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Purinergic regulation of duodenal surface pH and ATP concentration: Implications for mucosal defense, lipid uptake, and cystic fibrosis

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Abstract

The duodenum secretes HCO_3^- as part of a multi-layered series of defense mechanism against damage from luminal acid. In the 1980s, an alkaline surface layer was measured over the mucosa which correlated with the rate of HCO_3^- secretion. Since all biological processes are regulated, we investigated how the alkaline pH of the surface layer was maintained. Since the ecto-phosphorylase alkaline phosphatase (AP) is highly expressed in the duodenal brush border, we hypothesized that its extreme alkaline pH optimum (~pH 8-9) combined with its ability to hydrolyze regulatory purines such as ATP was part of an ecto-purinergic signalling system, consisting also of brush border P2Y receptors and CFTR-mediated HCO_3^- secretion. Extracellular ATP increases the rate of HCO_3^- secretion through this purinergic system. At high surface pH (pH_s), AP activity is increased, which then increases the rate of ATP hydrolysis, decreasing surface ATP concentration ($[\text{ATP}]_s$), with a resultant decrease of the rate of HCO_3^- secretion, which subsequently decreases pH_s . This feedback loop is thus hypothesized to regulate pH_s over the duodenal mucosa, and in several other HCO_3^- secretory organs. Since AP activity is directly related to pH_s , and since AP hydrolyzes ATP, $[\text{ATP}]_s$ and pH_s are co-regulated. Since many essential tissue functions such as ciliary motility and lipid uptake are dependent on $[\text{ATP}]_s$, dysregulation of pH_s and $[\text{ATP}]_s$ may help explain the tissue dysfunction characteristic of diseases such as cystic fibrosis.

Keywords

Alkaline phosphatase; ecto-ATPase; brush border membrane; HCO_3^- secretion; P2Y receptors; extracellular purines; mucosal defence; cystic fibrosis; intestinal lipid uptake

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Introduction

The location of the duodenal epithelium just distal to the gastric antrum and proximal to the pancreaticobiliary ducts uniquely exposes it to a highly variable pH environment due to peristaltically conveyed pulses of concentrated gastric acid combined with secreted HCO_3^- . Since the duodenum, unlike the stomach or esophagus is electrically leaky, it does not possess the structural integrity needed to prevent ion permeation into the tissue. Rather, the duodenum is protected by a coordinated, layered series of defenses which ultimately prevent irreversible acidification and necrosis of the epithelial cells when luminal pH is low.

The mucus- HCO_3^- layer is a barrier covering the duodenal epithelium, which helps prevent luminal H^+ from reaching the mucosa. Although HCO_3^- has long been implicated in the mucosal protection, the mechanism by which this secretion was protective was uncertain. In the 1980s, Flemström and others measured a surface alkaline pH at disequilibrium with the bulk luminal pH that correlated with the rate of HCO_3^- secretion and was associated with mucosal protection (**Fig. 1**) (Flemström *et al.*, 1983). The discovery of the alkaline layer was the basis for the current hypothesis of mucosal protection wherein luminal H^+ is thought to be neutralized by HCO_3^- secreted by the epithelial cells, preventing acidification of the underlying mucosa. This observation stresses the importance of regulation of surface pH (pH_s), of which, like pH_i , is likely to have physiological importance for the maintenance of normal organ function.

Purinergic Signalling

ATP and other purine nucleotides serve as neurotransmitters as part of a purinergic signalling system. Although purinergic neurotransmission was initially described in the 1970s, the concept that extracellular ATP (ATP_e) is an important non-neuronal regulatory molecule is relatively recent (Burnstock, 2007, Leipziger, 2003). Components of the system include exocytotic release of ATP from subsurface granules, interaction with well-defined purinergic G protein-coupled metabotropic cell surface receptors (P1 or P2Y) or ionotropic channels (P2X), and hydrolysis by ecto-hydrolases into ADP, AMP, and adenosine. Particularly in secretory epithelia, extracellular ATP and adenosine increase anion or HCO_3^- secretion through interaction with plasma membrane P1 and P2Y receptors. Indeed, selective P2Y agonists have been developed to overcome the airway anion secretory impairment characteristic of the disease cystic fibrosis (CF) (Clunes *et al.*, 2008).

