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Endogenous Luminal Surface Adenosine Signaling Regulates Duodenal Bicarbonate Secretion in Rats

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ABSTRACT

Luminal ATP increases duodenal bicarbonate secretion (DBS) via brush border P2Y receptors. Because ATP is sequentially dephosphorylated to adenosine (ADO) and the brush border highly expresses adenosine deaminase (ADA), we hypothesized that luminal [ADO] regulators and sensors, including P1 receptors, ADA, and nucleoside transporters (NTs) regulate DBS. We measured DBS with pH and CO_2 electrodes, perfusing ADO ± adenosine receptor agonists or antagonists or the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor $CFTR_{inh}$-172 on DBS. Furthermore, we examined the effect of inhibitors of ADA or NT on DBS. Perfusion of AMP or ADO (0.1 mM) uniformly increased DBS, whereas inosine had no effect. The $A_{1/2}$ receptor agonist 5′-(*N*-ethylcarboxamido)-adenosine (0.1 mM) increased DBS, whereas ADO-augmented DBS was inhibited by the potent A_{2B} receptor antagonist *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754) (10 μM). Other selective adenosine receptor agonists or antagonists had no effect. The A_{2B} receptor was immunolocalized to the brush border membrane of duodenal villi, whereas the A_{2A} receptor was immunolocalized primarily to the vascular endothelium. Furthermore, ADO-induced DBS was enhanced by 2′-deoxycoformycin (1 μM) and formycin B (0.1 mM), but not by *S*-(4-nitrobenzyl)-6-thioinosine (0.1 mM), and it was abolished by $CFTR_{inh}$-172 pretreatment (1 mg/kg i.p). Moreover, ATP (0.1 mM)-induced DBS was partially reduced by (1*R*,2*S*,4*S*,5*S*)-4–2-iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0] hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500) or 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB603) and abolished by both, suggesting that ATP is sequentially degraded to ADO. Luminal ADO stimulates DBS via A_{2B} receptors and CFTR. ATP release, ecto-phosphohydrolases, ADA, and concentrative NT may coordinately regulate luminal surface ADO concentration to modulate ADO-P1 receptor signaling in rat duodenum.

Introduction

Adenosine (ADO) is a purinergic signaling molecule that profoundly affects gut function, including motility, ion secretion, and the modulation of inflammation (Antonioli et al., 2008; Ye and Rajendran, 2009). Most of the research regarding extracellular ADO signaling in the gut has addressed enteric neurons, smooth muscle, afferent neurons, and the immune system, with relatively few studies addressing its effect on epithelial secretory function. Of these, even fewer

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ABBREVIATIONS: ADA, adenosine deaminase; DBS, duodenal bicarbonate secretion; ADO, adenosine; CFTR, cystic fibrosis transmembrane conductance regulator; CNT, concentrative nucleoside transporter; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CSC, 8-(3-chlorostyryl)caffeine; DCF, 2′-deoxycoformycin; ENT, equilibrative nucleoside transporter; ForB, formycin B; IAP, intestinal alkaline phosphatase; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; INO, inosine; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; NECA, 5′-(*N*-ethylcarboxamido)-adenosine; NT, nucleoside transporter; I_{sc}, short-circuit current; ENTPDase, ecto-nucleoside triphosphate diphosphohydrolase; MRS1754, *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide; MRS2500, (1*R*,2*S*,4*S*,5*S*)-4-[2-iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt; PSB603, 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate; CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine.

have reported the effect of luminal ADO on ion secretion in intact epithelia in vivo, because most published studies reported short-circuit current (I_{sc}) measured in intestinal T84 monolayers (Barrett et al., 1989; Strohmeier et al., 1995) or mounted mammalian intestinal tissues (Dobbins et al., 1984; Grasl and Turnheim, 1984; Ghanem et al., 2005). Because apical or basolateral ADO stimulated I_{sc}, ADO receptors were predicted to be expressed on both epithelial surfaces (Barrett et al., 1989; Strohmeier et al., 1995). Luminal AMP and ADO increase glucose transport in intact mouse small intestine in vitro and in vivo, consistent with apical ADO receptors (Kimura et al., 2005).

The duodenal enterocyte actively secretes bicarbonate as part of a system that protects the mucosa from acid injury. We have reported that duodenal bicarbonate secretion (DBS) is regulated by a luminal purinergic regulatory system comprised of the brush border intestinal alkaline phosphatase (IAP), nonlytic ATP release, and enterocyte P2Y receptors, which, when activated, augment epithelial DBS (Mizumori et al., 2009). Although not directly tested, this purinergic regulatory system probably functions to regulate duodenal surface microclimate pH, which prior studies had identified with the use of microelectrodes (Flemström et al., 1982; Flemström and Kivilaakso, 1983). We have also demonstrated in vivo that IAP activity at the epithelial surface is correlated with the rate of DBS (Akiba et al., 2007), further suggesting the importance of IAP in surface microclimate pH regulation. Despite strong evidence supporting the involvement of P2Y receptors in this regulatory system, potent P2Y receptor antagonists only partially inhibit DBS augmented by the perfusion of exogenous ATP or the inhibition of IAP (Mizumori et al., 2009). Because surface phosphohydrolases, including IAP and the combination of ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase or CD39) and 5′-nucleotidase (CD73), hydrolyze ATP to ADO, another known purinergic signaling compound, endogenously produced ADO may also mediate intestinal ion secretion. Unlike ATP, which activates P2Y $G_{q/11}$ receptors followed by the increase of cellular Ca^{2+}, ADO activates P1 G_i receptors (A_1 and A_3) or G_s receptors (A_{2A} and A_{2B}). The latter receptors increase adenylate cyclase activity, which elevates cellular cAMP, directly activating the cystic fibrosis transmembrane regulator (CFTR), an important component of active DBS (Hogan et al., 1997; Seidler et al., 1997; Hirokawa et al., 2004; Akiba et al., 2005). There is, however, no published study addressing the effect of luminal ADO on bicarbonate secretion.

