Mechanism of intestinal secretion: Effect of cyclic AMP on rabbit ileal crypt and villus cells

(regulation of secretion/bicarbonate secretion/intestinal secretagogues/intestinal electrolyte transport)

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Communicated by Gerhard Giebisch, April 8, 1991

ABSTRACT Cyclic AMP-dependent secretagogues such as cholera toxin inhibit the coupled absorption of Na^+ and Cl^- and stimulate the secretion of HCO_3^- and Cl^- in the ileum. Aside from Cl^- secretion, little is known about the mechanism of these cyclic AMP-mediated effects. We therefore determined the effect of forskolin, an agent known to increase intracellular cyclic AMP by stimulation of adenyl cyclase, on Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange in isolated crypt and villus cells from rabbit ileum. Forskolin increased cyclic AMP in the villus cells and decreased intracellular pH. The effect of forskolin on pH in villus cells was HCO_3^- independent, Na^+ dependent, and amiloride sensitive. Further, the rate of recovery from an acid load was decreased by forskolin. These data suggest that increasing cyclic AMP inhibits Na^+/H^+ exchange in villus cells. In crypt cells also, forskolin increased cyclic AMP; however, forskolin increased intracellular pH in these cells. The effect of forskolin in crypt cells was also HCO_3^- independent, Na^+ dependent, and amiloride sensitive. However, the rate of recovery from an acid load was increased by forskolin, the opposite effect of that seen in villus cells. These data suggest that increasing cyclic AMP in crypt cells stimulates Na^+/H^+ exchange. Inhibition of Na^+/H^+ exchange on the brush border membrane in villus cells would be expected to inhibit coupled NaCl absorption (which occurs by coupling of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange). Stimulation of Na^+/H^+ exchange in crypt cells, present only on the basolateral membrane, alkalinizes the cell, which would be expected to stimulate HCO_3^- secretion by stimulating the $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the brush border membrane. Thus, these results provide a mechanism for some of the previously unexplained *in vivo* and *in vitro* effects of cyclic AMP on ileal electrolyte transport.

Na^+ and Cl^- absorption in rabbit and human ileum occurs by the dual operation on Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers on the brush border membrane (BBM) of villus cells (1, 2). Crypt cells have no Na^+/H^+ exchanger on the BBM (1) and thus would not be expected to absorb Na^+ and Cl^- , a hypothesis supported by experimental observations (3–6). Both crypt and villus cells contain a Na^+/H^+ exchanger on the basolateral membrane (1) (Fig. 1).

The vectorial arrangement of a Na^+/H^+ exchanger on the basolateral membrane (BLM) and a $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the BBM suggests that crypt cells may secrete HCO_3^- (1). Stimulation of the BLM Na^+/H^+ exchange will alkalinize the cell, with subsequent stimulation of the BBM $\text{Cl}^-/\text{HCO}_3^-$ exchange resulting in net luminal HCO_3^- secretion. While HCO_3^- secretion occurs in normal human ileum and is stimulated by cholera *in vivo*, the mechanism is unknown (8–11).

Coupled Na^+ and Cl^- absorption, and presumably HCO_3^- secretion, in mammalian ileum is regulated by intracellular second messengers (12–16). We have recently been studying the mechanism of this regulation, utilizing isolated crypt and

FIG. 1. A model of rabbit ileal crypt and villus cells, demonstrating the distribution of some of the transporters. Reprinted with permission from ref. 7 (copyright The American Society for Clinical Investigation, Inc.).

villus cells (7, 17). We have found that serotonin, thought to act by increasing intracellular Ca^{2+} , inhibits coupled Na^+ and Cl^- absorption by inhibiting $\text{Cl}^-/\text{HCO}_3^-$ exchange in villus cells (7). Serotonin stimulated Na^+/H^+ exchange in crypt cells and this stimulation would be expected to stimulate HCO_3^- secretion (7). In the current study, we have determined the effect of forskolin, an agent that stimulates adenyl cyclase and increases intracellular cyclic AMP, on Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange in isolated villus and crypt cells from rabbit ileum.

