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Intestinal alkaline phosphatase regulates protective surface microclimate pH in rat duodenum

Misa Mizumori[1], Maggie Ham[1], Paul H. Guth[3], Eli Engel[2], Jonathan D. Kaunitz[1,3,4] and Yasutada Akiba[1,3,4]

[1]*Department of Medicine, School of Medicine,* [2]*Department of Biomathematics, University of California Los Angeles,* [3]*Greater Los Angles Veterans Affairs Healthcare System and* [4]*Brentwood Biomedical Research Institute, Los Angeles, CA 90073, USA*

Regulation of localized extracellular pH (pH_o) maintains normal organ function. An alkaline microclimate overlying the duodenal enterocyte brush border protects the mucosa from luminal acid. We hypothesized that intestinal alkaline phosphatase (IAP) regulates pH_o due to pH-sensitive ATP hydrolysis as part of an ecto-purinergic pH regulatory system, comprised of cell-surface P2Y receptors and ATP-stimulated duodenal bicarbonate secretion (DBS). To test this hypothesis, we measured DBS in a perfused rat duodenal loop, examining the effect of the competitive alkaline phosphatase inhibitor glycerol phosphate (GP), the ecto-nucleoside triphosphate diphosphohydrolase inhibitor ARL67156, and exogenous nucleotides or P2 receptor agonists on DBS. Furthermore, we measured perfusate ATP concentration with a luciferin–luciferase bioassay. IAP inhibition increased DBS and luminal ATP output. Increased luminal ATP output was partially CFTR dependent, but was not due to cellular injury. Immunofluorescence localized the $P2Y_1$ receptor to the brush border membrane of duodenal villi. The $P2Y_1$ agonist 2-methylthio-ADP increased DBS, whereas the $P2Y_1$ antagonist MRS2179 reduced ATP- or GP-induced DBS. Acid perfusion augmented DBS and ATP release, further enhanced by the IAP inhibitor L-cysteine, and reduced by the exogenous ATPase apyrase. Furthermore, MRS2179 or the highly selective $P2Y_1$ antagonist MRS2500 co-perfused with acid induced epithelial injury, suggesting that IAP/ATP/P2Y signalling protects the mucosa from acid injury. Increased DBS augments IAP activity presumably by raising pH_o, increasing the rate of ATP degradation, decreasing ATP-mediated DBS, forming a negative feedback loop. The duodenal epithelial brush border IAP–P2Y–HCO_3^- surface microclimate pH regulatory system effectively protects the mucosa from acid injury.

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Corresponding author J. D. Kaunitz: Bldg 114, Suite 217, West Los Angeles VA Medical Center, 11301 Wilshire Blvd, Los Angeles, CA 90073, USA. Email: jake@ucla.edu

Abbreviations AP, alkaline phosphatase; ATPγS, adenosine 5′-[γ-thio]-triphosphate; BCECF, 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein; CFTR, cystic fibrosis transmembrane conductance regulator; DBS, duodenal bicarbonate secretion; ENTPDase, ecto-nucleoside triphosphate diphosphohydrolase; GP, glycerol phosphate; IAP, intestinal alkaline phosphatase; LDH, lactate dehydrogenase; 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; pH_i, intracellular pH; pH_o, extracellular pH; PI, propidium iodide.

The upper gastrointestinal mucosa is protected from potentially injurious cyclical pulses of strong luminal acid by robust defence mechanisms including an HCO_3^- secretion-generated alkaline zone abutting the microvilli in disequilibrium with bulk luminal pH in duodenum and stomach (Williams & Turnberg, 1981; Flemström & Kivilaakso, 1983; Allen & Flemström, 2005). The alkaline surface microclimate neutralizes luminal H^+ to CO_2, facilitating absorption of the gastric H^+ load while protecting the epithelial cells from injury (Garner *et al.* 1984; Mizumori *et al.* 2006).

Intestinal alkaline phosphatase (IAP) is a glycosylphosphatidylinositol (GPI) anchored ecto-enzyme highly expressed in the brush border membrane of duodenal epithelial cells, with expression declining along the proximal–caudal axis (Hietanen, 1973; Akiba *et al.* 2007). Despite its high brush border expression, its function in intestinal mucosal physiology remains uncertain, with no endogenous substrate conclusively identified. Furthermore, its high pH optimum (>8) (Humphreys & Chou, 1979), has never been adequately explained in a biological context. Since HCO_3^- secretion is frequently invoked as a primary duodenal defence mechanism against concentrated gastric acid, the enterocyte surface extracellular pH (pH_o), due to robust HCO_3^- secretion, may be close to the pH optimum of

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IAP. To test this, we reported that duodenal IAP activity measured *in vivo* is dependent on bulk luminal pH and importantly, on the rate of duodenal HCO_3^- secretion (Akiba *et al.* 2007). This supports our hypothesis that pH_o is alkaline during HCO_3^- secretion and that pH_o is equivalent to the pH at the IAP catalytic site. In addition to postulating that pH_o correlates with IAP activity, we have further hypothesized that extracellular purines, notably ATP, serve as endogenous substrates for IAP. To access the IAP catalytic site, ATP is presumably released from the enterocytes into the luminal space (Yegutkin, 2008; Zimmermann, 2008). In the presence of HCO_3^-, IAP has ATPase activity which is termed HCO_3^--ATPase activity (Humphreys & Chou, 1979). Enhancement of HCO_3^- secretion by extracellular ATP combined with enhanced ATPase activity of IAP in response to this augmented rate of HCO_3^- secretion suggests the presence of a negative feedback loop. We thus hypothesized that luminal ATP is an endogenous substrate for duodenal brush border IAP, that pH_o is regulated by the balance between ATP-mediated activation of HCO_3^- secretion and the pH_o-dependent rate of ATP hydrolysis, and that purinergic regulation of pH_o is an important means by which the underlying epithelium resists damage due to luminal acid. Since other purine nucleotide hydrolases such as ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase) are also expressed in the enterocyte brush border (Yegutkin, 2008), we also tested the role of ENTPDase in ATP degradation compared with IAP.

Here, we show for the first time that luminal ATP is a substrate for brush border IAP in rat duodenum, that IAP inhibition predominantly 'unmasks' non-lytic endogenous ATP release from the mucosa into the lumen, that ATP release is partially cystic fibrosis transmembrane regulator (CFTR) dependent, that ATP-P2Y receptor signalling increases HCO_3^- secretion in rat duodenum, and that increased HCO_3^- secretion upregulates IAP activity, decreasing luminal ATP concentration with consequent diminished P2Y receptor signalling. We conclude that, on the basis of these data, the ecto-purinergic signalling system comprised of IAP, non-lytic ATP release, ATP-dependent HCO_3^- secretion and P2Y receptors regulates pH_o of the duodenal enterocyte, which in turn is important for mucosal protection from acid injury.

Methods

Chemicals and animals

$CFTR_{inh}$-172 was synthesized by Dr Samedy Ouk in the Department of Chemistry, UCLA (Akiba *et al.* 2005). INS45973 was kindly provided from Inspire Pharmaceuticals, Inc. (Durham, NC, USA). 2′,7′-*Bis*(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), its acetoxy methyl ester (BCECF-AM) and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR, USA). MRS2500 was obtained from Tocris Bioscience (Ellisville, MO, USA). L-Cysteine, glycerol phosphate (GP), ATP, ADP, UTP, UDP, 2-methylthio-ATP (2MeSATP), 2-methylthio-ADP (2MeSADP), adenosine 5′-[γ-thio]-triphosphate (ATPγS), ARL67156, MRS2179, apyrase, lactate dehydrogenase (LDH), Hepes and other chemicals were obtained from Sigma Chemical (St Louis, MO, USA). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 $CaCl_2$, and 10 Hepes at pH 7.0. All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee (VA IACUC). Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fasted overnight, but had free access to water. At the end of the experiments, all animals were euthanized by intravenous injection of sodium pentobarbital (50 mg kg^{-1}), followed by thoracotomy, or by terminal exsanguination under deep isoflurane anaesthesia.

Measurement of duodenal HCO_3^- secretion

Duodenal loops were prepared and perfused as previously described (Mizumori *et al.* 2006; Akiba *et al.* 2007). Under isoflurane anaesthesia (1.5–2.0%), the proximal duodenal loop (perfused length 2 cm) was perfused with pH 7.0 normal saline or Krebs buffer by using a peristaltic pump (Fisher Scientific, Pittsburgh, PA, USA) at 1 ml min^{-1}. The perfusate was bubbled with 100% O_2, and stirred and warmed at 37°C with a heating stirrer (Barnstead Int., Dubuque, IA, USA). To eliminate the buffer action of agonists or antagonists, which would over- or under-estimate the titration volume 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. Since 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 previously described (Mizumori *et al.* 2006; Akiba *et al.* 2007). The effluent was collected every 5 min in a sterilized tube on ice for ATP output measurement. After stabilization with continuous perfusion of pH 7.0 saline for ~30 min, the time was set as $t = 0$. The duodenal loop was perfused with pH 7.0 saline from $t = 0$ min until $t = 10$ min (basal period). The perfusate was then changed to pH 7.0 Krebs buffer from $t = 10$ min until $t = 35$ min (challenge period), with or without agonists or antagonists.

