine is amiloride sensitive in both cell types. When amiloride is removed from the villus cells, the rate of recovery from this acid load was faster in the presence of clonidine (Fig. 5 *C*; 0.07±0.01 dpH/min in control and 0.15±0.01 in clonidine, $n = 5$, $P < 0.001$). In contrast, in the crypt cells when amiloride is removed, the rate of recovery from the acid load was slower

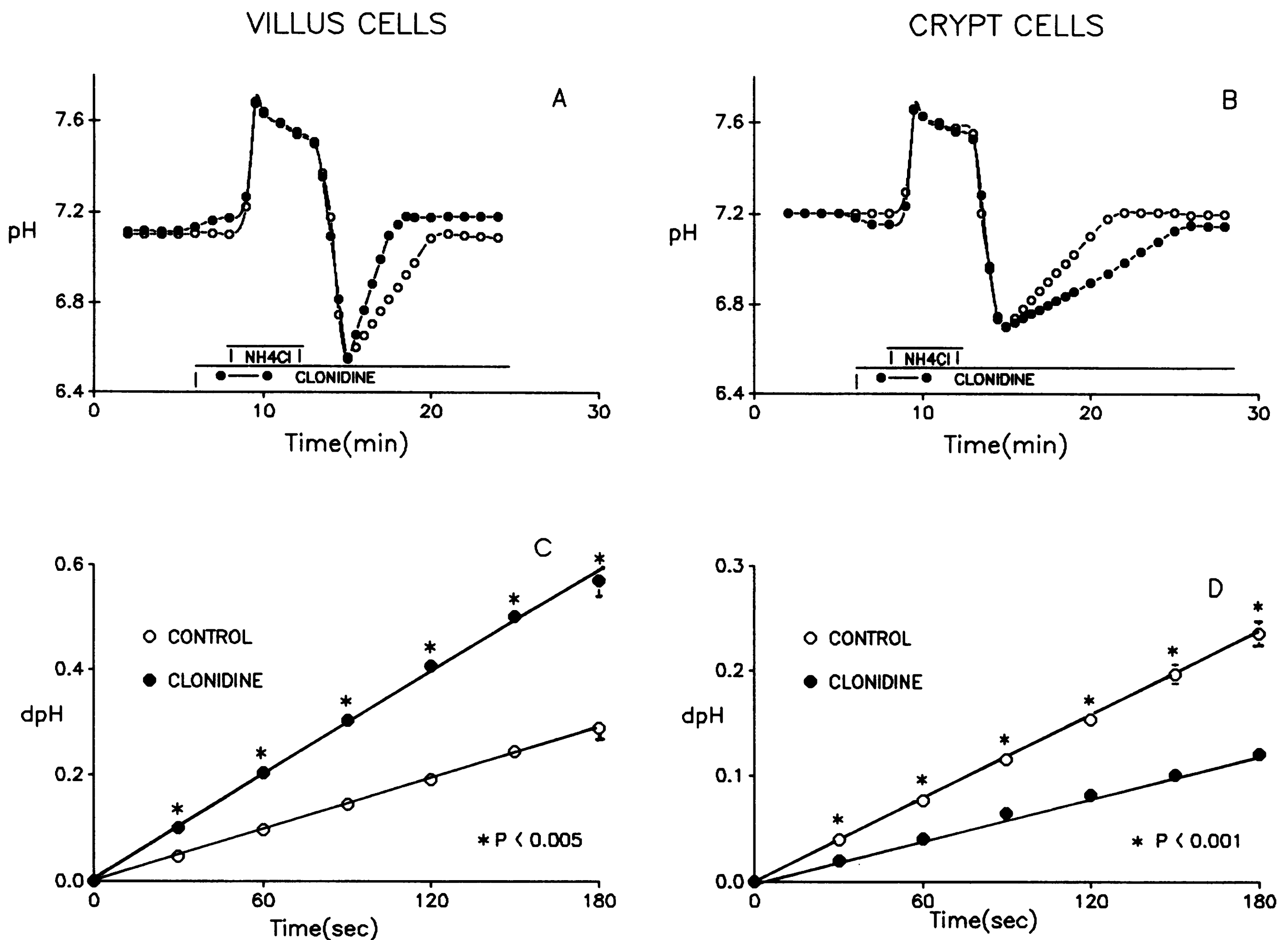

Figure 3. Effect of clonidine on recovery from an acid load induced by NH_4Cl in villus (*A*) and crypt (*B*) cells. The cells are first perfused with the standard Na-Hepes solution and then, with 30 mM NH_4Cl replacing 30 mM of NaCl, in the presence (●) or in the absence (○) of 10 μM clonidine during the time period shown. The rate of recovery, dpH/d*t*, from an acid load is faster in the presence of clonidine in villus (*C*; 0.10±0.01 dpH/min in control and 0.21±0.01 in clonidine, $n = 4$, $P < 0.005$), whereas it is slower in crypt cells (*D*; 0.08±0.01 dpH/d*t* in control and 0.04±0.01 in clonidine, $n = 4$, $P < 0.001$). *A* and *B* are representative experiments. In *C* and *D*, mean±SEM is shown, and where indicated (*), the dpH/d*t* at any given time is also significantly different from that of control.

in the presence of clonidine (Fig. 5 *D*; 0.02±0.01 dpH/min in control and 0.01±0.01 in clonidine, $n = 4$, $P < 0.001$). These data also confirm that clonidine stimulates Na:H exchange in villus cells, whereas it inhibits Na:H exchange in crypt cells.

Discussion