Even though many secretory epithelia, such as vas deferens, oviduct, airway, cholangiocyte, and pancreatic duct have ATP and adenosine-dependent anion secretion, consistent with ecto-purinergic regulation of ion transport, only recently has the purinergic system been described in the duodenum (Akiba *et al.*, 2007, Mizumori *et al.*, 2009). Every required component of the purinergic signalling system is expressed in the duodenum including surface purinergic metabotropic P2Y receptors, surface ectophosphohydrolases such as alkaline phosphatase (AP) and ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase or CD39), an ATP release mechanism, and luminal ATP-enhanced HCO_3^- secretion { Akiba, 2007 4129 /id; Dong, 2009 4931 /id; Mizumori, 2009 4800 /id }; **Fig. 2**. Our more recent studies support the presence of surface P1 receptors and adenosine-stimulated HCO_3^- in rat duodenum (Akiba *et al.*, 2009). Although the presence of an epithelial ecto-

purinergic system regulating Cl^- and HCO_3^- secretion is not new, its function to regulate pH_s has not been previously described.

How pH_s and $[\text{ATP}]_s$ are regulated—Ecto-purinergic regulation of HCO_3^- secretion exists on numerous organs, such as bile duct, seminiferous tubules, pancreatic duct, and airway. In these organs, exogenous purine nucleotides such as ATP and adenosine increased Cl^- or HCO_3^- secretion. In many cases, P1 and P2Y metabotropic purinoreceptors are localized to the epithelial cell apical membrane. Recently, we and others described that perfusion of ATP and other purine nucleotides increased the rate of HCO_3^- secretion in rodent proximal duodenum {Mizumori, 2009 4800 /id; Dong, 2009 4931 /id}. In rat, immunohistochemically localization and pharmacological characterization was consistent with activation of P2Y₁ receptors (**Fig. 2A**), whereas in mice P2Y₂ receptors appeared to be dominant. Along with increased HCO_3^- secretion, duodenum (and other HCO_3^- secreting tissues) also expresses AP in high abundance in the brush border membrane (**Fig. 2B**). Since AP has a high pH optimum (~8-9) and has ATPase activity, we hypothesized that AP serves as a pH-sensitive ATP ecto-phosphohydrolase at the intestinal surface. Coupled with purinergically regulated HCO_3^- secretion, this would serve as a negative feedback system in the regulation of pH_s and $[\text{ATP}]_s$. If pH_s is high, AP activity is high since its catalytic site is closer to its pH optimum. Consequently, the rate of ATP hydrolysis is high which then lowers $[\text{ATP}]_s$, which then decreases activation of surface P2Y receptors, decreasing the rate of HCO_3^- secretion, lowering pH_s . The system also regulates $[\text{ATP}]_s$ by the same mechanism: when $[\text{ATP}]_s$ increases due to lower AP activity at low pH_s , the rate of HCO_3^- secretion increases due to P2Y activation, raising pH_s , increasing the hydrolytic rate of AP. Another component of the ecto-purinergic pH_s regulatory system is the cystic fibrosis transmembrane regulator (CFTR), which is localized in the villous cells brush border membrane (**Fig. 2C**) (Collaco *et al.*, 2010)