For acid challenge, the perfusate was changed to pH 2.2 acid saline with or without inhibitors (challenge period; $t = 10$ min to $t = 20$ min). During the challenge period, the solution was perfused with a syringe pump, followed

by the perfusion of pH 7.0 saline with a peristaltic pump through a reservoir from $t = 20$ to $t = 45$ min (recovery period). HCO_3^- secretion was measured using a pH-stat (Radiometer Analytical, Lyon, France) and read at every 4 min in a 5 min interval, followed by the collection of the 1 ml effluent for 1 min at the end of every 5 min interval for ATP output measurement.

Measurement of ATP output in perfusate through duodenal loop *in vivo*

ATP concentration in the duodenal effluent was measured using a luciferin–luciferase-based bioluminescence assay kit (Kinase-Glo Luminescent Kinase Assays, Promega Co., Madison, WI, USA) with a Synergy 2 multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Fifty microlitres of an ATP standard solution (0.05–50 nM) or the sample effluent was placed in a 96-well plate in duplicate, followed by adding 50 μl of luciferin–luciferase solution using an auto injector. ATP output was expressed as pmol min^{-1} cm^{-1}. The lower limit of this ATP measurement is ~0.1 nM. We have confirmed that any chemical used in ATP output experiments in this study has no direct effect on the ATP-induced luminescence.

Since injured cells release ATP, we measured the release of the cytosolic enzyme LDH in response to IAP inhibition with GP, CFTR inhibition with $CFTR_{inh}$-172, or acid perfusion using a cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany). LDH output was expressed as U min^{-1} cm^{-1}. Since LDH output was undetectable during acid perfusion, and since acid phosphatase has been used as a cellular injury marker in rat small intestine (Pinto *et al.* 1989), acid-resistant acid phosphatase was measured in the effluents as another measure of cellular injury using a commercial kit (acid phosphatase assay kit, Sigma). Acid phosphatase output was expressed as mU min^{-1} cm^{-1}.

Perfusion effects of IAP inhibition and P2Y receptor antagonists

In order to examine the effect of IAP function on duodenal HCO_3^- secretion and ATP release, we first examined the effect of the inhibition of IAP on duodenal HCO_3^- secretion and ATP release. The duodenal loop was perfused with a non-selective, competitive AP inhibitor GP (10 mM) (Akiba *et al.* 2007) or the specific ENTPDase inhibitor ARL67153 (0.1 mM) (Levesque *et al.* 2007) dissolved in pH 7.0 Krebs buffer during the challenge period. Note that although GP is a non-selective AP inhibitor, since IAP is predominantly and highly expressed in duodenal brush border, luminal perfusion of GP through duodenal loops functionally exerts IAP inhibition (Akiba *et al.* 2007).

To determine which P2 receptor subtype is involved in duodenal HCO_3^- secretion, we examined the effect of perfusion of P2Y receptor agonists on duodenal HCO_3^- secretion: ATP (0.1 mM), ADP (0.1 mM), a $P2Y_1$ agonist 2MeSADP (0.1 mM), a broad P2X/potent $P2Y_1$ agonist 2MeSATP (0.1 mM), a selective $P2Y_2$ agonist INS45973 (0.01 mM), a $P2Y_2/Y_4$ agonist UTP (0.1 mM), a $P2Y_6$ agonist UDP (0.1 mM), and a broad P2X agonist ATPγS (0.1 mM) (Lambrecht, 2000; von Kügelgen, 2006). Furthermore, a selective $P2Y_1$ receptor antagonist MRS2179 (0.1 mM) was co-perfused with GP (10 mM) or ATP (0.1 mM).

Some animals were pre-treated with the potent selective CFTR inhibitor $CFTR_{inh}$-172 (1 mg kg^{-1}, I.P.) 1 h prior to the experiments. Pre-treatment with $CFTR_{inh}$-172 at this dose eliminates acid-induced HCO_3^- secretion in rat duodenum (Akiba *et al.* 2005).

To investigate the role of luminal ATP release in acid-induced HCO_3^- secretion, the IAP inhibitor L-cysteine (10 mM) (Akiba *et al.* 2007) or exogenous ATPase apyrase (1 U ml^{-1}) was perfused with pH 2.2 acid during challenge period.

Effects of P2Y receptor inhibition on intracellular acidification and cellular injury during acid exposure in rat duodenum

To further test our hypothesis that ATP-P2Y receptor signalling regulates a protective mechanism in response to luminal acid exposure, we examined the effect of the $P2Y_1$ receptor antagonist MRS2179 or the highly selective $P2Y_1$ antagonist MRS2500 (Hechler *et al.* 2006) on acid-induced intracellular acidification and cellular injury using *in vivo* microscopy. Intracellular pH (pH_i) of duodenal epithelial cells was measured using a fluorescence ratio technique as previously described (Akiba *et al.* 2006). Furthermore, epithelial cellular injury was assessed by *in vivo in situ* propidium iodide (PI) staining as previously reported (Akiba *et al.* 2001). Every 5 min, BCECF paired images at 495 and 450 nm excitation were captured, followed by capturing PI images at 535 nm excitation using a 10x objective lens. pH_i and the number of PI-positive dots corresponding to injured cell nuclei were analysed in the recorded images.

After BCECF loading followed by stabilization with pH 7.0 Krebs superfusion, the mucosa was superfused with pH 7.0 Krebs for 10 min (basal period, $t = 0$–10 min), followed by superfusion of pH 2.2 acid solution with or without MRS2179 (0.1 mM) or MRS2500 (10 μM) for 10 min (challenge period, $t = 10$–20 min), then with pH 7.0 Krebs for 15 min (recovery period, $t = 20$–35 min). All solutions contained PI (1 μM) to stain the nuclei of injured cells.

Expression of P2Y receptor subtypes

Immunofluorescence staining was performed as previously described (Akiba *et al.* 2006) on the cryostat sections of proximal duodenum using primary antibodies for $P2Y_1$, Y_2, Y_4, and Y_6 receptors (rabbit polyclonal, 1 : 100, Alomone Labs, Ltd, Jerusalem, Israel). Negative controls were examined by omitting the primary antibody or by the pre-incubation of antibody with the immunized peptide. The sections were observed using a confocal laser microscope (Leica TCS-SP Inverted, Leica Microsystems, Germany) or under a Zeiss fluorescence microscope.

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 ANOVA followed by Fischer's least significant difference test. P values of 0.05 were taken as significant.

Results

Effect of IAP inhibition on duodenal HCO_3^- secretion and ATP output

We initially determined our ability to measure ATP output into the duodenal lumen and the effect of inhibition of IAP and ENTPDase activities on ATP output. At baseline, during perfusion of pH 7.0 Krebs buffer following saline perfusion, HCO_3^- secretion (measured as total CO_2 output) was stable, and ATP output was stable but quite low ($\sim$0.2 pmol min^{-1} cm^{-1}) (Fig. 1*A* and *B*). A competitive AP inhibitor, GP (10 mM) increased duodenal HCO_3^- secretion (Fig. 1*A*) as previously described (Akiba *et al.* 2007), accompanied by an $\sim$25-fold increase in ATP output (Fig. 1*B*, inset). Furthermore, the addition of ARL67156 (0.1 mM) initially augmented HCO_3^- secretion (Fig. 1*A*) accompanied by a parallel enhancement of ATP output (Fig. 1*B*), suggesting that luminal ATP is degraded by IAP and ENTPDase, consistent with a prior study (Schweickhardt *et al.* 1995). ARL67156 alone also increased HCO_3^- secretion with increased ATP output, but less effectively than did GP (Fig. 1*A* and *B*). This result supports our hypothesis that luminal ATP is an endogenous substrate of IAP as well as of ENTPDase in the duodenum.

Effect of CFTR inhibition on GP-induced duodenal HCO_3^- secretion and ATP output

Next, we examined the effect of CFTR inhibition on IAP inhibition-induced HCO_3^- secretion and ATP output augmented by IAP inhibition in the duodenum, testing the hypothesis that CFTR function facilitates luminal ATP release. Pre-treatment with $CFTR_{inh}$-172 (1 mg kg^{-1}, I.P.) reduced GP-induced HCO_3^- secretion (Fig. 2*A*) and GP-induced ATP output (Fig. 2*B*), suggesting that HCO_3^- secretion and ATP output during IAP inhibition are partially CFTR dependent. LDH output gradually declined over time, and was unaffected by IAP or CFTR inhibition (Fig. 2*C*), strongly consistent with non-lytic ATP release.

Expression of P2Y receptors in the duodenal epithelium

Immunofluorescence for P2Y receptors demonstrated that $P2Y_1$ receptors were expressed on the brush border membrane of duodenal villous cells (Fig. 3*A* and *B*). $P2Y_2$ or Y_4 receptor was not recognized (Fig. 3*C* and *D*), whereas diffuse staining for $P2Y_6$ receptor was observed in the villous cells and in the interstitium (Fig. 3*E*). Pre-absorbed antibody with the immunizing peptide or omission of the primary antibody revealed no staining.