METHODS

Cell Isolation. Villus and crypt cells were separated from rabbit ileum by a calcium-chelation technique (17) utilizing 0.1 mM EDTA and were maintained in short-term culture. Six fractions of cells were sequentially collected, and fraction 1 was used as villus cells and fraction 6 as crypt cells. Enzyme markers, morphology, and transporter specificity were utilized to assure good separation of crypt and villus cells. The cells were maintained in short-term culture for up to 6–8 hr. Viability was assessed by trypan blue exclusion, linear incorporation of leucine into protein, Na^+ -stimulated glucose uptake (villus cells), and preserved cell structure in electron microscopy. The presence of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers and their role in the regulation of intracellular pH have been demonstrated in both cell types (17).

Intracellular pH Measurements. The cells were loaded with 10 μM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein ac-

Abbreviations: BBM, brush border membrane; BLM, basolateral membrane; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, acetoxymethyl ester of BCECF.

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FIG. 2. Effect of 10 μM forskolin on villus (A) and crypt (B) cells. Cells were perfused with HCO_3^- -containing ($\circ$, $\bullet$) or HCO_3^- -free (Δ , $\triangle$) solutions containing 10 μM forskolin ($\bullet$, Δ) or 10 μM dideoxyforskolin ($\circ$, $\triangle$) during the period shown by the bar.

etoxymethyl ester (BCECF-AM) from a 10 mM stock in dimethyl sulfoxide for 10 min at 37°C. A coverslip with a subconfluent monolayer of cells was mounted in a thermostatically controlled cuvette (37°C) in a Perkin-Elmer 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 by using the high- K^+ nigericin technique (17). All experiments were performed in $\text{CO}_2/\text{HCO}_3^-$ or NaHepes (HCO_3^- -free) solutions. The standard $\text{CO}_2/\text{HCO}_3^-$ 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 was gassed with 5% $\text{CO}_2/95\%$ O_2 ; pH was 7.4 at 37°C. The NaHepes solution contained (in mM) NaCl, 130; KCl, 4.5; KH_2PO_4 , 1.2; MgSO_4 , 1; CaCl_2 , 1.25; Hepes, 20; and was gassed with 100% O_2 ; pH was 7.4 at 37°C. BCECF-AM was purchased from Molecular Probes; serotonin was purchased from Sigma.

Measurement of Cyclic AMP. Cells (1.5 ml, 0.2–0.4 mg of protein) were added to a microcentrifuge tube and kept at 37°C. After exposure to 10 μM forskolin or dideoxyforskolin for various time intervals, the tubes were centrifuged at high speed for 15 sec. The supernatant was removed and the cells were frozen in acetone/dry ice. Immediately, 100 μl of 3% (wt/wt) perchloric acid was added to the pellet of frozen cells, and cyclic AMP levels were determined by a cyclic [^3H]AMP competitive binding assay with kits purchased from Diagnostic Products (Los Angeles). Forskolin was purchased from Sigma, and dideoxyforskolin was from Calbiochem.

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 was performed on cells isolated from a single rabbit. Where representative examples are shown experiments were performed on three to seven separate cell preparations from different animals. The rate of recovery of pH (dpH/dt) was determined by linear regression

and is shown as the slope $\pm$ SE. The paired Student *t* test was used for statistical analysis.

RESULTS

Fig. 2A illustrates that 10 μM forskolin but not dideoxyforskolin causes a reversible acidification of villus cells, independent of the presence of HCO_3^- . Baseline pH decreased from 7.18 ± 0.01 to 7.13 ± 0.01 ($n = 6$, $P < 0.005$) in the presence of HCO_3^- and from 7.11 ± 0.01 to 7.04 ± 0.02 ($n = 6$, $P < 0.01$) in the absence of HCO_3^- . In contrast, forskolin but not dideoxyforskolin caused an alkalinization of crypt cells in both the presence and the absence of HCO_3^- (Fig. 2B). In the presence of HCO_3^- the pH increased from 7.29 ± 0.01 to 7.34 ± 0.01 , and in the absence of HCO_3^- it increased from 7.21 ± 0.01 to 7.27 ± 0.01 ($n = 6$, $P < 0.005$).