This hypothesis is supported by several observations. First, as already discussed, the rate of HCO_3^- secretion is enhanced by luminal perfusion of purine nucleotides such as ATP and adenosine. Second, measurement of AP activity in vivo using the fluorogenic substrate ELF-97 indicated that AP activity positively correlates with the rate of HCO_3^- secretion and perfusate pH (Akiba *et al.*, 2007). The importance of this observation is that it is the first reported measurement of AP activity in vivo in an intact tissue. As a consequence, AP activity measured in vitro, particularly preparations incapable of vectorial HCO_3^- transport, may be discordant with what occurs physiologically in situ. Conditions predicted to lower pH_s , such as perfusion with a strongly acidic solution in the presence of a potent inhibitor of CFTR, CFTR_{inh}-172, which virtually abolished duodenal HCO_3^- secretion, strongly impaired AP activity, (Akiba *et al.*, 2007). Third, the perfused rat duodenum releases ATP at baseline and increases release in response to a prosecretory signals such as acid perfusion, enhanced by AP inhibitors. Fourth, inhibition of AP or other duodenal ecto phosphohydrolases such as ectonucleotide triphosphodephosphorylase (ENTPDase) increases the rate of HCO_3^- secretion. Fifth, potent pharmacological inhibitors of CFTR impair ATP release (Mizumori *et al.*, 2009). These data indicate that pH_s and $[\text{ATP}]_s$ are correlated due to the pH-sensitive activity of AP, which in effect acts as a pH_s sensor and regulator.

Although data unequivocally support the hypothesis that a functioning CFTR is needed for normal duodenal HCO_3^- secretion {Seidler, 1997 842 /id;Hogan, 1997 924 /id}, its interaction with the purinergic signalling system is somewhat problematic. Although duodenal luminal perfusion with ATP increased HCO_3^- transport, P2Y_1 and P2Y_2 receptors, for which ATP and ADP, or UTP are ligands, are of the G_q subtype that signal through the protein kinase C (PKC)/ Ca^{++} pathway (Lazarowski *et al.*, 2009). In contrast, adenosine is a ligand for the P1 receptor, which, in intestine is generally the A_{2b} (G_s) subtype (Kolachala *et al.*, 2006, Strohmeier *et al.*, 1995) which interacts with the PKA/cAMP pathway, directly activating CFTR (Clarke *et al.*, 1998, Illek *et al.*, 1997). Since ATP is hydrolyzed to adenosine by AP and other surface hydrolytic enzymes, and since CFTR may also be stimulated by the PKC/ Ca^{++} pathway (Hogan *et al.*, 1997) the relative contributions of the P1 and P2 receptors towards ATP-induced HCO_3^- secretion is currently unknown.

Functional implications

Duodenal host defense—If duodenal pH_s and $[\text{ATP}]_s$ are regulated, what are the functional consequences of such regulation? Since the duodenum must withstand acid pulses from the stomach, perhaps one important function is protection of the underlying mucosa from irreversible acidification. Many laboratories have studied the importance of HCO_3^- secretion in mucosal defense; many hormones and mediators, such as vasoactive intestinal peptide, cAMP, and E-type prostaglandins increase the rate of HCO_3^- secretion (Aihara *et al.*, 2007, Allen *et al.*, 2005, Hogan *et al.*, 1994). The relation of HCO_3^- secretion stimulated by these hormones and mediators to that stimulated by purinergic analogs is however unclear. We recently demonstrated that disruption of the ecto-purinergic pH_s regulatory system with perfusion of a P2Y_1 antagonist in the presence of physiological concentrations of acid markedly increased epithelial injury, underscoring the importance of purinergic pH_s regulation while confirming the importance of HCO_3^- secretion in mucosal protection (**Fig. 3**) (Mizumori *et al.*, 2009). Furthermore, activation of the adenosine A_{2b} receptors increases cAMP in airway (Clancy *et al.*, 1999) and duodenal HCO_3^- secretion via CFTR activation (Akiba *et al.*, 2009). Since we have found that ATP is released immediately on luminal acid exposure, but is then degraded rapidly to ADP, AMP and adenosine by surface phosphohydrolases, P2Y signalling is likely predominant during luminal acid exposure, whereas P1 signalling occurs after the acid pulse. Nevertheless, the duodenum, upper intestine, and other HCO_3^- organs also have other functions which may be affected by dysregulation of pH_s or $[\text{ATP}]_s$.