In the villus cells clonidine increases intercellular pH. This effect of clonidine is bicarbonate independent, Na dependent, amiloride sensitive and is characterized by an accelerated recovery from an acid load induced by three different methods. Recovery from an alkaline load is unaffected. These data are consistent with stimulation of Na:H exchange in the villus cells by clonidine (Fig. 6).

In the crypt cells, unlike the villus cells, clonidine decreases intracellular pH. This effect is also bicarbonate independent, Na dependent, and amiloride sensitive. However, in the crypt cells, unlike the villus cells, the effect of clonidine is characterized by a slowed recovery from an acid load induced by three different methods. Recovery from an alkaline load is also not affected. These data are consistent with inhibition of Na:H exchange in crypt cells by clonidine (Fig. 6).

The effects of clonidine demonstrated in this study are most likely specific for the $\alpha 2$ agonist clonidine. The receptor for this agonist has been demonstrated on rabbit ileal villus and crypt cell membranes (11). Further, yohimbine, an $\alpha 2$ antagonist, blocked the effect of clonidine in both villus and crypt cells (Fig. 1).

It has been previously reported in intact tissue studies from the rabbit ileum that clonidine stimulates coupled NaCl absorption that occurs by the dual operation of Na:H and $Cl:HCO_3$ exchange on the BBM of villus cells (12–14). However, the mechanism of stimulation of NaCl absorption by this $\alpha 2$ agonist was not known. This study demonstrates that the mechanism by which clonidine increases coupled NaCl absorption is a result of the stimulation of Na:H exchange in villus cells.

Functionally, two Na:H exchangers have been demonstrated in villus cells (Fig. 6), one each on the BBM and the BLM