Exposure of villus cells to 10 μM forskolin resulted in a 2-fold increase in cyclic AMP at 30 sec, and levels were significantly elevated at all remaining times tested (Fig. 3A). At 11 min, cyclic AMP levels were increased 7-fold (3.21 ± 0.076 to 23.37 ± 2.43 pmol/mg of protein). In contrast, 10 μM dideoxyforskolin had no effect on cyclic AMP levels. While qualitatively similar results were obtained with crypt cells, the effect of 10 μM forskolin was more dramatic, resulting in a 26-fold increase in cyclic AMP at 11 min (3.26 ± 0.61 to 85.11 ± 13.11 pmol/mg of protein, Fig. 3B). Dideoxyforskolin had no effect on cyclic AMP levels in crypt cells. Basal levels of cyclic AMP were not different in crypt and villus cells. Addition of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) increased the forskolin-stimulated increase of cyclic AMP from 16.71 ± 0.65 to 43.32 ± 2.19 pmol/mg of protein ($n = 2$) at 8 min in villus cells but increased cyclic AMP only from 49.58 ± 4.42 to 52.40 ± 15.6 pmol/mg of protein ($n = 2$) at 8 min in crypt cells.

The acidification of villus cells by forskolin in the absence of HCO_3^- is unlikely to be due to stimulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange (operating as a Cl^-/OH^- exchanger), because OH^- is a

FIG. 3. Changes in intracellular cyclic AMP levels in villus (A) and crypt (B) cells. Cells were exposed to 10 μM forskolin ($\bullet$), 10 μM dideoxyforskolin ($\circ$), or nothing ($\square$) for the times shown.

FIG. 4. Effect of 1 mM amiloride (A) and Na⁺-free solutions (C) on forskolin-induced acidification in villus cells. Cells were perfused with amiloride-containing or Na⁺-free solutions during the period shown by the bar. Na⁺ removal was accomplished by substituting tetramethylammonium chloride for Na⁺. (B) After amiloride withdrawal the dpH/dt (pH unit/min) for control cells (○) is 0.093 ± 0.013 and for cells exposed to forskolin (●) it is 0.056 ± 0.005 ($n = 3$, $P < 0.05$). (D) With Na⁺ readdition the dpH/dt for control cells (○) is 0.133 ± 0.036 and for cells exposed to forskolin (●) it is 0.072 ± 0.026 ($n = 7$, $P < 0.005$). A and C are representative experiments. In B and D, where indicated (*), the dpH/dt at any given time is also significantly different from that of the control.

poor substrate for the Cl⁻/HCO₃⁻ exchanger (18). Addition of amiloride or removal of Na⁺ acidifies villus cells (Fig. 4A and C) by inhibiting Na⁺/H⁺ exchange (17). These maneuvers prevented further acidification by forskolin in villus cells (Fig. 4A and C). When amiloride was removed or Na⁺ was added, however, the recovery from this acidification was slower in the

presence of forskolin (Fig. 4B and D). These results suggest that forskolin inhibits Na⁺/H⁺ exchange in villus cells. Recovery from an acid load induced by an NH₄Cl pulse was also delayed in the presence of forskolin (Fig. 5).