Effect of ATP analogues on duodenal HCO_3^- secretion

Brush border P2Y receptors were next characterized pharmacologically by examining the effect of perfusion of several agonists with different selectivity on duodenal HCO_3^- secretion. ATP or ADP (0.1 mM) increased duodenal HCO_3^- secretion (Fig. 4*A*). The potent $P2Y_1$ receptor agonist 2MeSATP or 2MeSADP (0.1 mM) rapidly increased HCO_3^- secretion, whereas a P2X agonist ATPγS (0.1 mM) gradually increased HCO_3^- secretion (Fig. 4*B*). Nevertheless, a selective $P2Y_2$ agonist INS45973 (0.01 mM), the $P2Y_2/Y_4$ agonist UTP (0.1 mM) or the $P2Y_6$ agonist UDP (0.1 mM) had no effect on HCO_3^- secretion (Fig. 4*C*). These results suggest that $P2Y_1$ receptors help regulate luminal purine-stimulated HCO_3^- secretion in rat duodenum.

Effect of a $P2Y_1$ antagonist on ATP- or GP-induced HCO_3^- secretion

To further test the involvement of $P2Y_1$ receptors on duodenal HCO_3^- secretion, a selective $P2Y_1$ receptor antagonist MRS2179 (0.1 mM) was perfused with ATP (0.1 mM) or GP (10 mM). ATP-induced HCO_3^- secretion was reduced by the co-perfusion of MRS2179 (Fig. 5*A*). Furthermore, MRS2179 reduced GP-induced HCO_3^- secretion (Fig. 5*B*). These results suggest that luminal ATP stimulates HCO_3^- secretion via $P2Y_1$ receptors, and IAP inhibition increases HCO_3^- secretion by increasing ATP content which activates $P2Y_1$ receptors.

Effect of acid exposure on duodenal HCO_3^- secretion and ATP output

Since luminal acidification is the most accepted physiological stimulant of duodenal HCO_3^- secretion (Akiba *et al.* 2001, 2005, 2007), we measured acid-induced HCO_3^- secretion with the pH-stat technique. Perfusion with a pH 2.2 acid solution increased duodenal HCO_3^- secretion during the post-acid-stress recovery period (Fig. 6*A*), and increased luminal ATP output only during acid exposure (Fig. 6*B*). An IAP inhibitor L-cysteine (10 mM) co-perfused with the acid solution enhanced acid-induced duodenal HCO_3^- secretion (Fig. 6*A*) as previously described (Akiba *et al.* 2007), and enhanced luminal ATP output during the acid challenge period (Fig. 6*B*). In contrast, co-perfusion of an exogenous ATPase apyrase (1 U ml^{-1}) impaired acid-induced HCO_3^- secretion (Fig. 6*A*), with inhibition of ATP output during acid exposure (Fig. 6*B*). These results suggest that luminal purinergic signalling during

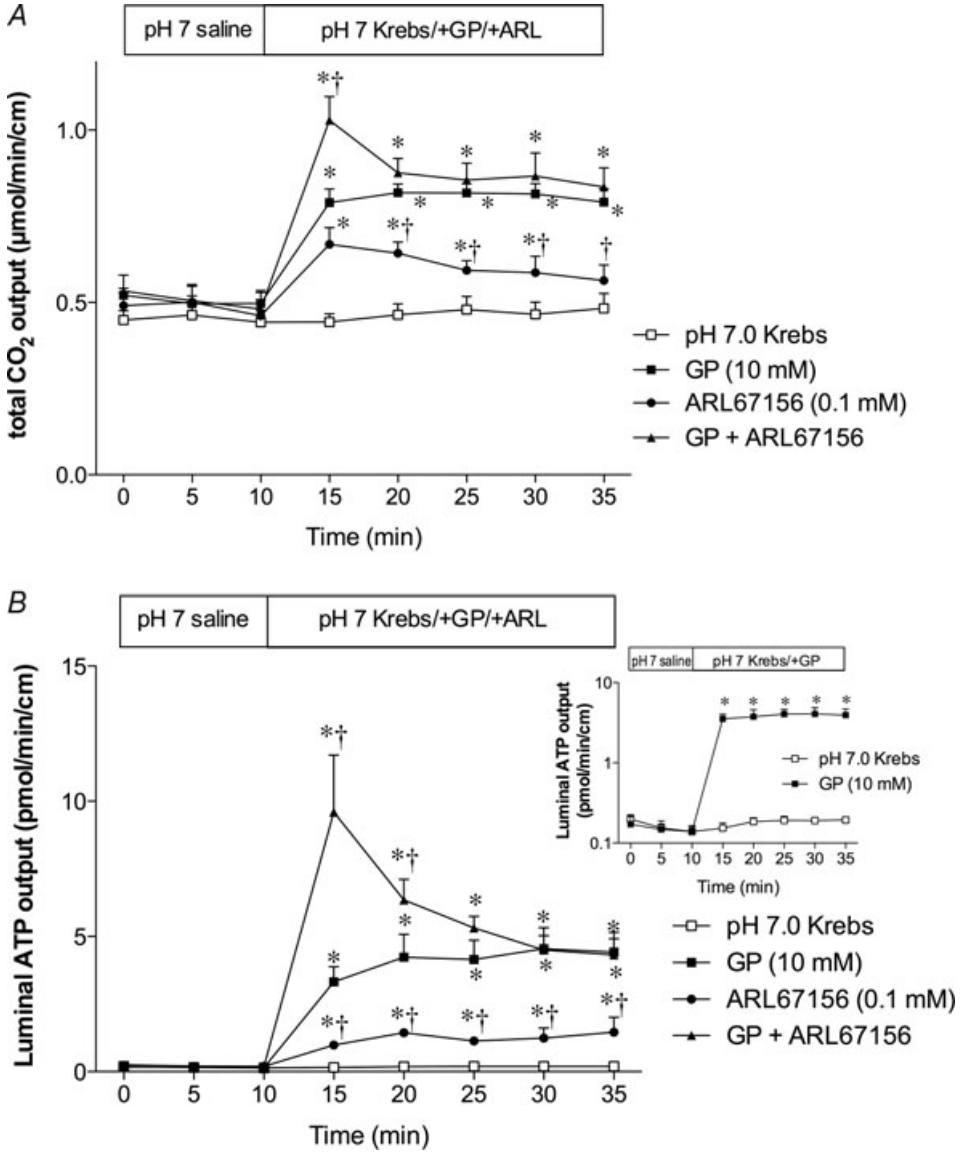

Figure 1. Effect of intestinal alkaline phosphatase (IAP) or ecto-ATPase inhibition on HCO_3^- and ATP output in rat duodenum

A, total CO_2 output, corresponding to the duodenal HCO_3^- secretion, was measured with flow-through pH and CO_2 electrodes. Perfusion of glycerol phosphate (GP, 10 mM) increased total CO_2 output and ARL67156 (0.1 mM) temporally enhanced the effect of GP. *B*, luminal ATP output was measured in the effluents with bioluminescence. Inset, log replot of ATP output under IAP inhibition. Each data point represents mean ± S.E.M. ($n = 6$ rats). *$P < 0.05$ *vs* pH 7.0 Krebs group, †$P < 0.05$ *vs* GP group.