Intestinal fat absorption—Intestinal lipid uptake is one such function. Genetic deletion of the mouse paralog of the intestinal AP gene *Akp3* enhances enterocyte lipid uptake after a lipid meal, suggesting that AP activity slows lipid uptake from lumen into the enterocyte or from enterocyte into the lymph (Narisawa *et al.*, 2003). The reason for this, however, is unexplained. Since AP absence presumably dysregulates pH_s and $[\text{ATP}]_s$, perhaps enterocyte lipid uptake is optimal at a certain pH_s or $[\text{ATP}]_s$ range. Although limited data are currently available, it appears that lipid uptake is enhanced at acidic pH_s (Shiau *et al.*, 1980, Shiau *et al.*, 1990), although in mouse models of CFTR dysfunction or lack, in which the duodenum is acidified, triglyceride absorption is delayed (Bijvelds *et al.*, 2005). It is difficult, however to predict pH_s in the absence of AP function. Presumably, the lack of ATP

hydrolysis would increase the rate of HCO_3^- secretion which would raise pH_s , slowing lipid uptake. A more plausible explanation for the observed acceleration of enterocyte lipid uptake in *Akp3* knockout mice bears on $[\text{ATP}]_s$ dysregulation. CD36 is a surface glycoprotein implicated in the uptake of enterocyte long chain fatty acids (Drover *et al.*, 2005, Drover *et al.*, 2008), which has a paralog expressed in platelet membranes (Guthmann *et al.*, 2002). $[\text{ATP}]_s$ affects lipid uptake into platelets in a U-shaped relationship, with an uptake rate nadir of $\sim 10 \mu\text{M}$ (Guthmann *et al.*, 2002). In the absence of IAP function, $[\text{ATP}]_s$ increases many-fold, probably at least 2 log orders (Mizumori *et al.*, 2009), which would increase the rate of lipid uptake, suggesting that intestinal lipid uptake may as well have an optimal $[\text{ATP}]_s$, thus helping explain enhanced lipid uptake in *Akp3* knockout mice.

Extraintestinal organs: Implications for cystic fibrosis—Outside the gut, tissues that have ecto-purinergically regulated HCO_3^- secretion and express P2Y receptors, AP, and CFTR would be predicted also to have processes dependent on regulation of pH_s and $[\text{ATP}]_s$. The male and female reproductive tracts must maintain an alkaline luminal pH in their capacitive and reservoir portions in order to prevent sperm activation (Chan *et al.*, 2006, Hihnala *et al.*, 2006, Pastor-Soler *et al.*, 2005, Rodriguez-Martinez, 2007). CFTR dysfunction impairs the rate of epithelial HCO_3^- secretion presumably by its effect on anion exchangers or by transporting HCO_3^- itself (Hug *et al.*, 2003, Ko *et al.*, 2002, Reddy *et al.*, 2003, Tang *et al.*, 2009, Zsembery *et al.*, 2002). According to our hypothesis and to our duodenal measurements, impaired HCO_3^- secretion inadequately alkalinizes the catalytic site of AP, impairing its activity, which in turn increases $[\text{ATP}]_s$ (Mizumori *et al.*, 2009). Nevertheless, in duodenum, as discussed above, acute CFTR inhibition with the potent and selective CFTR inhibitor CFTR_{inh}-172 not only impairs AP activity during luminal acid challenge, but also decreases $[\text{ATP}]_s$ for unclear reasons (Akiba *et al.*, 2007, Mizumori *et al.*, 2009), perhaps due to direct participation of CFTR in the ATP release mechanism (Cantiello, 2001, Grygorczyk *et al.*, 1997). Thus, CFTR dysfunction presumably dysregulates pH_s and $[\text{ATP}]_s$ by not only impairing AP activity due to acidification of its catalytic site, but also by impairing ATP release from the epithelial cells.