In crypt cells, removal of Na⁺ or addition of amiloride results in acidification (Fig. 6A and C), which is due to

FIG. 5. Effect of forskolin on recovery from an acid load in villus cells. The cells are first perfused with the standard NaHepes solution, then with 30 mM NH₄Cl and 100 mM NaCl instead of the 130 mM NaCl in the standard solution and allowed to recover to baseline, then the NH₄Cl pulse was repeated in the presence (A) or absence (C) of 10 μM forskolin. In the control-forskolin experiment (B) the dpH/dt (unit/min) before forskolin (○) is 0.106 ± 0.010 and for forskolin (●) it is 0.059 ± 0.007 ($n = 5$, $P < 0.005$). In the control-control experiment (D), dpH/dt after the first NH₄Cl pulse (○) is 0.110 ± 0.006 and after the second NH₄Cl pulse (●) it is 0.128 ± 0.005 ($n = 3$, P not significant). A and C are representative experiments. In B, where indicated (*), the dpH/dt at any given time is also significantly different. When rate of recovery for forskolin in B is compared with that of the two controls in D, it is significantly different from both ($P < 0.01$).

FIG. 6. Effect of 1 mM amiloride (A) and Na⁺-free (C) solutions on forskolin-induced alkalinization in crypt cells. Experiments were performed as outlined in the legend of Fig. 4. (B) After amiloride removal the dpH/dt (unit/min) for control cells (○) is 0.028 ± 0.003 and for cells exposed to forskolin (●) it is 0.052 ± 0.008 ($n = 3$, $P < 0.05$). (D) With Na⁺ readdition the dpH/dt for control cells (○) is 0.079 ± 0.014 , and for cells exposed to forskolin (●) it is 0.125 ± 0.010 ($n = 4$, $P < 0.025$). A and C are representative experiments. In B and D, where indicated (*), the dpH/dt at any given time is also significantly different.

inhibition of Na⁺/H⁺ exchange (17). These maneuvers blocked the alkalinization induced by forskolin seen in Fig. 2B. When amiloride was removed or Na⁺ was added, however, the recovery from this acidification was faster in the presence of forskolin (Fig. 6B and D), an effect opposite that seen in villus cells (Fig. 4B and D). Recovery from an acid load induced by an NH₄Cl pulse was also faster in the

presence of forskolin (Fig. 7). These results suggest that forskolin stimulates Na⁺/H⁺ exchange in crypt cells.

DISCUSSION

These studies indicate that forskolin inhibits Na⁺/H⁺ exchange in villus cells and stimulates Na⁺/H⁺ exchange in

FIG. 7. Effect of forskolin on recovery from an acid load in crypt cells. Experiments were performed as outlined in the legend of Fig. 5. (B) In the control-forskolin experiment the dpH/dt (unit/min) before forskolin (○) is 0.072 ± 0.012 and for forskolin (●) it is 0.114 ± 0.007 ($n = 4$, $P < 0.05$). (D) In the control-control experiment dpH/dt after the first NH₄Cl pulse (○) is 0.076 ± 0.003 and after the second NH₄Cl pulse (●) it is 0.077 ± 0.004 ($n = 3$, $P = \text{not significant}$). A and C are representative experiments. In B, where indicated (*), the dpH/dt at any given time is also significantly different. When rate of recovery for forskolin in (B) is compared with that of the two controls in D, it is significantly different from both ($P < 0.01$).

crypt cells. These effects of forskolin are most likely secondary to its effect on cyclic AMP levels, since an analog of forskolin that has no effect on adenylyl cyclase, dideoxyforskolin, had no effect on intracellular pH in villus or crypt cells (Fig. 2). Semrad and Chang (19) have reported that cyclic AMP inhibits Na^+/H^+ exchange in enterocytes from chicken small intestine. They did not distinguish between crypt and villus cells; however, judging from our observations, they were probably studying villus cells.

Acidification of villus cells was greatest with Na^+ removal, intermediate with amiloride, and least with forskolin. Na^+ removal should result in complete inhibition of Na^+/H^+ exchange. Amiloride is a competitive antagonist, thus inhibition will be dependent on the concentration of Na^+ and amiloride. These results indicate that forskolin is causing only partial inhibition of the Na^+/H^+ exchanger.

Although forskolin increased cyclic AMP in both crypt and villus cells, the effect was much more prominent in crypt cells (Fig. 3). This observation is a further indicator that there are differences between villus and crypt cells and that we have successfully separated these two cell types (7, 17). Preliminary data demonstrate that the phosphodiesterase inhibitor isobutylmethylxanthine increased forskolin stimulation of cyclic AMP in villus but not crypt cells, suggesting that the lower cyclic AMP response to forskolin in villus cells may be due in part to greater phosphodiesterase activity.