An ADO-degrading enzyme adenosine deaminase (ADA) is highly expressed and active in the enterocyte brush border (Witte et al., 1991; Mohamedali et al., 1993). Furthermore, luminal ADO is absorbed via the enterocyte nucleoside transporters (NTs), such as concentrative NT (CNT or SLC28) and the equilibrative NT (ENT or SLC29) (Pastor-Anglada et al., 2007). These observations suggest that luminal ADO signaling activity may be affected by ADA and NT activity, as reported in the airway epithelium (Hirsh et al., 2007). Furthermore, apical CNT activity is thought to mediate ADO uptake into the epithelial cells, where it can be recycled to ATP, metabolized, or released across the basolateral membrane (Ritzel et al., 1998).

On the basis of these observations, we formulated a hypothesis that, in addition to ATP-P2Y purinergic signaling, DBS is regulated by a related ADO-based system, in which ADO is generated from released ATP by brush border phosphohydrolases and interacts with P1 receptors on the enterocyte brush border. Brush border ADA and NT activity may regulate luminal surface ADO concentration, affecting luminal surface ADO signaling. We thus studied the presence and mechanism of a luminal surface ADO signaling system in rat duodenum, testing the hypothesis that DBS is regulated by ADO. We further hypothesized that ADO-P1 signaling complements ATP-P2Y signaling in the coregulation of DBS. Because physiological, endogenous ADO signaling in the gut has not been reported previously and orally ingested P1 receptor ligands are of current interest in the therapy of intestinal inflammation, an integrative study of intestinal brush border ADO signaling is likely to be of current interest.

Materials and Methods

Chemicals and Animals. $CFTR_{inh}$-172 was synthesized by Dr. Samedy Ouk in the Department of Chemistry, University of California, Los Angeles (Akiba et al., 2005). Formycin B (ForB) was purchased from Berry and Associates, Inc. (Dexter, MI). 2′-Deoxycoformycin (DCF), (1*R*,2*S*,4*S*,5*S*)-4-[2-iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy) bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500), and 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)-phenyl]]-1-propylxanthine (PSB603) were obtained from Tocris Bioscience (Ellisville, MO). ADO, AMP, ATP, inosine (INO), N^6-cyclopentyladenosine (CPA), 5′-(*N*-ethylcarboxamido)-adenosine (NECA), N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS21680), 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), 8-(3-chlorostyryl)caffeine (CSC), *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1754), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523), HEPES, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Krebs' solution contained 136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES at pH 7.0. All studies were performed with the approval of the Veterans Affairs Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 200 to 250 g (Harlan, Indianapolis, IN) were fasted overnight, but had free access to water.

Measurement of DBS. Duodenal loops were prepared and perfused as described previously (Mizumori et al., 2006; Akiba et al., 2007). Under isoflurane anesthesia (1.5–2.0%), the proximal duodenal loop (perfused length 2 cm) was perfused with pH 7.0 Krebs buffer by using a peristaltic pump (Thermo Fisher Scientific, Waltham, MA) at 1 ml/min. The perfusate was bubbled with 100% O_2 and stirred and warmed at 37°C with a heating stirrer (Barnstead International, Dubuque, IA). To eliminate the buffer action of agonists or antagonists, which would overestimate or underestimate the titration volume by using pH-stat, two sets of flow-through pH and CO_2 electrodes were connected in the perfusion loop where pH and CO_2 concentration ($[CO_2]$) were simultaneously and continuously measured. Because the input (perfusate) $[CO_2]$ is ~0, the effluent $[CO_2]$ and pH were used to calculate the total CO_2 output equivalent to the secreted HCO_3^- as described previously (Mizumori et al., 2006; Akiba et al., 2007). After stabilization with continuous perfusion of pH 7.0 Krebs buffer for ~30 min, the time was set as $t = 0$. The duodenal loop was perfused with pH 7.0 Krebs buffer from $t = 0$ min until $t = 10$ min (basal period). The perfusate was then changed to pH 7.0 Krebs buffer containing agonists or antagonists from $t = 10$ min until $t = 35$ min (challenge period), with or without agonists or antagonists.

Experimental Protocol. We first examined the effect of perfusion of AMP, ADO, or INO on DBS. The duodenal loop was perfused with AMP, ADO, or INO (0.1 mM), the same concentration used for ATP-induced stimulation of DBS (Mizumori et al., 2009), dissolved in pH 7.0 Krebs buffer during the challenge period. Some animals were

pretreated with the potent selective CFTR inhibitor $CFTR_{inh}$-172 (1 mg/kg i.p) 1 h before the experiments. Pretreatment with $CFTR_{inh}$-172 at this dose eliminates acid-induced HCO_3^- secretion in rat duodenum (Akiba et al., 2005).