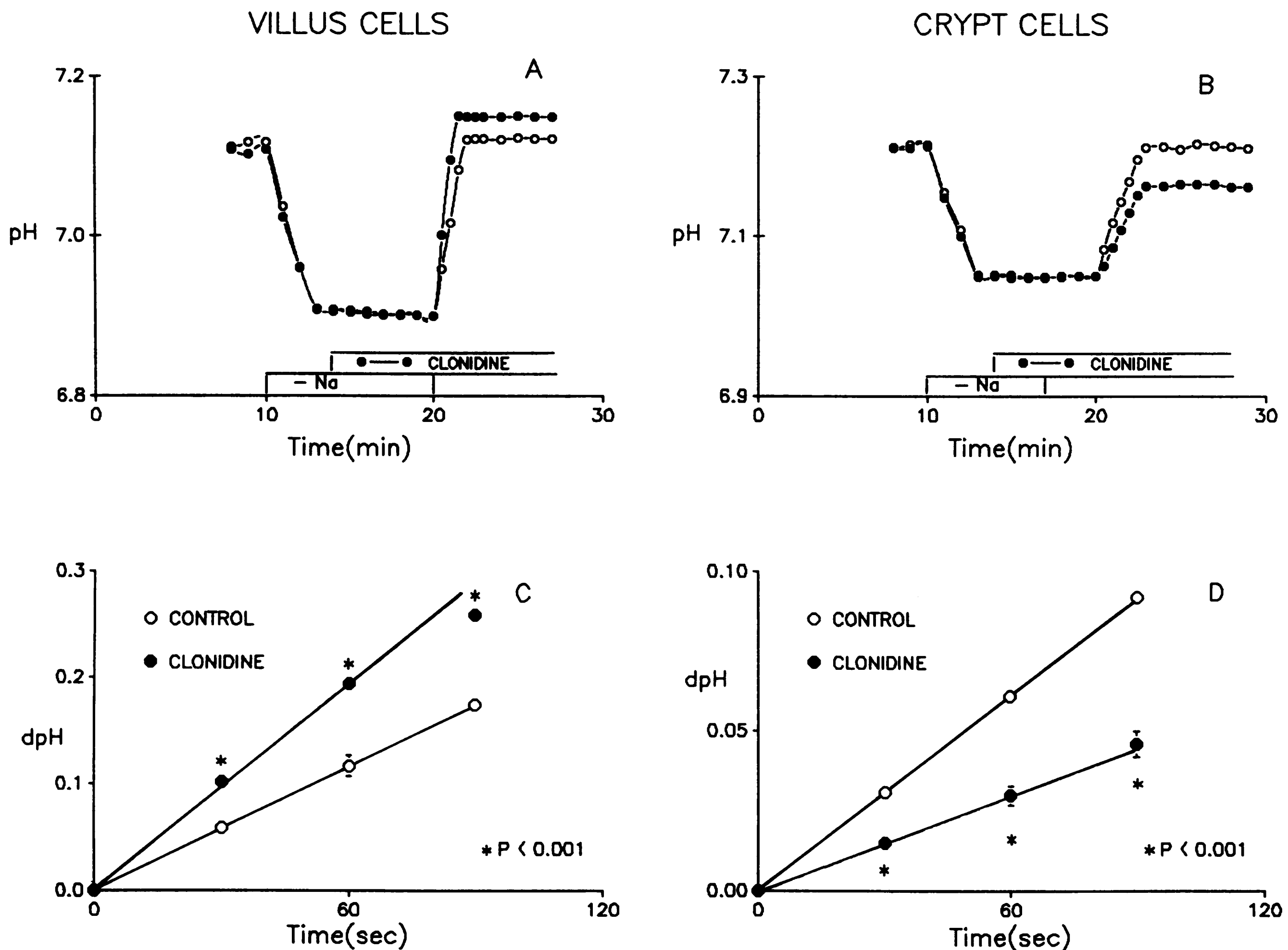

Figure 4. Effect of clonidine in Na-free solution and on recovery from an acid load induced by Na removal in villus (*A*) and crypt (*B*) cells. The cells were first perfused with the standard Na-Hepes solution and then with Na-free solution (Na removal was accomplished by substitution with tetramethyl ammonium) during the period shown by the bar. Cells were then exposed to and allowed to recover to baseline in the presence (●) or absence (○) of 10 μM clonidine. Clonidine has no effect on villus or crypt cells in the absence of Na. However, with Na re-addition the rate of recovery, dpH/d*t*, from Na-removal–induced acid load is faster in the presence of clonidine in villus cells (*C*; 0.12±0.01 dpH/min in control and 0.19±0.01 in clonidine, $n = 5$, $P < 0.001$), whereas it is slower in crypt cells (*D*; 0.06±0.01 dpH/min in control and 0.03±0.01 in clonidine, $n = 5$, $P < 0.001$). *A* and *B* are representative experiments. In *C* and *D*, mean±SEM is shown, and where indicated (*), the dpH/d*t* at any given time is also significantly different from that of control.

(1). With our technique of measuring Na:H exchange activity by monitoring intracellular pH in isolated cells, we cannot tell whether clonidine is stimulating the BBM, the BLM, or both Na:H exchangers. However, because clonidine stimulates coupled NaCl absorption in intact tissue studies from the rabbit ileum, it is likely that clonidine stimulates the BBM Na:H exchange in villus cells (12–14).