We have previously demonstrated that serotonin also stimulates Na^+/H^+ exchange in crypt cells (7). Serotonin, however, has no effect on cyclic AMP levels in crypt cells (data not shown), and current evidence suggests that serotonin works via Ca^{2+} -mediated pathways (13, 20). Thus it would appear that increasing intracellular Ca^{2+} or cyclic AMP will stimulate the Na^+/H^+ exchanger on the BLM of crypt cells (Fig. 1).

As stated previously, stimulation of the Na^+/H^+ exchanger on the BLM of crypt cells would be expected to stimulate HCO_3^- secretion by activating the $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the BBM (Fig. 1). Although serotonin has not been shown to stimulate HCO_3^- secretion in intact tissue, cholera toxin, which increases cyclic AMP, has been shown to stimulate HCO_3^- secretion *in vivo* (8–10). Our results provide possible insight into the mechanism of this cyclic AMP-stimulated HCO_3^- secretion. Cyclic AMP will also stimulate Cl^- secretion in rabbit ileum, presumably by activating a Cl^- channel on the BBM (12, 21). This Cl^- channel would facilitate HCO_3^- secretion via $\text{Cl}^-/\text{HCO}_3^-$ exchange by enabling Cl^- to recycle across the BBM.

Forskolin inhibited Na^+/H^+ exchange in villus cells, whereas serotonin inhibited $\text{Cl}^-/\text{HCO}_3^-$ exchange in villus cells (7). Thus in villus cells it would appear that increasing intracellular cyclic AMP inhibits Na^+/H^+ exchange and increasing intracellular Ca^{2+} inhibits $\text{Cl}^-/\text{HCO}_3^-$ exchange. Either effect would be expected to block coupled Na^+ and Cl^- absorption, since this occurs by dual operation of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange on the BBM (Fig. 1). Indeed, serotonin and agents that increase cyclic AMP have been shown to inhibit coupled Na^+ and Cl^- absorption in rabbit ileum *in vitro* (12, 13, 16, 22).

There are two Na^+/H^+ exchangers on villus cells (Fig. 1), one on the BBM and one on the BLM. With our technique of measuring Na^+/H^+ exchange activity by monitoring intracellular pH in isolated cells we cannot tell whether forskolin is inhibiting the BBM, the BLM, or both exchangers. It seems unlikely that forskolin is inhibiting only the BLM exchanger, as the resulting intracellular acidification would be expected to stimulate Na^+/H^+ exchange on the BBM and thus trans-epithelial Na^+ absorption. As stated previously, agents that increase cyclic AMP inhibit Na^+ and Cl^- absorption in rabbit

ileum *in vitro* (12, 16, 22). Therefore it seems likely that forskolin is inhibiting the Na^+/H^+ exchanger on the BBM. Finally, forskolin could be stimulating the Na^+/H^+ exchanger on the BLM (as it does in the crypt cell), but this effect is masked by an even greater inhibition of the BBM exchanger.

It must be kept in mind that we are studying isolated cells that do not have tight junctions. It is therefore possible that response to agonists may not be identical to that seen in intact tissue. Nevertheless, these studies provide a mechanism for some of the previously unexplained *in vivo* and *in vitro* effects of cyclic AMP on ileal electrolyte transport. Further, since forskolin stimulated Na^+/H^+ exchange in crypt cells and inhibited Na^+/H^+ exchange in villus cells, these results further emphasize the need to separate these two cell populations in dissecting the regulation of ion transport pathways in the intestine.

This work was supported by National Institutes of Health Research Grant AM 31969. U.S. is the recipient of Senior Research Fellow Award from the American Gastroenterological Association, a Fellowship Award from the National Foundation for Ileitis and Colitis, and a New Investigator Award from the American Heart Association (CT).

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