To determine which P1 adenosine receptor subtype (A_1, A_{2A}, A_{2B}, or A_3) is involved in DBS, we examined the effect of perfusion of P1 receptor agonists at concentrations close to the ED_{50} for each receptor on DBS: a selective A_1 receptor agonist CPA (0.1 mM), a potent A_{2A} receptor agonist CGS21680 (10 μM), a nonselective A_1/A_2 receptor agonist NECA (0.1 mM), or a selective A_3 receptor agonist IB-MECA (10 μM). Furthermore, a potent P1 receptor antagonist was coperfused with ADO (0.1 mM), a selective A_1 receptor antagonist DPCPX (0.1 mM), a selective A_{2A} receptor antagonist CSC (0.1 mM), a potent A_{2B} receptor antagonist MRS1754 (10 μM), or a selective A_3 receptor antagonist MRS1523 (10 μM). Antagonist concentrations were chosen to be at concentrations near the ID_{50} of each receptor.

To test the contribution of the ADO-degrading enzyme ADA and the ADO-absorbing CNT or ENT to DBS, we perfused a highly potent ADA inhibitor DCF (1 μM), a CNT inhibitor ForB (0.1 mM), or an ENT inhibitor NBTI (0.1 mM) with or without ADO (0.1 mM).

Because we have shown that luminally released ATP from duodenal mucosa stimulates HCO_3^- secretion partially via $P2Y_1$ receptor activation (Mizumori et al., 2009) and ATP is degraded to ADO by IAP and ENTPDase/5′-nucleotidase (Zimmermann, 2000), we examined the effect of a highly potent $P2Y_1$ receptor antagonist MRS2500 (1 μM) or a highly selective A_{2B} receptor antagonist PSB603 (10 μM) on ATP (0.1 mM)-induced HCO_3^- secretion to clarify the contribution of ATP-P2Y and ADO-P1 signals to the ATP-induced DBS.

Expression of P1 Receptor Subtypes in Rat Duodenum. Immunofluorescence staining was performed as described previously (Akiba et al., 2006) on the cryostat sections of proximal duodenum fixed with 4% paraformaldehyde, using primary antibodies for A_1, A_{2A}, A_{2B}, and A_3 receptors (rabbit polyclonal, 1:100; Alomone Labs, Jerusalem, Israel), CFTR (M3A7 mouse monoclonal, 1:50; Thermo Fisher Scientific, Waltham, MA), or ADA (goat polyclonal, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Negative controls were examined by omitting the primary antibody or preincubating antibody with the immunized peptide. The sections were observed under a fluorescence microscope (Carl Zeiss GmbH, Jena, Germany), and the images were captured and recorded with a charge-coupled device color video camera (Hamamatsu Photonics, Hamamatsu, Japan) with imaging software, Simple PCI (Compix Inc. Imaging Systems, Cranberry Township, PA) or a Zeiss confocal laser scanning microscope (LSM 710).

Statistics. All data are expressed as means ± S.E.M. Data were derived from six rats in each group. Comparisons between groups were made by one-way analysis of variance followed by Fischer's least significant difference test. P values <0.05 were taken as significant.

Results

Effect of ADO on Duodenal HCO_3^- Secretion. To determine nucleotide or nucleoside specificity, we initially examined the effect of AMP, ADO, or INO (0.1 mM) on DBS. During perfusion of pH 7.0 Krebs buffer, HCO_3^- secretion (measured as total CO_2 output) was stable over time (Fig. 1). AMP and ADO uniformly increased HCO_3^- secretion, whereas INO had no effect (Fig. 1A), suggesting that ADO is a predominant signaling molecule among the three for HCO_3^- secretion.

To test the role of CFTR in ADO-induced DBS, rats were pretreated with $CFTR_{inh}$-172 (1 mg/kg i.p). CFTR inhibition abolished ADO-induced HCO_3^- secretion (Fig. 1B), suggesting that ADO-induced DBS is mediated via CFTR.

Effect of P1 Receptor Agonists or Antagonists on Duodenal HCO_3^- Secretion. Next, we examined the effect of P1 receptor agonists on DBS. The A_1/A_2 receptor agonist NECA (0.1 mM) increased HCO_3^- secretion, whereas CPA (A_1, 0.1 mM), CGS21680 (A_{2A}, 10 μM), or IB-MECA (A_3, 10 μM) had no effect (Fig. 2).

We also examined the effect of P1 receptor antagonists on ADO-induced HCO_3^- secretion. The selective A_1 receptor antagonist DPCPX (0.1 mM) or selective A_{2A} receptor antagonist CSC (0.1 mM) failed to affect ADO-induced HCO_3^- secre-