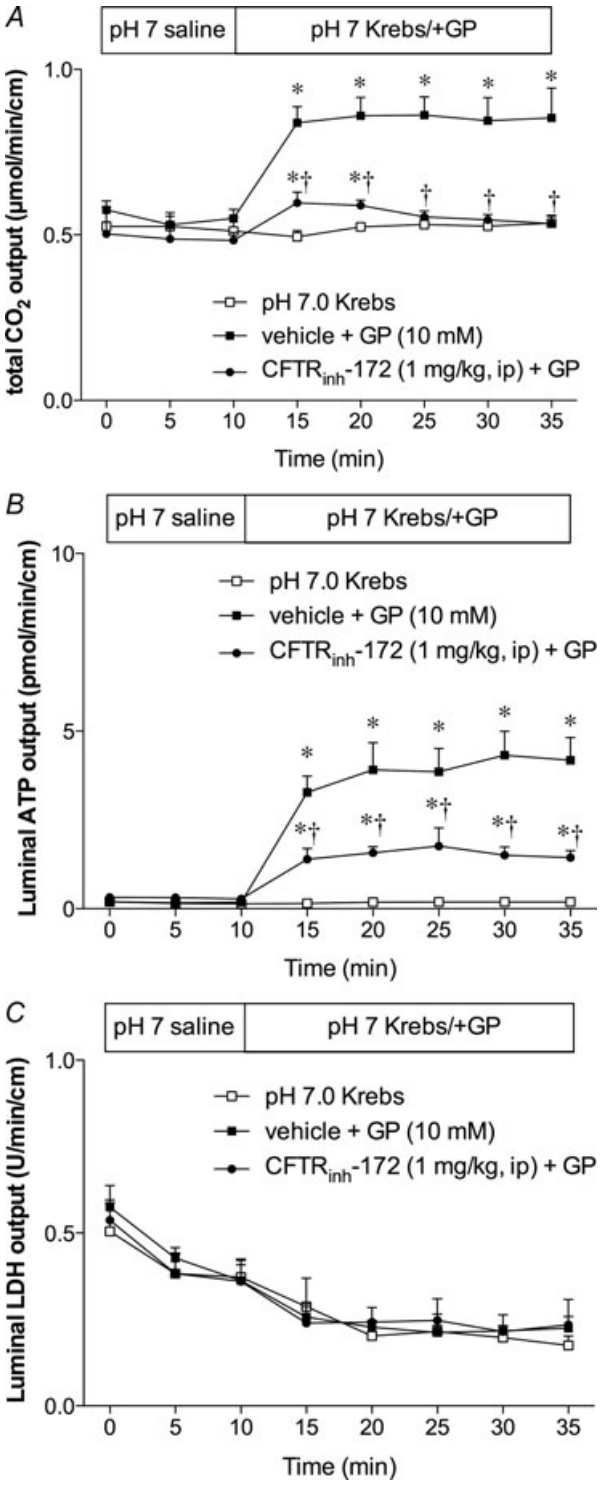

Figure 2. Effect of CFTR inhibition on alkaline phosphatase inhibition-induced HCO_3^- and ATP output in rat duodenum
Animals were pre-treated with $CFTR_{inh}$-172 (1 mg kg^{-1}, I.P.) or vehicle 1 h prior to the experiments. *A*, glycerol phosphate (GP, 10 mM)-induced increased HCO_3^- secretion was reduced by $CFTR_{inh}$-172 pre-treatment. *B*, GP-induced increased ATP output was reduced by $CFTR_{inh}$-172 pre-treatment. *C*, lactate dehydrogenase (LDH) activity was measured in the effluents. Perfusion of GP with or without $CFTR_{inh}$-172 pre-treatment had no effect on LDH output. Each data point represents mean ± S.E.M. ($n = 6$ rats). *$P < 0.05$ *vs* pH 7.0 Krebs group, †$P < 0.05$ *vs* vehicle + GP group.

acid exposure may be an important regulatory system for duodenal HCO_3^- secretion, and that IAP function is present even when the bulk luminal pH is 2.2 as previously described (Akiba *et al.* 2007). Furthermore, acid phosphatase output decreased during acid perfusion, followed by a recovery to baseline during following acid perfusion, consistent with non-lytic ATP release (Fig. 6*C*).

Effect of $P2Y_1$ antagonism on mucosal injury

To further test our hypothesis that ATP-P2Y receptor signalling regulates a protective response to luminal acid exposure, we measured the effect of the $P2Y_1$ receptor antagonist MRS2179 or the more selective and potent antagonist MRS2500 on acid-induced intracellular acidification and cellular injury using *in vivo* microscopy.

Perfusion of a pH 2.2 solution rapidly lowered pH_i followed by pH_i recovery to values above baseline (overshoot) as previously described (Akiba *et al.* 2001) (Fig. 7*A*), unaccompanied by an increase of PI-positive cells (Fig. 7*B*), consistent with prior measurements of injury using histological assessment (Akiba *et al.* 2001), and further consistent with our cellular enzyme release data (Fig. 6*C*). MRS2179 co-perfusion with acid solution enhanced intracellular acidification during acid challenge and impaired pH_i recovery (Fig. 7*A*) accompanied by a progressive increase in the number of PI-positive cells during and after acid exposure (Fig. 7*B*, inset). Furthermore, a highly selective $P2Y_1$ antagonist MRS2500 increased the cell injury with further intracellular acidification. This result suggests that ATP-P2Y receptor signalling is activated during acid exposure in order to protect the epithelial cells from acid-induced injury.

Discussion

We demonstrated that IAP inhibition increased duodenal HCO_3^- secretion, coincident with increased luminal ATP output and that luminal ATP stimulates HCO_3^- secretion via $P2Y_1$ receptor activation in rat duodenum. ATP output was non-lytic and was partially CFTR dependent. ATP output was also augmented by the inhibition of IAP and ENTPDase function, and was augmented by luminal acid. IAP function remained intact at a bulk luminal pH of 2.2, 5 logs lower than its *in vitro* pH optimum. This is the first study to measure luminal ATP output in the duodenum *in vivo* and to examine its function as a component of a purinergic pH_o regulatory system. The marked enhancement of ATP output by IAP and ENTPDase inhibition supports their function in an ecto-purinergic signalling system. Furthermore, this is the first study to test the hypothesis that IAP, as part of an ecto-purinergic signalling system, is an alkaline chemosensor essential for

the regulation of the pH_o of the protective alkaline surface layer.

The observed marked increase of the rate of ATP release associated with ecto-phosphorylase inhibition strongly supports our hypothesis that IAP and ENTPDase are integral components of the duodenal surface ecto-purinergic signalling system. ATP may be constitutively released from duodenal epithelial cells by non-lytic processes, as reported in epithelial and non-epithelial cell models (Yegutkin, 2008), although its mode of release is still debated (Fitz, 2007). P2Y receptor-mediated HCO_3^- secretion enhances IAP activity, presumably by bringing it closer to its pH optimum, which then reduces extracellular ATP concentration ($[ATP]_o$), forming a negative feedback loop (Fig. 8*A*). ATP output declined while HCO_3^- secretion increased after acid perfusion, further supporting the presence of negative feedback. In this fashion, IAP activity appears to serve as a pH_o sensor, regulating $[ATP]_o$ according to pH_o. The marked increase of ATP release during acid exposure may be due to decreased IAP activity attributable to a lower pH_o (Fig. 8*B*), or enhanced ATP release in response to acid by exocytosis. This latter supposition is supported by the observed marked exocytosis of mucus in response to acid perfusion (Akiba *et al.* 2000) combined with the observation that ATP release may be exocytotic (Okada *et al.* 2006; Sawada *et al.* 2008). The high rate of ATP release in acidic conditions compared with the lower rate observed with phosphorylase inhibition

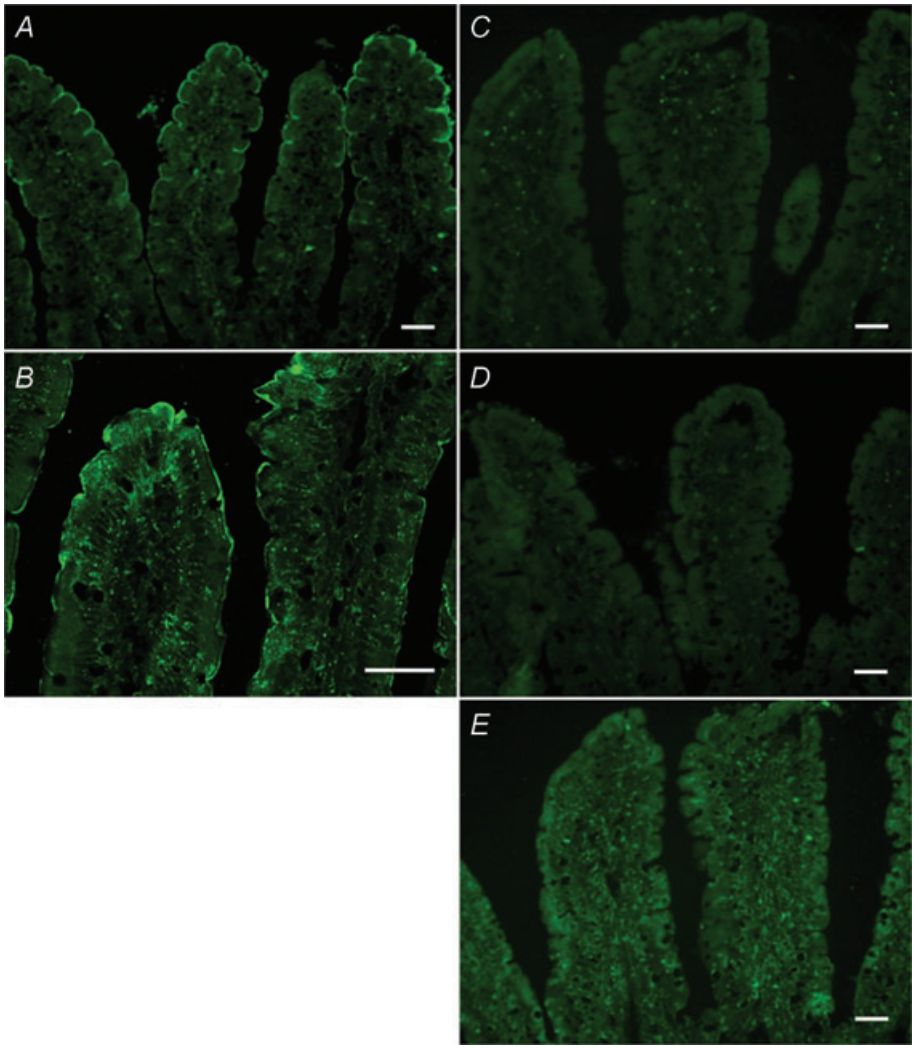

Figure 3. Expression of P2Y receptors in rat duodenal epithelium
Cryostat sections of fixed rat duodenum were reacted with P2Y receptor antibodies. $P2Y_1$ receptor-like immunoreactivity was strongly recognized on the brush border membranes of villous cells (*A* and *B*). No specific staining was observed for $P2Y_2$ (*C*) and $P2Y_4$ (*D*) receptors, whereas $P2Y_6$ receptor was diffusely positive in the villous cells and interstitium (*E*). Scale bar, 50 μm. *A*, *C*–*E*: conventional microscope images, *B*: confocal microscope image.