Recently Tse et al. (15) have reported the presence of two isoforms of Na:H exchange, NHE2 and NHE3, on the BBM in the rabbit ileum. Our studies do not permit speculation as to which of these isoforms of Na:H exchange is primarily affected by clonidine in the villus cells.

In crypt cells clonidine inhibits Na:H exchange. Knickelbein et al. (1) have demonstrated that only Cl:HCO_3 exchange is present on the BBM of crypt cells, whereas Na:H exchange is found on the BLM. Based on the orientation of transporters in these cells, either the direct stimulation of the BBM Cl:HCO_3 exchange and/or the stimulation of BLM Na:H exchange, which will alkalinize the cell and may subsequently stimulate the BBM Cl:HCO_3 exchange, should result in the secretion of HCO_3 by the crypt cells. Conversely, the inhibition of Na:H exchange (i.e., by clonidine in this study) in crypt cells will acidify the cell, which may inhibit the Cl:HCO_3 exchange on the BBM, resulting in the inhibition of HCO_3 secretion.

Inhibition of HCO_3 secretion by $\alpha 2$ agonists has been well described in intact tissue studies from the stomach, duodenum, and ileum (8, 12–14, 16–19). However, in all these tissues the mechanism of HCO_3 secretion and the inhibition of this process by $\alpha 2$ agonists is poorly understood. Current evidence would suggest that secretion of HCO_3 occurs most likely via Cl:HCO_3 exchange in the stomach and duodenum. An ion channel and a paracellular pathway may also be involved in the duodenum. How clonidine inhibits HCO_3 secretion in these tissues is not known.