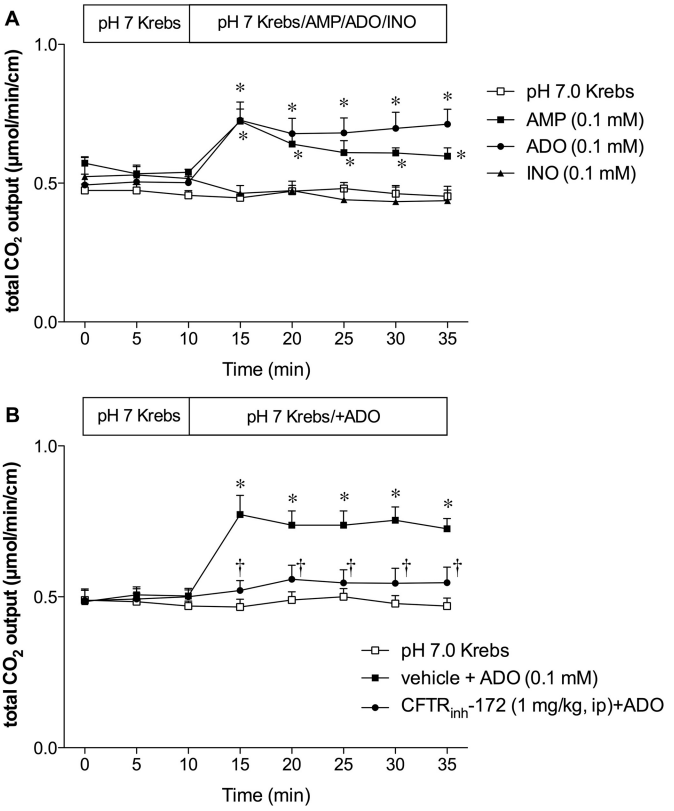

Fig. 1. Effect of ADO on duodenal HCO_3^- secretion in rats. A, duodenal HCO_3^- secretion was measured as total CO_2 output with flow-through pH and CO_2 electrodes. Perfusion of AMP (0.1 mM) or ADO (0.1 mM) similarly increased total CO_2 output, whereas INO (0.1 mM) had no effect. Data represent mean ± S.E.M. (n = 6 rats). *, $p < 0.05$ versus pH 7.0 Krebs group. B, CFTR was inhibited by $CFTR_{inh}$-172 (1 mg/kg i.p) 1 h before the experiment. CFTR inhibition abolished the ADO effect. Data represent mean ± S.E.M. (n = 6 rats). *, $p < 0.05$ versus pH 7.0 Krebs group; †, $p < 0.05$ versus ADO group.

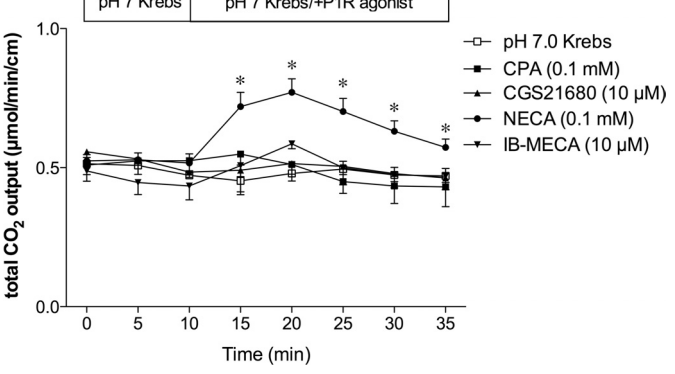

Fig. 2. Effect of P1 receptor agonists on duodenal HCO_3^- secretion in rats. Perfusion of CPA (0.1 mM), CGS21680 (10 μM), or IB-MECA (10 μM) had no effect, whereas NECA (0.1 mM) increased HCO_3^- secretion. Data represent mean ± S.E.M. (n = 6 rats). *, $p < 0.05$ versus pH 7.0 Krebs group.

tion (Fig. 3A). The potent A_{2B} receptor antagonist MRS1754 (10 μM) abolished ADO-induced HCO_3^- secretion, whereas the selective A_3 receptor antagonist MRS1523 (10 μM) had no effect (Fig. 3B). To confirm the inhibitory selectivity for A_{2B} receptors, a highly selective A_{2B} receptor antagonist PSB603 (1 or 10 μM) was perfused with ADO. PSB603 dose-dependently inhibited ADO-induced HCO_3^- secretion (Fig. 3C). These results suggest that A_{2B} receptor is involved in ADO-induced DBS.

Expression of P1 Receptors in the Duodenal Epithelium. Immunofluorescence for P1 receptors showed that A_{2B} receptor was expressed on the brush border membrane of duodenal villous cells (Fig. 4C). A_{2A} receptor was recognized predominantly in the endothelium in the lamina propria of villi (Fig. 4B). A_1 or A_3 receptor was not observed in the villi (Fig. 4, A and D), whereas A_1 receptor was recognized in the myenteric plexus (Fig. 4A inset). This result supports our hypothesis that luminal ADO stimulates duodenal HCO_3^- secretion via the A_{2B} receptor. To further demonstrate the presence of the components of luminal ADO-P1 signaling on the duodenal brush border, immunostaining for CFTR, A_{2B} receptor, and ADA was also examined. The brush border membranes of duodenal villous cells expressed CFTR (Fig. 4E), A_{2B} receptor (Fig. 4F), and ADA (Fig. 4G), further supporting our hypothesis.