The human CF phenotype includes pancreatic dysfunction, male and female infertility, severe pulmonary disease, and metabolic bone disease. (Durie *et al.*, 2004, Rowntree *et al.*, 2003). Pulmonary disease, however, is usually the most serious and ultimately fatal complication of cystic fibrosis (Lazarowski and Boucher, 2009). Although dysfunctional CFTR mutations are unquestionably causative of the disease CF, the actual connection between CFTR function and the clinical phenotype is not fully understood. Prior to the cloning of the CFTR, one prevailing view was that CF is a disease of delayed pulmonary clearance. Many groups reported that the serum of CF patients contained an inhibitor of ciliary activity to support this hypothesis, since CF phenotypically, particularly in its advanced stages, resembles diseases of primary ciliary dysfunction (Kollberg *et al.*, 1978, Miller, 1973, Waring, 1973). After the cloning of the CFTR, hypotheses shifted towards impairment of Cl^- secretion, since the CFTR was identified as a Cl^- channel. An attractive and logical hypothesis which prevails to the present is that CFTR dysfunction impairs Cl^- secretion which decreases airway surface liquid (ASL) volume. Decreased ASL volume is thought then to produce the characteristic viscous mucous and increased infection

susceptibility associated with the disease (Clunes and Boucher, 2008, Com *et al.*, 2009, Lazarowski and Boucher, 2009) (Boucher, 2004, Mall, 2008). Nevertheless, the extreme small volumes of the periciliary fluid have impaired full characterization of airway surface liquid (ASL) pH and nucleotide content, although sophisticated methods such as X-ray microanalysis have been instrumental in the measurement of ASL electrolyte concentrations (Vanthanouvong *et al.*, 2006). ASL pH is arguably acidic in CF, which has been thought to impair the rheological properties of mucus and increase mucus viscosity, increase interactions between mobile and surface-tethered airway mucus, impair the function of airway immune cells, and impair airway bacterial clearance (Coakley *et al.*, 2001, McShane *et al.*, 2003).

In view of our findings regarding the regulation of epithelial $[ATP]_s$ and pH_s , we would like to propose a hypothesis which could help explain the observed CF phenotype. With CF dysfunction, HCO_3^- secretion is impaired, reducing AP activity, with consequent decreased ATP hydrolysis. Decreased ATP hydrolysis is countered by decreased ATP release, as discussed above. In either case, $[ATP]_s$ control is dysregulated. Several key epithelial functions are directly dependent on $[ATP]_s$. Ciliary function, for example, like CD36-mediated lipid uptake, depends on $[ATP]_s$ with an inverted U-shaped relationship, with optimal $[ATP]_s \approx 10 \mu M$ (Tarasiuk *et al.*, 1995). Thus, dysregulation of $[ATP]_s$ impairs ciliary function, suggesting a means by which CFTR dysfunction, with it consequent impairment of AP activity and ATP release, alters $[ATP]_s$. Although ciliary function can be measured in vitro from cells shed from the nasal epithelium, only a few reports exist of ciliary function in CF subjects. Delayed ciliary transit with normal ciliary beat frequency was reported in an oft-cited study, the latter a likely artifact of measuring beat frequency in vitro in ATP-containing medium (Rutland *et al.*, 1981). Another consequence of $[ATP]_s$ dysregulation is altered purinergic receptor activation, in particular, $P2Y_2$ receptors. In the lung, $P2Y_2$ receptors mediate increased chloride secretion and inhibit Na^+ absorption via the epithelial Na^+ channel ENaC. Low $[ATP]_s$ decreases periciliary fluid volume by decreasing Cl^- secretion while increasing ENaC-mediated Na^+ uptake (Com and Clancy, 2009), an observation on which CF therapy with exogenous purinergic agonists is based (Clunes and Boucher, 2008, Picher *et al.*, 2004, Schreiber *et al.*, 2005). An important indirect consequence of pH_s dysregulation is the detoxification of bacterial lipopolysaccharide (LPS). One of the hallmarks of the CF pulmonary phenotype and the probable cause of early mortality is the persistent infection with *Pseudomonas aeruginosa* and related organisms. *P. aeruginosa* exerts much of its virulence via secretion of LPS, which, as its name implies, is a compound composed of multiple lipophilic aliphatic carbon chains linked to carbohydrate moieties (Buchanan *et al.*, 2009, Pier, 2007). The toxic effects of LPS are associated with phosphorylation of the protein A moiety of LPS. Dephosphorylation of LPS by exogenous AP has already been used therapeutically for bacterial-associated inflammatory disorders (Beumer *et al.*, 2003). As discussed above, AP activity, when measured in vivo, is impaired in the presence of CFTR dysfunction, probably due to a lack of an alkaline environment at the catalytic site of the enzyme. Notably, AP activity, and ATP hydrolysis, when measured in vitro in cells isolated from CF tissues, is increased, possibly due to compensatory increased AP expression due to impaired AP function in vivo. (Picher *et al.*, 2004). Again, AP activity measured in vivo in intact tissue is likely to differ from AP activity measured in