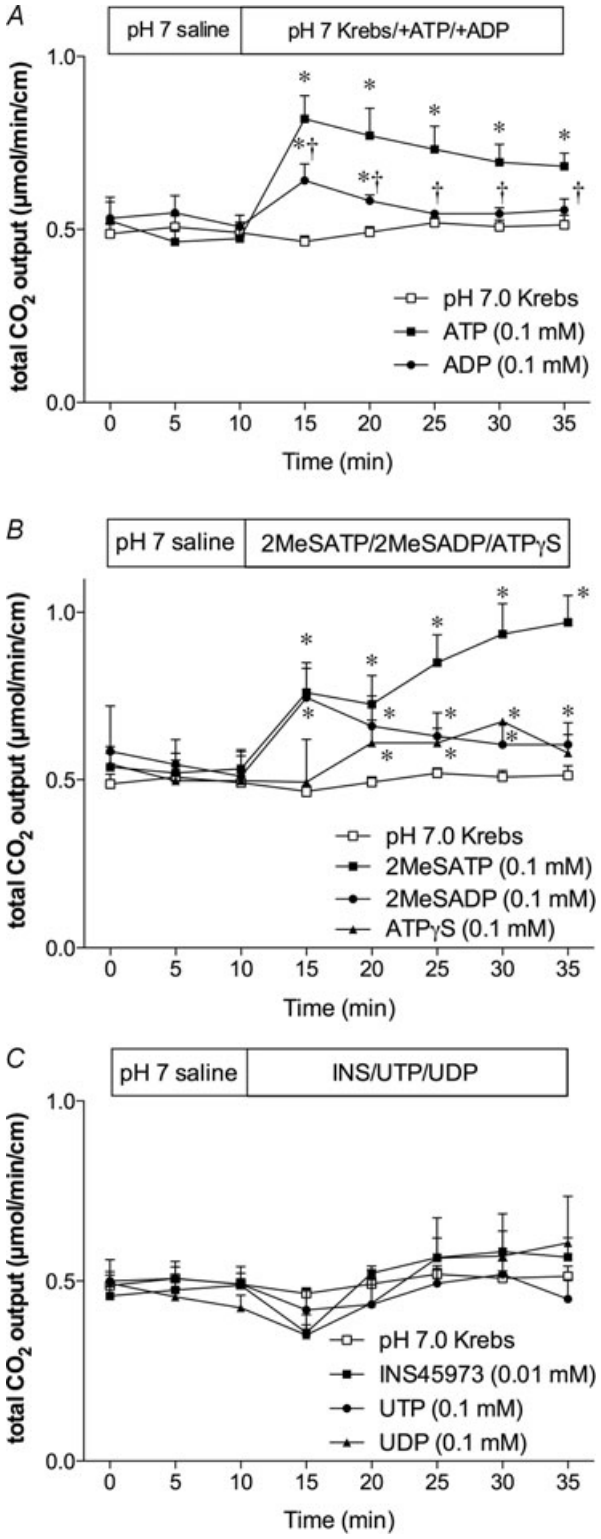

Figure 4. Effects of P2Y receptor agonists on duodenal HCO_3^- secretion in rats
A, perfusion of ATP and ADP (0.1 mM) increased total CO_2 output in duodenum. *B*, $P2Y_1$ receptor agonists, 2MeSATP and 2MeSADP (0.1 mM) or P2X agonist ATPγS (0.1 mM) increased total CO_2 output. *C*, perfusion of INS45973 (0.01 mM), UTP (0.1 mM) or UDP (0.1 mM) had no effect on total CO_2 output. Each data point represents mean ± S.E.M. ($n = 6$ rats). $^*P < 0.05$ *vs* pH 7.0 Krebs group.

alone suggests that both mechanisms are present. In either case, luminal acidification triggers epithelial HCO_3^- secretion, which we hypothesize rapidly corrects pH_o via the ecto-purinergic system as a 'pH_o-sensing emergency signal' (Fig. 8*B*), while more slowly increasing the rate of bulk luminal alkalinization via submucosal signalling systems (Kaunitz & Akiba, 2006). Since interruption of the ecto-purinergic pathway with a $P2Y_1$ receptor antagonist markedly increases injury susceptibility, whereas impairment of enhanced bulk HCO_3^- secretion with CFTR dysfunction decreases injury susceptibility (Akiba *et al.* 2001; Hirokawa *et al.* 2004;

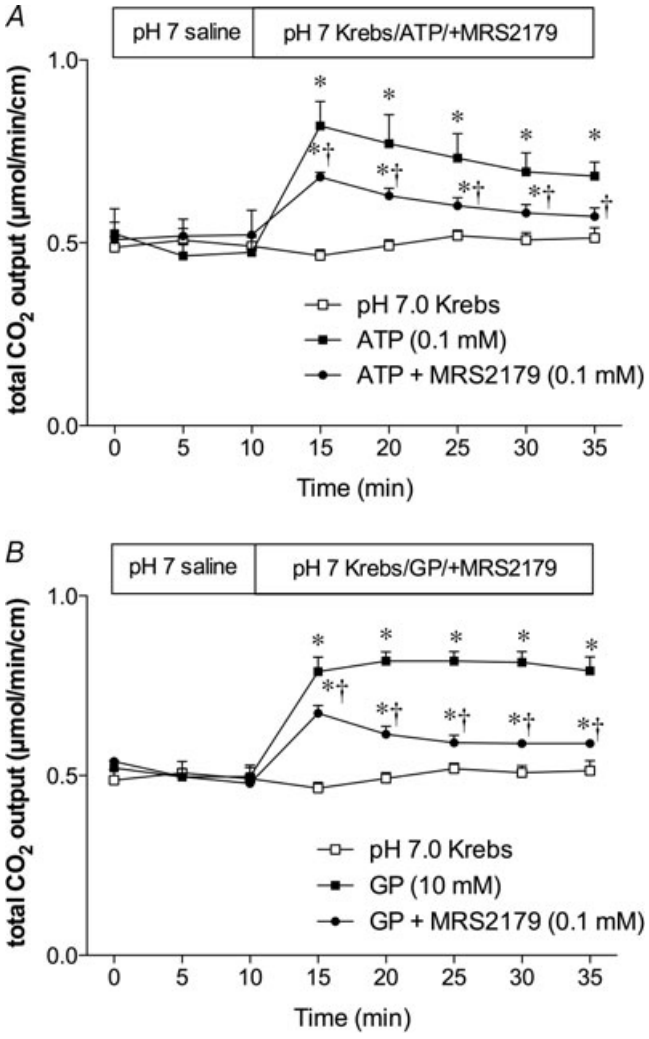

Figure 5. Effect of $P2Y_1$ receptor antagonist on the stimulated HCO_3^- secretion in rat duodenum
A, a selective $P2Y_1$ receptor antagonist MRS2179 (0.1 mM) reduced the ATP-induced duodenal HCO_3^- secretion. Each data point represents mean ± S.E.M. ($n = 6$ rats). $^*P < 0.05$ *vs* pH 7.0 Krebs group, †$P < 0.05$ *vs* ATP group. *B*, glycerol phosphate (GP)-induced duodenal HCO_3^- secretion was reduced by MRS2179. Each data point represents mean ± S.E.M. ($n = 6$ rats). $^*P < 0.05$ *vs* pH 7.0 Krebs group, †$P < 0.05$ *vs* GP group.