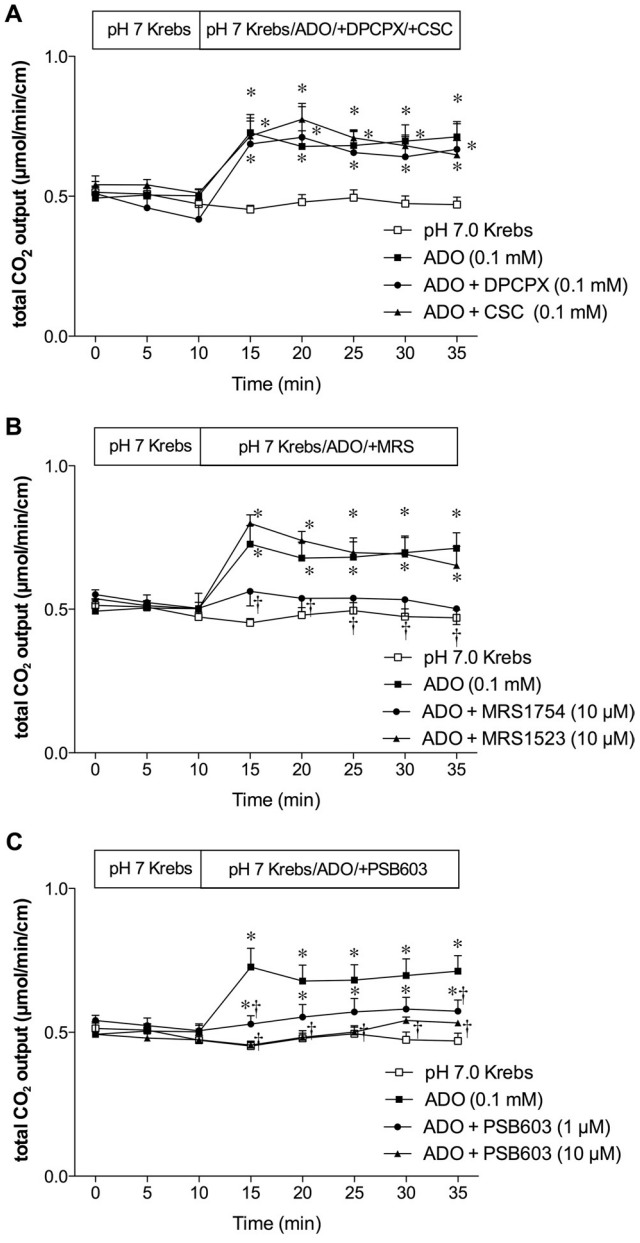

Fig. 3. Effect of P1 receptor antagonists on ADO-induced augmented HCO_3^- secretion in rat duodenum. A, coperfusion of DPCPX (0.1 mM) or CSC (0.1 mM) had no effect on ADO-induced increase of HCO_3^- secretion. B, coperfusion of MRS1754 (10 μM) abolished the ADO effect, but MRS1523 (10 μM) had no effect. C, coperfusion of PCB603 (1 or 10 μM) inhibited the effect of ADO. Data represent mean ± S.E.M. (n = 6 rats). *, $p < 0.05$ versus pH 7.0 Krebs group; †, $p < 0.05$ versus ADO group.

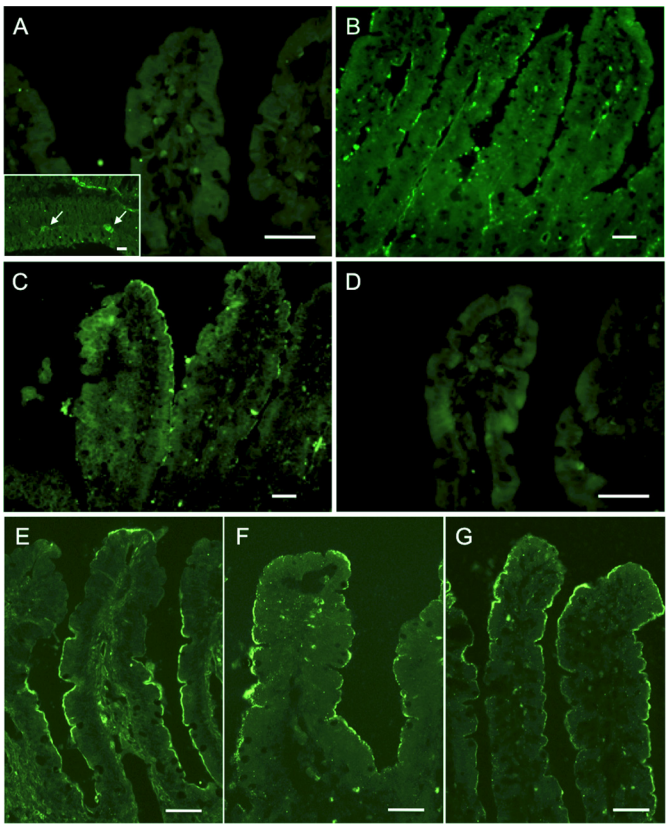

Fig. 4. Expression of P1 receptors, CFTR and ADA, in rat duodenal mucosa. A–D, conventional microscopic images. E–G, confocal laser scanning microscopic images. Cryostat sections of fixed rat duodenum were reacted with primary antibodies for P1 receptors, CFTR and ADA. A and D, no specific staining was observed for A_1 (A) or A_3 (D) receptor in the villi. A_1 receptor-like immunoreactivity was recognized in the myenteric plexus (arrows, A, inset). B, A_{2A} receptor-like immunoreactivity was observed mainly on the endothelial cells of vasculature. C and F, A_{2B} receptor-like immunoreactivity was recognized on the brush border membranes of villous cells. E–G, CFTR (E), A_{2B} receptor (F), and ADA (G) were coexpressed on the enterocyte brush border. Bars, 50 μm.