vitro if active HCO_3^- secretion is not present, since the catalytic site of AP would not be adequately alkalinized. Thus, reduced LPS detoxification secondary to reduced AP activity due to impaired HCO_3^- secretion with acidic ASL may help explain the marked susceptibility of CF subjects to infection with pathogens in which LPS is a major virulence factor for organisms that colonize CF subjects such as pseudomonas (Pier, 2007).

Summary

Dysregulation of pH_s and $[\text{ATP}]_s$ due to dysfunction of components of the ecto-purinergic pH_s and $[\text{ATP}]_s$ regulatory system can have profound effects on organ function. Acidification of the duodenal surface alkaline layer by a P2Y receptor antagonist can increase the susceptibility of the underlying epithelial cells to luminal acid injury. AP lack or dysfunction is associated with accelerated lipid uptake. Some of the components (e.g. P2Y receptors) are already therapeutic targets; we hope that other components can be targeted therapeutically as well in the quest to ameliorate serious diseases such as CF.

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Fig. 1. Measurement of rat duodenal pH_s in vivo

The bulk luminal pH was 5. An antimony microelectrode was slowly advanced through the mucus layer. Note the sudden and linear increase of pH as the probe traverses the mucus layer, reaching a pH near the epithelial surface of ~ 7.5 prior to entering the epithelial cell, represented by electrical noise and instability of the signal near the end of the trace. Adapted from (Flemström and Kivilaakso, 1983).

Fig. 2. Expression of P2Y receptors in rat duodenal epithelium

Cryostat sections of fixed rat duodenum were reacted with P2Y receptor antibodies. P2Y₁ receptor-like immunoreactivity was strongly recognized on the brush border membranes of villous cells (**A**). Alkaline phosphatase activity, as recognized by ELF-97 fluorescence, is strongly expressed on the brush border membrane of the duodenal villous epithelial cells (**B**). CFTR is also highly expressed in the enterocyte brush border of rat jejunum (**C**) (Modified and adapted from Akiba *et al.*, 2007, Collaco *et al.*, 2010, and Mizumori *et al.*, 2009).

Fig. 3. P2Y₁ receptor inhibition acidifies enterocytes with subsequent injury in rat duodenum in vivo

Intracellular pH (pH_i) was microscopically measured with the pH-sensitive, intracellular fluorescent dye BCECF in A. Epithelial cell injury was measured with the fluorescent conditionally impermeant nuclear dye propidium iodide (PI) in B. A: The P2Y₁ antagonists MRS2179 (0.1 mM) or MRS2500 (10 μM) exaggerated acid-induced intracellular acidification while inhibiting pH_i recovery after acid exposure compared with vehicle controls. B: Acid co-perfused with either P2Y₁ antagonist injured the mucosa, as measured by the increased number of PI-positive cells. Inset, PI staining of duodenal epithelial cells showing PI positive nuclei in the damaged epithelial cells in the pH 2.2 + MRS 2179 group. Adapted from: (Mizumori *et al.*, 2009).