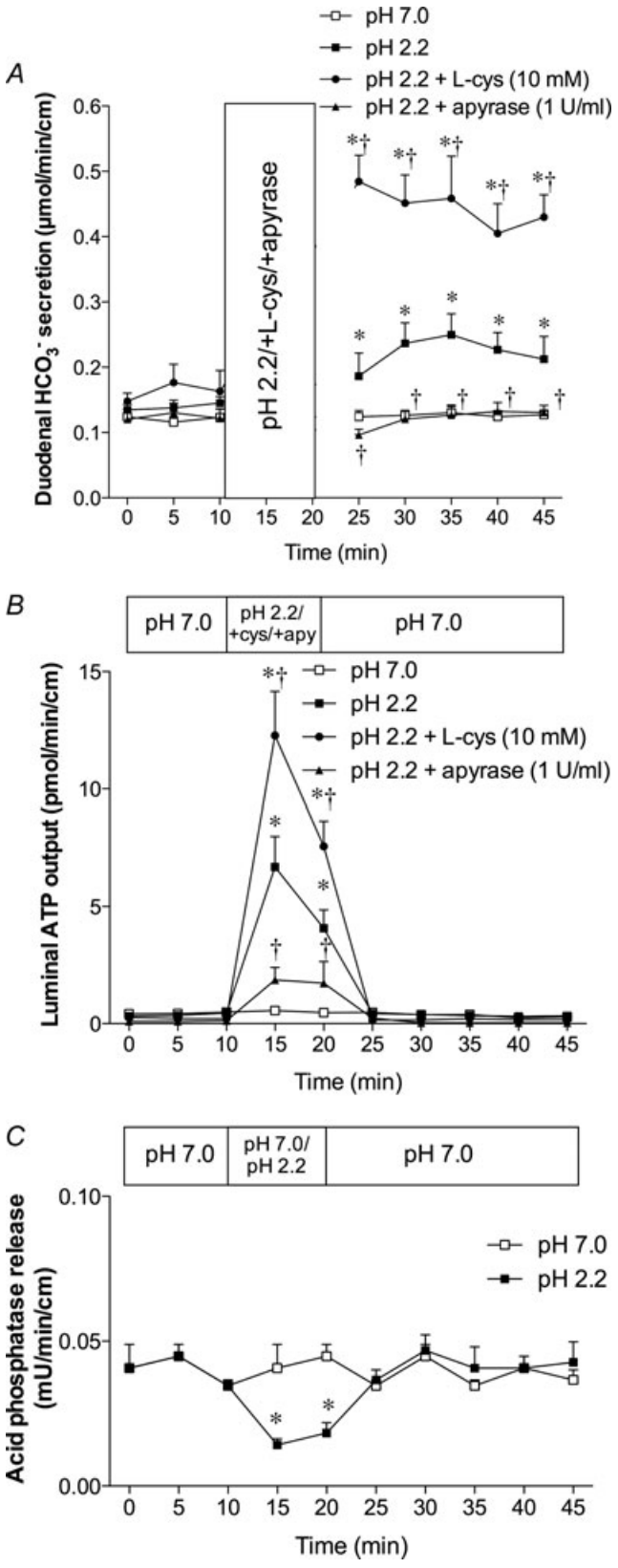

Figure 6. Effect of L-cysteine or apyrase on acid-induced duodenal HCO_3^- secretion and ATP output in rats
A, duodenal HCO_3^- secretion was measured by pH-stat. Perfusion of pH 2.2 saline increased duodenal HCO_3^- secretion. L-Cysteine (L-cys, 10 mM) enhanced, but apyrase (1 U ml^{-1}) inhibited acid-induced augmented HCO_3^- secretion. Data are expressed as means ± S.E.M. ($n = 6$). *$P < 0.05$ *vs* pH 7.0 saline group, †$P < 0.05$ *vs* pH 2.2 saline group. *B*, ATP content in the effluents was measured with bioluminescence. Perfusion of pH 2.2 saline increased luminal ATP output. L-Cysteine (L-cys, 10 mM) enhanced, but apyrase diminished acid-induced ATP output. Data are expressed as means ± S.E.M. ($n = 6$). *$P < 0.05$ *vs* pH 7.0 saline group, †$P < 0.05$ *vs* pH 2.2 saline group. *C*, acid phosphatase activity was measured in the effluents. Data are expressed as means ± S.E.M. ($n = 6$). *$P < 0.05$ *vs* pH 7.0 saline group.

Akiba *et al.* 2005), the pH_o regulatory mechanism may be dominant for mucosal protection from acid injury.

In other HCO_3^--secreting epithelia such as airway, bile duct, oviduct and seminiferous tubule that highly express apical AP, CFTR, and P2Y receptors, a similar mechanism may be in place where precise control of localized alkaline pH_o is essential for organ function (Marteau *et al.* 1990; Rodriguez-Martinez, 2007). Airway non-ciliated cells (Clara cells) express $P2Y_2$ receptors, CFTR, and AP, increase I_{sc} in response to extracellular purines and increase $[Ca^{2+}]_i$ in response to ATP released by adjacent neuroepithelial bodies, all consistent with paracrine purinergic regulation of HCO_3^- secretion in airway. Dysregulation of airway pH_o in CF mice, however, is controversial (Fischer & Widdicombe, 2006). The high correlation between organ distribution of ecto-purinergically regulated, CFTR-dependent HCO_3^- secretion and the CF phenotype underscores the possible importance of ecto-purinergic pH_o regulation in the functioning of CFTR-expressing organs (Durie *et al.* 2004; Pastor-Soler *et al.* 2005; Rodriguez-Martinez, 2007; Haston *et al.* 2008).

Duodenal brush border membranes have at least two distinct ecto-ATPase pathways that degrade ATP into adenosine (Schweickhardt *et al.* 1995). IAP degrades ATP to ADP, AMP and adenosine, whereas ENTPDase (CD39 family) degrades ATP to ADP and AMP. 5′-Nucleotidase (CD73) then degrades AMP to adenosine (Zimmermann, 2000). Luminal ATP stimulates ion secretion from epithelia via the activation of G-protein-coupled P2Y purinergic receptors (Leipziger, 2003). Ecto-ATPase activity, although studied in other epithelia with secretion under ecto-purinergic control (Zimmermann, 2000), has never been previously studied in the context of the regulation of duodenal HCO_3^- secretion. Increased ATP output during IAP or ENTPDase inhibition is consistent with basal ecto-ATPase activity of IAP and ENTPDase in the duodenal brush border membrane (Schweickhardt *et al.* 1995). Since ENTPDase inhibition augmented HCO_3^- and ATP output less effectively than did IAP inhibition, IAP appears to be the primary ecto-ATPase in duodenum. Dual inhibition of IAP and ENTPDase

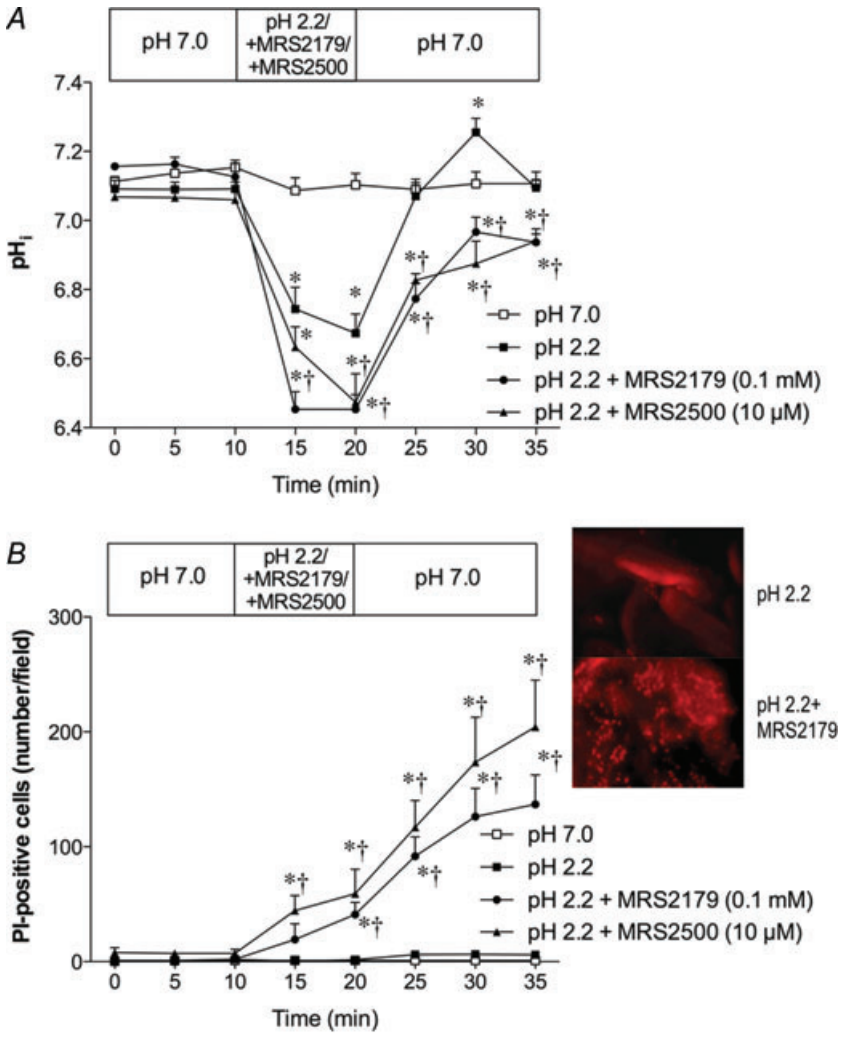

Figure 7. Effect of P2Y$_1$ receptor inhibition on acid-induced intracellular acidification and epithelial injury in rat duodenum
A, intracellular pH (pH$_i$) of duodenal epithelial cells was measured with *in vivo* microscopy. MRS2179 (0.1 mM) or MRS2500 (10 μM) enhanced acid-induced intracellular acidification and inhibited pH$_i$ recovery after acid exposure. Data are expressed as means ± S.E.M. ($n = 6$). *$P < 0.05$ *vs* pH 7.0 group, †$P < 0.05$ *vs* pH 2.2 group. *B*, injured cells were assessed with propidium iodide (PI) staining *in vivo*. MRS2179 or MRS2500 perfused with acid increased the number of PI-positive cells. Inset, PI staining of duodenal epithelial cells imaged at $t = 35$ min in pH 2.2 and pH 2.2 + MRS2179 groups. Data are expressed as means ± S.E.M. ($n = 6$). *$P < 0.05$ *vs* pH 7.0 group, †$P < 0.05$ *vs* pH 2.2 group.

additively enhanced HCO_3^- and ATP output, followed by a decline to the level of HCO_3^- and ATP output observed with IAP inhibition alone, suggesting that either ENTPDase was incompletely inhibited, that another ecto-ATPase was present, or that ATP output substantially declined over time. Since ARL67153 preferentially inhibits ENTPDase 1 and 3 (Levesque *et al.* 2007), ARL67153 may have incompletely inhibited ENTPDase activity.