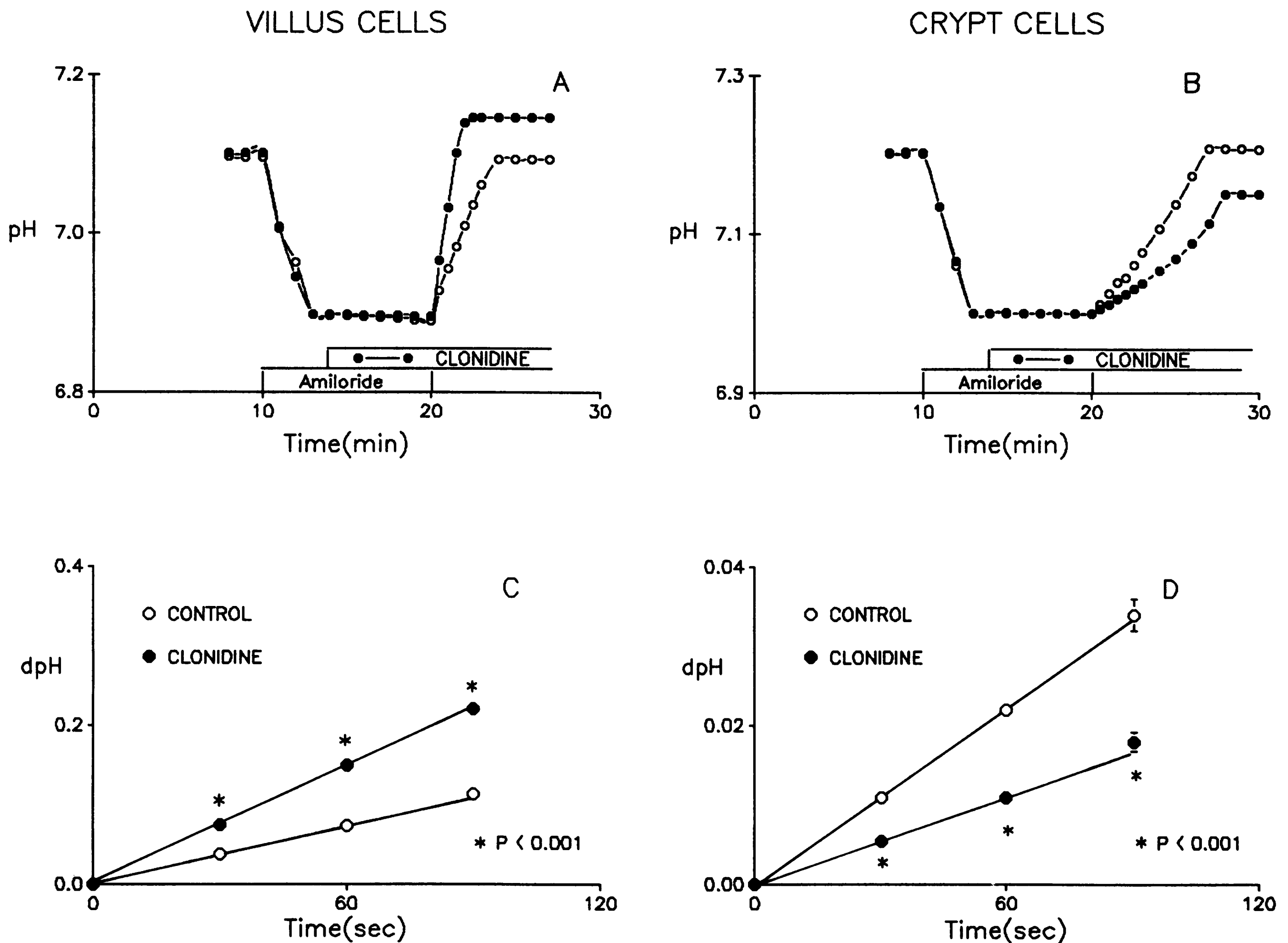

Figure 5. Effect of clonidine in amiloride-containing solution and on recovery from an acid load induced by amiloride (1 mM) in villus (*A*) and crypt (*B*) cells. The cells were first perfused with the standard Na-Hepes solution and then with amiloride during the period shown by the bar. The cells were then exposed to and allowed to recover to baseline in the presence (●) or absence (○) of 10 μM clonidine. Clonidine has no effect on villus or crypt cells in the presence of amiloride. However, after amiloride withdrawal the rate of recovery, dpH/d*t*, from an amiloride-induced acid load is faster in the presence of clonidine in villus cells (*C*; 0.07±0.01 dpH/min in control and 0.15±0.01 in clonidine, $n = 5$, $P < 0.001$), whereas it is slower in crypt cells (*D*; 0.02±0.01 dpH/min in control and 0.01±0.01 in clonidine, $n = 5$, $P < 0.001$). *A* and *B* are representative experiments. In *C* and *D*, mean±SEM is shown, and where indicated (*), the dpH/d*t* at any given time is also significantly different from that of control.

In the ileum, HCO_3 secretion has been well described in in vivo and in vitro studies (6). Inhibition of HCO_3 secretion by $\alpha 2$ agonists has also been described in intact tissue studies (6, 17). However, the mechanism of HCO_3 secretion or the inhibition of this secretion in the ileum is not known.

The recent findings of Minhas and co-workers (20, 21) based on intact tissue studies of the rabbit ileum would support the hypothesis that crypt cells secrete HCO_3. The orientation of transporters in the crypt cell, Cl:HCO_3 exchange on the BBM and Na:H exchange on the BLM (Fig. 6), would also suggest that these cells secrete HCO_3 in the ileum. We have previously demonstrated that two well-known agonists of secretion in the ileum that mediate their action via different intracellular second messengers (calcium and cAMP) stimulate Na:H exchange in crypt cells (3, 4). As previously discussed this stimulation of the BLM Na:H exchange may result in the secretion of HCO_3. Apart from stimulation of BBM Cl:HCO_3 exchange, other possible mechanisms of HCO_3 secretion include a Na:HCO_3 cotransporter or a HCO_3 channel. However, there is presently no evidence for the presence of these later two transport pathways in the rabbit ileum.

In contrast to the effect of secretagogues, clonidine, an absorptogogue, inhibits Na:H exchange in crypt cells. The inhibition of BLM Na:H exchange in crypt cells results in the acidification of these cells, which would subsequently inhibit the BBM Cl:HCO_3 exchange, resulting in the inhibition of HCO_3 secretion, a phenomenon previously unexplained in intact tissue studies.