Effect of Inhibition of ADA or NT on DBS. The highly potent ADA inhibitor DCF (1 μM) increased HCO_3^- secretion, whereas a CNT inhibitor ForB (0.1 mM) gradually increased HCO_3^- secretion (Fig. 5). Furthermore, coperfusion of DCF and ForB additively enhanced HCO_3^- secretion (Fig. 5). This result suggests that endogenous ADO stimulates HCO_3^- secretion and luminal surface ADO concentration is regulated by the brush border ADA activity and CNT.

Furthermore, we examined the role of ENT and CNT in ADO-induced HCO_3^- secretion. The ENT inhibitor NBTI (0.1 mM) had no effect on ADO-induced HCO_3^- secretion (Fig. 6A), whereas the CNT inhibitor ForB (0.1 mM) en-

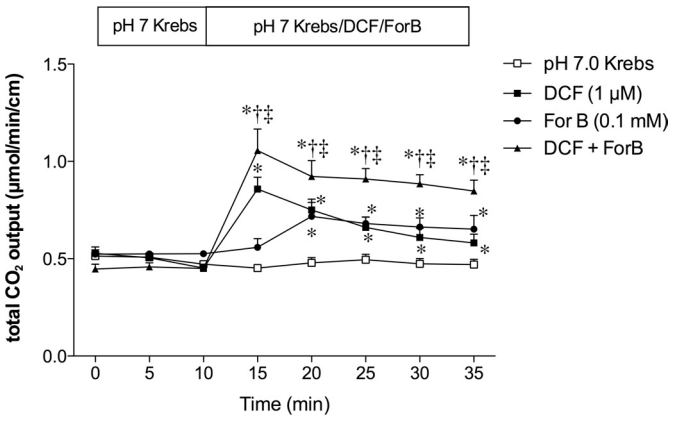

Fig. 5. Effect of inhibitors of ADA or CNT on duodenal HCO_3^- secretion in rats. DCF (1 μM), ForB (0.1 mM), or both was perfused. DCF and ForB augmented HCO_3^- secretion. *, $p < 0.05$ versus pH 7.0 Krebs group; †, $p < 0.05$ versus DCF group; ‡, $p < 0.05$ versus ForB group.

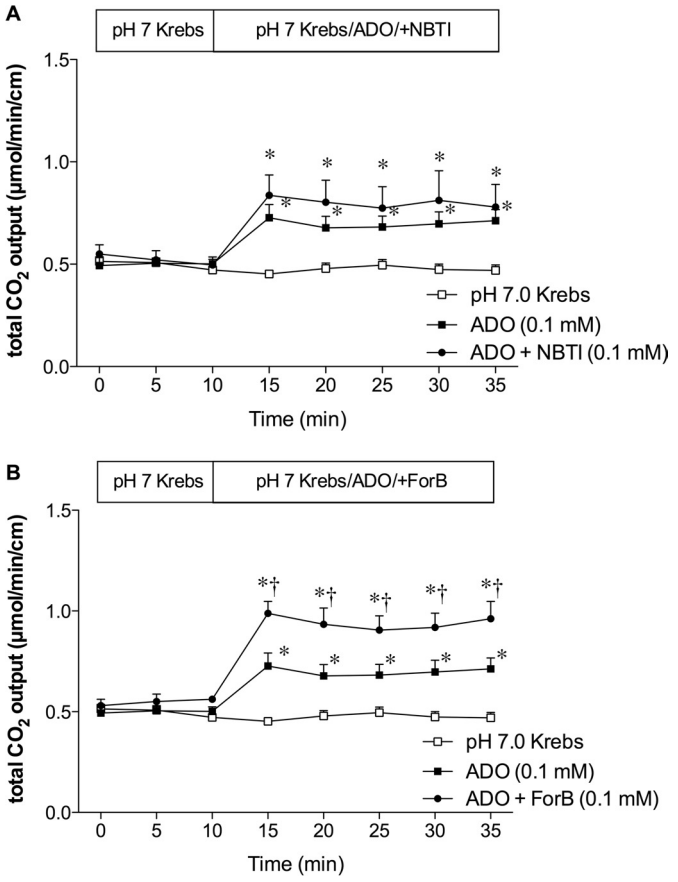

Fig. 6. Effect of ENT or CNT on ADO-induced HCO_3^- secretion in rat duodenum. A, the ENT inhibitor NBTI (0.1 mM) had no effect on ADO-induced HCO_3^- secretion. B, ADO-induced HCO_3^- secretion was enhanced by the addition of ForB (0.1 mM). Data represent mean ± S.E.M. ($n = 6$ rats). *, $p < 0.05$ versus pH 7.0 Krebs group; †, $p < 0.05$ versus ADO group. Data represent mean ± S.E.M. ($n = 6$ rats).

hanced the effect of ADO (Fig. 6B), further suggesting that CNT, not ENT, regulates luminal surface ADO-induced HCO_3^- secretion in rat duodenum.