Our finding that IAP inhibition markedly (>25-fold) increased ATP output, underscores the importance of the ecto-ATPase activity of IAP. Although the rapid cell turnover in intestinal villi may spontaneously release ATP, little ATP release was observed at baseline. Although shear stress developed as a consequence of rapid perfusion may increase ATP release, ATP output also increased in response to IAP inhibition with GP in non-perfused duodenal loops (data not shown). Our study also confirmed no parallel increase of cellular enzyme release with ATP output.

IAP inhibition-associated enhanced ATP output was partially inhibited by CFTR inhibition, suggesting that ATP release is, at least in part, regulated via CFTR function. Since CFTR function is necessary for stimulated duodenal, pancreatic and biliary HCO_3^- secretion (Hogan *et al.* 1997; Akiba *et al.* 2005), the regulation of ATP release, ATP-P2Y receptor signalling and AP activity might be closely related to CFTR function. Indeed, diminished ATP release is an explanation offered for the observed impairment of HCO_3^- secretion with CFTR dysfunction (Hogan *et al.* 1997). It is possible that the stimulated HCO_3^- secretion is concomitant with ATP release. Nevertheless, epithelial ATP release may occur by exocytosis, and via 'ATP anion channels' (Sabirov & Okada, 2004), although their CFTR dependence measured in model systems is controversial (Grygorczyk & Hanrahan, 1997; Braunstein *et al.* 2001; Okada *et al.* 2006). Furthermore, the role of constitutively released ATP is also unknown. Since ATP is degraded to adenosine, which is a secretagogue and an anti-inflammatory mediator

(Hasko *et al.* 2008), we assume that the baseline ATP release is related to adenosine signalling in intestine. Thus, IAP and ENTPDase inhibition increases $[ATP]_o$, but decreases adenosine signalling, presumably affecting the adenosine-mediated maintenance or homeostasis of mucosal functions.

Although we immunolocalized $P2Y_1$ receptors to the brush border membrane, the functional data are fully consistent with sole $P2Y_1$ receptor involvement. Rapid dephosphorylation by ecto-ATPases of exogenous ATP to ADP or ADP to AMP may affect the local concentration of ADP at the $P2Y_1$ receptor. Dong *et al.* (2009) recently reported that $P2Y_2$, but not $P2Y_4$ receptors were expressed in mouse duodenum, and that the $P2Y_2$ agonist UTP increased I_{sc} in Ussing-chambered murine duodenum in $P2Y_2$ wild-type but not in null mice. The discrepancy from our results may be due to species differences, or may be due to the action of submucosal P2 receptors, since serosal exposure to UTP was equally efficacious. Indeed, the full ATP effect may involve P2X receptor activation, consistent with the delayed increase of HCO_3^- secretion with perfusion of the P2X receptor agonists 2MeSATP and ATPγS. The $P2X_1$ receptor is localized in the interstitium of the villous core and $P2X_7$ is present in the villous cells in rat duodenum (Gröschel-Stewart *et al.* 1999), suggesting that the delayed response may reflect additional time for the agonist to diffuse to the villous interior (Takeuchi *et al.* 2007). Although $P2Y_1$ null mice have been used to study the well-known ecto-purinergic regulation of platelet aggregation and other conditions, characterization of their epithelial secretion has not been performed (Hohenstein *et al.* 2007; Gachet, 2008).

Despite its high proximal expression, the role of IAP is undefined. IAP may degrade ATP released from ingested food into adenosine and other absorbable non-phosphorylated purines, and may also facilitate or inhibit intestinal fat absorption possibly by dysregulating pH_o, which is important for fatty acid uptake across the brush border (Eliakim *et al.* 1989; Shiau, 1990; Narisawa *et al.* 2003; Narisawa *et al.* 2007). IAP may also degrade and detoxify luminal lipopolysaccharide, reducing bacterial translocation through the small intestinal mucosa (Goldberg *et al.* 2008), suggesting an alternative function in mucosal defence from bacterial pathogens (Geddes & Philpott, 2008). IAP also appears to be identical to thiamine diphosphatase, which converts ingested thiamine phosphates to the absorbable form of the vitamin (Schaller & Holler, 1975). The thiamine-deficient phenotype of presumptive reduced clinical IAP expression underscores the importance of IAP in thiamine absorption (Luong & Nguyen, 2005).

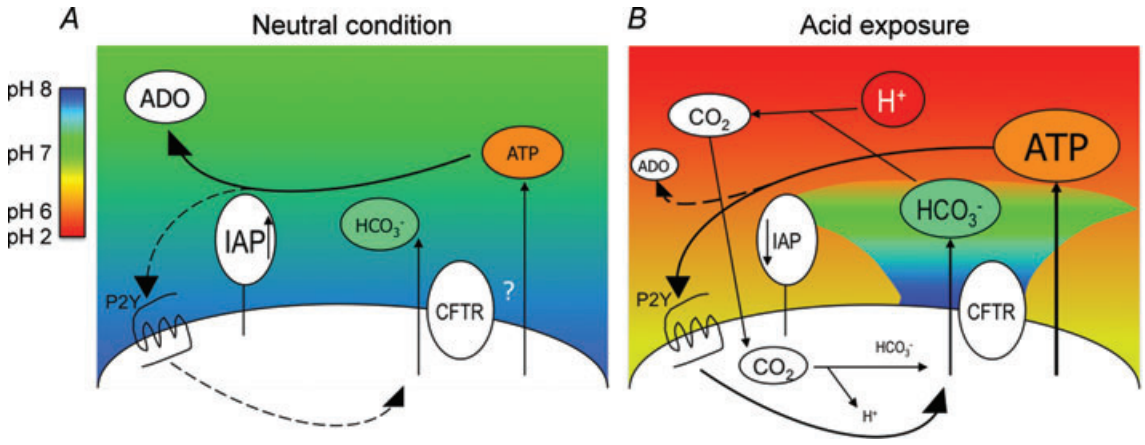

Figure 8. Proposed mechanisms of IAP/ATP/P2Y signalling in regulation of surface microclimate pH in rat duodenum

A, bulk pH = 7. IAP activity is high due to an alkaline microclimate. IAP degrades luminally released ATP into adenosine (ADO), reducing P2Y receptor activation, suppressing HCO_3^- secretion, decreasing microclimate pH, reducing IAP activity (negative feed-back). The mechanism by which CFTR function partially contributes to ATP release is unknown. *B*, bulk pH = 2. Acid exposure lowers microclimate pH, thereby reducing IAP activity, inhibiting ATP degradation, activating P2Y receptor, increasing HCO_3^- secretion. Increased HCO_3^- secretion alkalinizes microclimate pH and neutralizes luminal H^+ to generate CO_2, facilitated by ecto-carbonic anhydrase (CA) activity. Liberated CO_2 traverses apical membrane into the epithelial cells where it is re-converted into H^+ and HCO_3^- by cytosolic CA activity. Intracellular H^+ lowers intracellular pH; HCO_3^- is secreted across the apical membrane into the microclimate, completing the Jacob–Stewart cycle between microclimate and the cytoplasm. Secreted HCO_3^- increases microclimate pH, increasing IAP activity, reducing extracellular ATP concentration, reducing ATP-P2Y receptor signalling. These mechanisms explain the rapid, luminal pH-sensitive regulation of surface microclimate pH by IAP. IAP, intestinal alkaline phosphatase; ADO, adenosine; CFTR, cystic fibrosis transmembrane conductance regulator.

In conclusion, duodenal brush border IAP degrades ATP released from the epithelium, stimulating HCO_3^- secretion via P2Y receptor activation. HCO_3^- secretion increases the local pH of IAP, increasing its ecto-ATPase activity, decreasing ATP, which decreases HCO_3^- secretion. This ecto-purinergic system may thus regulate cell-surface pH, maintaining a protective alkaline microclimate during acid stress.

References

Akiba Y, Furukawa O, Guth PH, Engel E, Nastaskin I, Sassani P, Dukkipatis R, Pushkin A, Kurtz I & Kaunitz JD (2001). Cellular bicarbonate protects rat duodenal mucosa from acid-induced injury. *J Clin Invest* **108**, 1807–1816.

Akiba Y, Ghayouri S, Takeuchi T, Mizumori M, Guth PH, Engel E, Swenson ER & Kaunitz JD (2006). Carbonic anhydrases and mucosal vanilloid receptors help mediate the hyperemic response to luminal CO_2 in rat duodenum. *Gastroenterology* **131**, 142–152.

Akiba Y, Guth PH, Engel E, Nastaskin I & Kaunitz JD (2000). Dynamic regulation of mucus gel thickness in rat duodenum. *Am J Physiol Gastrointest Liver Physiol* **279**, G437–G447.

Akiba Y, Jung M, Ouk S & Kaunitz JD (2005). A novel small molecule CFTR inhibitor attenuates HCO_3^- secretion and duodenal ulcer formation in rats. *Am J Physiol Gastrointest Liver Physiol* **289**, G753–G759.

Akiba Y, Mizumori M, Guth PH, Engel E & Kaunitz JD (2007). Duodenal brush border intestinal alkaline phosphatase activity affects bicarbonate secretion in rats. *Am J Physiol Gastrointest Liver Physiol* **293**, G1223–G1233.

Allen A & Flemström G (2005). Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *Am J Physiol Cell Physiol* **288**, C1–C19.