Thus, the findings of this study and our previous studies suggest that in the ileum HCO_3 transport occurs via Cl:HCO_3 exchanger on the BBM of the crypt cells. Agents mediate HCO_3 secretion and inhibition of secretion by first affecting the BLM Na:H exchanger, which by altering intracellular pH either stimulates or inhibits the BBM Cl:HCO_3 exchange, resulting in the

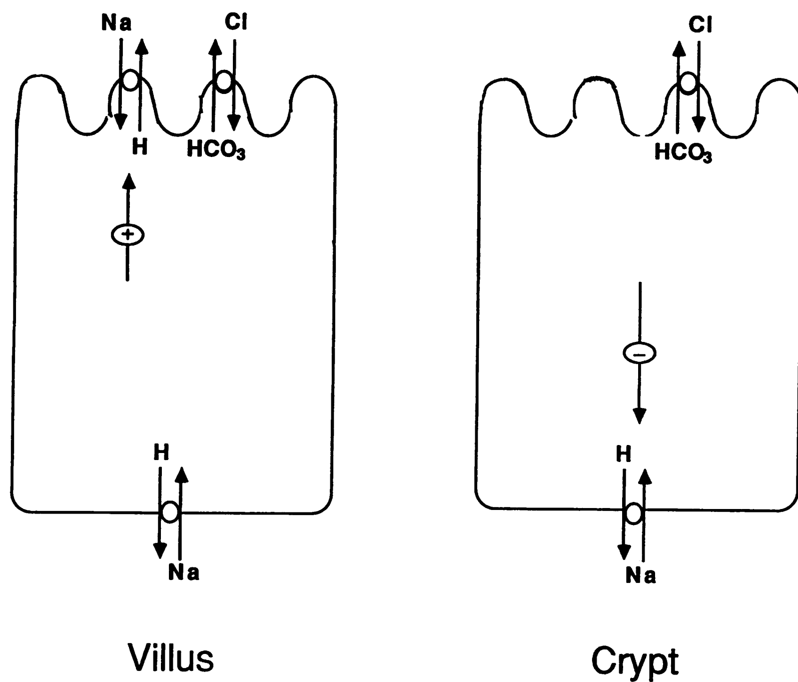

Figure 6. A model of rabbit ileal villus and crypt cells illustrating the transporters and demonstrating the effect of clonidine on the transporters.

stimulation or inhibition of HCO_3 secretion by the crypt cell (Fig. 6). This hypothesis is supported by the finding of Minhas and co-workers (20, 21) that crypt cells secrete HCO_3 and the orientation of transporters in the crypt cell (1).

In the crypt cell, the distribution of transporters, Cl:HCO_3 on the BBM and Na:H on the BLM, may be questioned based on a recent preliminary study by Hoogerwerf et al. (22). In this study they reported that the message for all three isoforms of Na:H exchange (NHE1, thought to be on the BLM, and NHE2 and NHE3, thought to be on the BBM in the ileum) are found in villus and crypt cells (22). Inadequate cell separation criteria resulting in the contamination of villus with crypt cells and vice versa could account for this finding. Also, because Knickelbein et al. (1) could not demonstrate Na:H exchange activity in the BBM of crypt cells, Hoogerwerf et al.'s finding of the message for the various forms of Na:H exchange needs to be correlated with the presence of functional proteins in BBM and BLM in the crypt cells. Further, Hoogerwerf et al.'s findings are also in contrast to the findings of Bookstein et al. (23). They have, by immunolocalization as well as in situ hybridization, demonstrated that only NHE1 is present in the rat ileal crypt cells, whereas both NHE1 and NHE3 are present in the villus cells. Although this controversy has yet to be settled, it is interesting to note that even if all of the same isoforms of Na:H exchange are present in villus and crypt cells, they respond markedly differently in these cells to not only secretogogues as we previously demonstrated (4) but also to absorptogogues as illustrated in this study.

In conclusion, these studies provide a mechanism for some of the previously unexplained in vivo and in vitro effects of an intestinal absorptogogue on ileal electrolyte transport. Further, because clonidine stimulated Na:H exchange in villus cells and inhibited Na:H exchange in crypt cells, these results also emphasize the need to separate these two cell populations in dissecting the regulation of ion transport pathways in the intestine. Not only are the transport pathways different in crypt and villus cells, but the regulation of these transporters by secretagogues and absorptogogues also differs.

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