Effect of $P2Y_1$ or A_{2B} Receptor Antagonist on ATP-Induced DBS. We next tested our hypothesis that endogenous ADO is produced by brush border phosphohydrolases, because ATP is released from the duodenal mucosa in response to physiological secretory stimuli such as luminal acid perfusion, or ATP release is unmasked under the inhibition of IAP or ENTPDase (Mizumori et al., 2009). Furthermore, exogenous ATP activates $P2Y_1$ receptors on the enterocyte brush border, augmenting DBS (Mizumori et al., 2009). We hence studied the sequential effect of exogenous luminal ATP on DBS. Luminal perfusion of ATP (0.1 mM) increased HCO_3^- secretion (Fig. 7) as reported previously (Mizumori et al., 2009). ATP-induced HCO_3^- secretion was partially reduced by the addition of a highly potent $P2Y_1$ receptor antagonist MRS2500 (1 μM). A highly selective A_{2B} receptor antagonist PSB603 (10 μM) also reduced ATP-induced HCO_3^- secretion. Coperfusion of MRS2500 and PSB603 abolished ATP-induced HCO_3^- secretion (Fig. 7). These data suggest that luminal ATP stimulates HCO_3^- secretion partially via the $P2Y_1$ receptor; simultaneously sequential degradation of ATP by brush border phosphohydrolases supplies luminal surface ADO in situ to activate the A_{2B} receptors. ATP release thus seems to be the sole source of extracellular purines, which sequentially activate brush border ATP and ADO receptors.

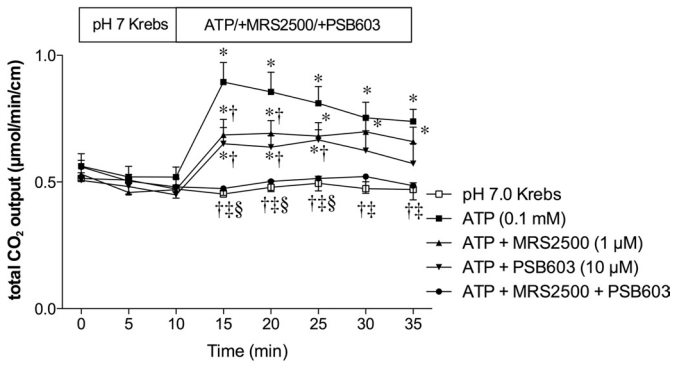

Fig. 7. Effect of $P2Y_1$ or A_{2B} receptor antagonist on ATP-induced HCO_3^- secretion in rat duodenum. The potent $P2Y_1$ receptor antagonist MRS2500 (1 μM) or selective A_{2B} receptor antagonist PSB603 (10 μM) was coperfused with ATP (0.1 mM). ATP-induced HCO_3^- secretion was partially via $P2Y_1$ or A_{2B} receptor. Data represent mean ± S.E.M. ($n = 6$ rats). *, $p < 0.05$ versus pH 7.0 Krebs group; †, $p < 0.05$ versus ATP group; ‡, $p < 0.05$ versus ATP + MRS2500 group; §, $p < 0.05$ versus ATP + PSB603 group.

Discussion

Exogenous luminal AMP and ADO, not INO, augmented DBS, consistent with the activation of brush border P1 receptors. Studies with selective P1 agonists and antagonists were consistent with brush border A_{2B} receptors mediating ADO-induced DBS. The presence of A_{2B} receptors, CFTR, and ADA at the enterocyte brush border was confirmed by immunohistochemistry. Additive augmentation of DBS by the ADA inhibitor DCF and the CNT inhibitor ForB implicated these proteins in the regulation of luminal surface ADO abundance, whereas the ENT does not seem to affect luminal ADO concentrations. Finally, DBS augmented by perfusion of exogenous ATP was abolished by coperfusion of P1 and P2 receptor antagonists, but partially by perfusion of either antagonist singly, providing the novel observation that luminal ADO is derived from ATP in the intestine. This is the first study demonstrating that luminal ADO stimulates DBS measured in vivo in an intact preparation, ADO-induced DBS is mediated in intestine by brush border A_{2B} receptors

and CFTR, and ADO is endogenously generated in the duodenal lumen.

Despite its considerable importance to neuromuscular signaling, differentiation, and inflammatory modulation, few in vivo descriptions of ADO effects on epithelial secretory function exist. In the 1970s and 1980s, ATP but not ADO was reported to increase intestinal ion secretion (Kohn et al., 1970; Gerencser and Armstrong, 1972; Korman et al., 1982). The earliest descriptions of the prosecretory effect of ADO in intestine were reported in 1984, in which basolateral addition of ~0.5 to 1.0 mM ADO to chambered rabbit ileum or colon in vitro elicited an increase in electrogenic net Cl^- secretion (Dobbins et al., 1984; Grasl and Turnheim, 1984). More recently, numerous groups have reported the prosecretory effects of ADO in cultured epithelial cells or mounted intestinal tissues through the activation of A_2 or A_1 receptors, respectively (Ghanem et al., 2005; Novak et al., 2008; Wang et al., 2008; Rajagopal and Pao, 2010).