Braunstein GM, Roman RM, Clancy JP, Kudlow BA, Taylor AL, Shylonsky VG, Jovov B, Peter K, Jilling T, Ismailov II, Benos DJ, Schwiebert LM, Fitz JG & Schwiebert EM (2001). Cystic fibrosis transmembrane conductance regulator facilitates ATP release by stimulating a separate ATP release channel for autocrine control of cell volume regulation. *J Biol Chem* **276**, 6621–6630.

Dong X, Smoll EJ, Ko KH, Lee J, Chow JY, Kim HD, Insel PA & Dong H (2009). P2Y receptors mediate Ca^{2+} signalling in duodenocytes and contribute to duodenal mucosal bicarbonate secretion. *Am J Physiol Gastrointest Liver Physiol* **296**, G424–G432.

Durie PR, Kent G, Phillips MJ & Ackerley CA (2004). Characteristic multiorgan pathology of cystic fibrosis in a long-living cystic fibrosis transmembrane regulator knockout murine model. *Am J Pathol* **164**, 1481–1493.

Eliakim R, Schryver-Kecskemeti K, Nogee L, Stenson WF & Alpers DH (1989). Isolation and characterization of a small intestinal surfactant-like particle containing alkaline phosphatase and other digestive enzymes. *J Biol Chem* **264**, 20614–20619.

Fischer H & Widdicombe JH (2006). Mechanisms of acid and base secretion by the airway epithelium. *J Membr Biol* **211**, 139–150.

Fitz JG (2007). Regulation of cellular ATP release. *Trans Am Clin Climatol Assoc* **118**, 199–208.

Flemström G & Kivilaakso E (1983). Demonstration of a pH gradient at the luminal surface of rat duodenum *in vivo* and its dependence on mucosal alkaline secretion. *Gastroenterology* **84**, 787–794.

Gachet C (2008). P2 receptors, platelet function and pharmacological implications. *Thromb Haemost* **99**, 466–472.

Garner A, Flemström G, Allen A, Heylings JR & McQueen S (1984). Gastric mucosal protective mechanisms: roles of epithelial bicarbonate and mucus secretions. *Scand J Gastroenterol Suppl* **101**, 79–86.

Geddes K & Philpott DJ (2008). A new role for intestinal alkaline phosphatase in gut barrier maintenance. *Gastroenterology* **135**, 8–12.

Goldberg RF, Austen WG Jr, Zhang X, Munene G, Mostafa G, Biswas S, McCormack M, Eberlin KR, Nguyen JT, Tatlidede HS, Warren HS, Narisawa S, Millan JL & Hodin RA (2008). Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proc Natl Acad Sci U S A* **105**, 3551–3556.

Gröschel-Stewart U, Bardini M, Robson T & Burnstock G (1999). P2X receptors in the rat duodenal villus. *Cell Tissue Res* **297**, 111–117.

Grygorczyk R & Hanrahan JW (1997). CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *Am J Physiol Cell Physiol* **272**, C1058–C1066.

Hasko G, Linden J, Cronstein B & Pacher P (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* **7**, 759–770.

Haston CK, Li W, Li A, Lafleur M & Henderson JE (2008). Persistent osteopenia in adult cystic fibrosis transmembrane conductance regulator-deficient mice. *Am J Respir Crit Care Med* **177**, 309–315.

Hechler B, Nonne C, Roh EJ, Cattaneo M, Cazenave JP, Lanza F, Jacobson KA & Gachet C (2006). MRS2500 [2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate], a potent, selective, and stable antagonist of the platelet P2Y1 receptor with strong antithrombotic activity in mice. *J Pharmacol Exp Ther* **316**, 556–563.

Hietanen E (1973). Interspecific variation in the levels of intestinal alkaline phosphatase, adenosine triphosphatase and disaccharidases. *Comp Biochem Physiol A* **46**, 359–369.

Hirokawa M, Takeuchi T, Chu S, Akiba Y, Wu V, Guth PH, Engel E, Montrose MH & Kaunitz JD (2004). Cystic fibrosis gene mutation reduces epithelial cell acidification and injury in acid-perfused mouse duodenum. *Gastroenterology* **127**, 1162–1173.

Hogan DL, Crombie DL, Isenberg JI, Svendsen P, Schaffalitzky de Muckadell OB & Ainsworth MA (1997). Acid-stimulated duodenal bicarbonate secretion involves a CFTR-mediated transport pathway in mice. *Gastroenterology* **113**, 533–541.

Hohenstein B, Renk S, Lang K, Daniel C, Freund M, Leon C, Amann KU, Gachet C & Hugo CP (2007). P2Y1 gene deficiency protects from renal disease progression and capillary rarefaction during passive crescentic glomerulonephritis. *J Am Soc Nephrol* **18**, 494–505.

Humphreys MH & Chou LY (1979). Anion-stimulated ATPase activity of brush border from rat small intestine. *Am J Physiol Endocrinol Metab* **236**, E70–E76.

Kaunitz JD & Akiba Y (2006). Review article: duodenal bicarbonate – mucosal protection, luminal chemosensing and acid-base balance. *Aliment Pharmacol Ther* **24**, 169–176.

Lambrecht G (2000). Agonists and antagonists acting at P2X receptors: selectivity profiles and functional implications. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 340–350.

Leipziger J (2003). Control of epithelial transport via luminal P2 receptors. *Am J Physiol Renal Physiol* **284**, F419–F432.

Levesque SA, Lavoie EG, Lecka J, Bigonnesse F & Sevigny J (2007). Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. *Br J Pharmacol* **152**, 141–150.

Luong KV & Nguyen LT (2005). Adult hypophosphatasia and a low level of red blood cell thiamine pyrophosphate. *Ann Nutr Metab* **49**, 107–109.

Marteau C, Sastre B, Iconomidis N, Portugal H, Pauli AM & Gerolami A (1990). pH regulation in human gallbladder bile: study in patients with and without gallstones. *Hepatology* **11**, 997–1002.

Mizumori M, Meyerowitz J, Takeuchi T, Lim S, Lee P, Supuran CT, Guth PH, Engel E, Kaunitz JD & Akiba Y (2006). Epithelial carbonic anhydrases facilitate P_{CO_2} and pH regulation in rat duodenal mucosa. *J Physiol* **573**, 827–842.

Narisawa S, Hoylaerts MF, Doctor KS, Fukuda MN, Alpers DH & Millan JL (2007). A novel phosphatase upregulated in Akp3 knockout mice. *Am J Physiol Gastrointest Liver Physiol* **293**, G1068–G1077.

Narisawa S, Huang L, Iwasaki A, Hasegawa H, Alpers DH & Millan JL (2003). Accelerated fat absorption in intestinal alkaline phosphatase knockout mice. *Mol Cell Biol* **23**, 7525–7530.

Okada SF, Nicholas RA, Kreda SM, Lazarowski ER & Boucher RC (2006). Physiological regulation of ATP release at the apical surface of human airway epithelia. *J Biol Chem* **281**, 22992–23002.

Pastor-Soler N, Pietrement C & Breton S (2005). Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. *Physiology (Bethesda)* **20**, 417–428.

Pinto A, Calignano A, Mascolo N, Autore G & Capasso F (1989). Castor oil increases intestinal formation of platelet-activating factor and acid phosphatase release in the rat. *Br J Pharmacol* **96**, 872–874.

Rodriguez-Martinez H (2007). Role of the oviduct in sperm capacitation. *Theriogenology* **68**, S138–S146.

Sabirov RZ & Okada Y (2004). ATP-conducting maxi-anion channel: a new player in stress-sensory transduction. *Jpn J Physiol* **54**, 7–14.

Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H, Yamamoto A & Moriyama Y (2008). Identification of a vesicular nucleotide transporter. *Proc Natl Acad Sci U S A* **105**, 5683–5686.

Schaller K & Holler H (1975). Thiamine absorption in the rat. ii. intestinal alkaline phosphatase activity and thiamine absorption from rat small intestine in-vitro and in-vivo. *Int J Vitam Nutr Res* **45**, 30–38.

Schweickhardt C, Sabolic I, Brown D & Burckhardt G (1995). Ecto-adenosinetriphosphatase in rat small intestinal brush-border membranes. *Am J Physiol Gastrointest Liver Physiol* **268**, G663–G672.

Shiau YF (1990). Mechanism of intestinal fatty acid uptake in the rat: the role of an acidic microclimate. *J Physiol* **421**, 463–474.

Takeuchi T, Ham M, Mizumori M, Guth PH, Engel E, Kaunitz JD & Akiba Y (2007). Solute diffusion through stripped mouse duodenum. *J Physiol Pharmacol* **58**, 767–791.

von Kügelgen I (2006). Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* **110**, 415–432.

Williams SE & Turnberg LA (1981). Demonstration of a pH gradient across mucus adherent to rabbit gastric mucosa: evidence for a 'mucus-bicarbonate' barrier. *Gut* **22**, 94–96.

Yegutkin GG (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* **1783**, 673–694.

Zimmermann H (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 299–309.

Zimmermann H (2008). ATP and acetylcholine, equal brethren. *Neurochem Int* **52**, 634–648.

Author contributions

All authors contributed to this study for study design, experiments, analysis and interpretation of data, and preparation of the original and revised manuscripts. All authors approved the final version to be published. All parts of this study were conducted in West Los Angeles Veterans Affairs Medical Center.

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