The ready availability of potent and selective adenosine receptor agonists and antagonists enabled us to pharmacologically characterize ADO-induced DBS. CPA, CGS21680, and IB-MECA (A_1, A_{2A}, and A_3 selective agonists, respectively) did not affect DBS. Nevertheless, NECA increased DBS, indicating that either receptor A_1 or A_2 is involved in ADO-mediated DBS. Given the nonselective nature of NECA, and the lack of specific A_{2B} agonists, we used specific A_1-, A_{2A}-, A_{2B}-, and A_3-selective antagonists, DPCPX, CSC, MRS1754, and MRS1523, respectively. Of these antagonists, only MRS1754, the A_{2B} antagonist inhibited ADO-induced DBS. Immunostaining confirmed the presence of A_{2B} receptors on the villous brush border, consistent with the pharmacological observations. In contrast, A_{2A} receptors predominantly localized on the endothelial cells, consistent with the role of ADO in the regulation of blood flow via A_{2A} receptors (Pennanen et al., 1994; Belardinelli et al., 1998). A_1 or A_3 receptor-like immunoreactivity was not observed on the villous brush border, whereas A_1 receptors were located in the myenteric plexus, as reported previously in human jejunum (Christofi et al., 2001). No staining for A_3 receptor-like immunoreactivity was observed in the duodenum, although the antibody used recognized the A_3 receptor in the esophageal mucosa (data not shown).

DCF, a potent ADA inhibitor, and ForB, a CNT inhibitor, increased DBS. These data demonstrate the presence of endogenous ADO generation in the lumen. Furthermore, ForB, not NBTI, enhanced ADO-induced DBS, suggesting that brush border CNT, unlike ENT, is related to nucleoside transport from the lumen to the enterocytes as predicted (Pastor-Anglada et al., 2007). Present on the apical membrane of epithelial cells, CNTs may absorb luminal nucleosides into the enterocytes. Of the three known paralogs belonging to the SLC28A family, CNT1–3, CNT2 is the most likely duodenal brush border ADO transporter. Because no selective CNT inhibitor is available, data supporting this hypothesis include the high relative duodenal expression of CNT2 and the relative affinity of CNT2 for purines compared with CNT1 or CNT3 (Gray et al., 2004; Larráyoz et al., 2004; Lu et al., 2004; Kim et al., 2007). Similar to our data, an "adenosine scavenging" mechanism affects basolateral extracellular ADO concentrations in cultured T84 cell monolayers (Tally et al., 1996). Pharmacological inhibition of the proteins implicated in adenosine scavenging increases extracellular ADO concentration, increasing I_{sc} via the basolateral A_{2B} receptor. Since that report was published many of the proteins involved in the adenosine scavenging mechanism have been identified; the phenomenon described probably was caused by ENTs localized on the T84 cell basolateral membrane, which transport in either direction in a concentration-dependent fashion and may regulate intracellular nucleoside concentrations (Pastor-Anglada et al., 2007). Furthermore, our findings are consistent with the previous report that luminal ADA and CNT, not ENT, regulate luminal ADO concentrations in the human airway epithelia (Hirsh et al., 2007).

MRS2500, a $P2Y_1$ antagonist, and PSB603, an A_{2B} antagonist, each decreased DBS augmented by luminal perfusion of ATP, and coperfusion of both antagonists completely suppressed ATP-induced DBS. Therefore, luminal ATP and its metabolite ADO may simultaneously activate P2Y and P1 receptors, respectively. We have reported previously that IAP inhibition increases DBS as a probable consequence of increasing ATP output into the lumen because of reduced ATP hydrolysis. This ATP release is partially reduced by CFTR inhibition (Mizumori et al., 2009), whereas CFTR inhibition abolished ADO-induced DBS, suggesting that the CFTR-dependent effect of ATP on DBS may be explained by ADO-A_{2B} signaling. Nevertheless, CFTR inhibition abolished the secretory effect of ADO, which is fully consistent with ADO-induced CFTR activation via A_{2B} G_s receptors that increases intracellular cAMP (Dobbins et al., 1984).

The source of endogenous ADO is controversial. The nonlytic release of ATP from many epithelia has been reported in numerous publications (Woo et al., 2008; Seminario-Vidal et al., 2009). The released ATP is enzymatically hydrolyzed by ENTPDase to AMP, which is then converted by ecto-5′-nucleotidase to ADO (Zimmermann, 2000). Alternatively, ATP is sequentially dephosphorylated to ADO by alkaline phosphatase (Yegutkin, 2008). ADO can then be further degraded to INO by ADA. We have previously reported nonlytic ATP release and brush border IAP and CD39 activity in rat duodenum (Mizumori et al., 2009), suggesting that luminal surface ADO is derived from released ATP, as predicted earlier in T84 cell monolayers (Stutts et al., 1995). Our data in the present study support this hypothesis, because luminal ATP-induced DBS was inhibited by the A_{2B} receptor antagonist. Another possible source of extracellular ADO is the extracellular cAMP–ADO pathway, consisting of cAMP transporter, ecto-phosphodiesterase, and ecto-5′-nucleotidase (Gödecke, 2008). This pathway is observed in the skeletal muscle (Chiavegatti et al., 2008) and ileal muscle strip (Giron et al., 2008). Perfusion of the rat duodenum with cAMP, however, did not increase the rate of DBS, inconsistent with the presence of the cAMP–ADO pathway in rat duodenum (unpublished observations).

In summary, we have reported for the first time that endogenously produced luminal surface ADO increases HCO_3^- secretion in an intact epithelium in vivo through the activation of A_{2B} receptors. Our data complement the "adenosine scavenging" hypothesis wherein extracellular ADO concentrations are regulated by apical nucleoside transporters and ADA, which in turn regulate intestinal anion secretion.

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