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Importance of bicarbonate transport in pH control during amelogenesis – need for functional studies

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Abstract

Dental enamel, the hardest mammalian tissue, is produced by ameloblasts. Ameloblasts show many similarities to other transporting epithelia although their secretory product, the enamel matrix, is quite different. Ameloblasts direct the formation of hydroxyapatite crystals, which liberate large quantities of protons that then need to be buffered to allow mineralization to proceed. Buffering requires a tight pH regulation and secretion of bicarbonate by ameloblasts. Many investigations have used immunohistochemical and knock-out studies to determine the effects of these genes on enamel formation, but up till recently very little functional data was available for mineral ion transport. To address this, we developed a novel 2D in vitro model using HAT-7 ameloblast cells. HAT-7 cells can be polarized and develop functional tight junctions. Furthermore, they are able to accumulate bicarbonate ions from the basolateral to the apical fluid spaces. We propose that in the future the HAT-7 2D system along with similar cellular models will be useful to functionally model ion transport processes during amelogenesis. Additionally, we also suggest that similar approaches will allow a better understanding of the regulation of the cycling process in maturation-stage ameloblasts, and the pH sensory mechanisms, which are required to develop sound, healthy enamel.

Keywords

ameloblast; enamel; amelogenesis; HAT-7; pH regulation; bicarbonate; in vitro; transwell; ion transport; functional model; microfluorometry; transepithelial resistance

Introduction

In the human body dental enamel is the hardest material as it has the highest mineral concentration. Its major disorders are related to environmental and genetic conditions. In

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both cases mineral formation is greatly impaired. As an example, fluorosis of the teeth is a developmental disturbance caused by intake of supraoptimal amounts of fluoride during early childhood (Aoba & Fejerskov, 2002; P. Denbesten & Li, 2011), and may result in the formation of more brittle enamel that is susceptible to fracture (Vieira, Hanocock, Eggertsson, Everett, & Grynpas, 2005). Dental caries and erosion are also important causes of enamel loss (West & Joiner, 2014). Fully formed enamel is acellular, and therefore can not regenerate. However, in recent years the finding that genetic influences alter enamel structure to affect hardness and caries susceptibility (Bayram et al., 2015; Shaffer, Carlson, et al., 2015; Shaffer, Wang, et al., 2015; Vieira et al., 2017) direct the need to better understand the mechanisms of enamel formation, which will allow further identification of risk factors and to optimal enamel formation.

Ameloblasts secrete enamel in a two-stage process. A moderately mineralized matrix structure is built first, and then the remodelling of this matrix leads to an extremely high level of mineralization. Ameloblasts differentiate from the inner enamel epithelium, which is originally derived from the oral epithelium. They take several distinct morphological forms corresponding to different functional states during their life cycle (Robinson, 2014). Importantly, the epithelial tight junctions of the ameloblasts seal the intercellular space and allow the maintenance of extreme concentration gradients between the apical and basolateral sides of the cells. Calcium and phosphate ions are transported actively through the cells into the mineralization space by a process which is only partially understood.

Histochemical evidence has been presented for the expression of specific ion transporters in the ameloblast membrane (Bronckers, 2017; Josephsen et al., 2010), but their exact role in crystal growth is not yet determined (Robinson, 2014; Varga, Kerémi, Bori, & Földes, 2015). In the secretory phase, the whole thickness of the enamel is formed but its mineralization is only approximately 20–30%. In the maturation stage, the morphology of ameloblasts changes and cyclically transforms between ruffle- and smooth-ended states. The likely importance of this cyclical modulation is that ameloblasts have dual functions: (1) to secrete calcium and phosphate, and neutralize the protons liberated during hydroxyapatite crystal growth, and (2) reabsorb and degrade amelogenin cleaved by matrix metalloproteinase-20 (MMP-20) and kallikrein-4 (Smith & Nanci, 1995; Zhu et al., 2014). Degradation of amelogenin and parallel crystal expansion continues until the whole organic matrix is eliminated and replaced by the tightly packed and practically impermeable crystal structure (Robinson, 2014; Zhu et al., 2014).

Acid/base balance plays a very important role during enamel hydroxyapatite formation since the crystal growth depends on a delicate cellular control of the ionic composition and pH of the extracellular fluid (Takagi et al., 1998). Namely, the formation of hydroxyapatite during the maturation stage of amelogenesis certainly generates a large quantity of protons. Therefore, to sustain crystal growth, these protons need to be neutralized (Josephsen et al., 2010; Lacruz, Nanci, Kurtz, Wright, & Paine, 2010; Smith, 1998) by the secretion of neutralizing ions, particularly bicarbonate, into the enamel space (Figure 1).

Unlike other luminal spaces of epithelial cells such as salivary glands, pancreas, kidney or liver, the enamel space is practically inaccessible to direct physiological examination.

However, studies of the formed enamel mineral in animal models with specific genetic defects can give us a type of fossil record of ion transport into the enamel matrix. Very recently three outstanding reviews have appeared covering the ion transport processes during amelogenesis (A. L. Bronckers, 2017; Lacruz, 2017; Lacruz, Habelitz, Wright, & Paine, 2017). In this review we direct our focus to the physiology of bicarbonate secretion as it may happen in ameloblasts, and the need for functional models to better understand the molecular physiology and pharmacology of pH control-related processes in enamel formation.

Bicarbonate: a buffer molecule and more

All living organisms have buffering systems to protect extracellular and intracellular fluids from substantial pH changes when acids or bases are added to them. The three major buffer systems that regulate blood pH are bicarbonate, phosphate and protein buffer systems. Of these systems, undoubtedly, bicarbonate is the most important because it is the only one that is coupled with the respiratory system, and also because its total functional buffering capacity is the highest in all organisms. When considering the whole scale of its physiological functions in the oral cavity, we must recognize some additional properties of bicarbonate besides being a buffering ion. These include the regulation of secretion and “unwrapping” of mucin molecules (Quinton, 2010) as well as control of soluble adenylate cyclase (sAC) activity both in eukaryotic and prokaryotic cells (Rahman, Buck, & Levin, 2013). Prior to exocytosis mucin molecules are stored in intracellular vesicles, in which calcium and proton concentrations are significantly higher than that of the cytosol. The high level of Ca^{2+} (>200 mM) and acidic environment (pH<6) are required to shield negative charges of the mucin molecules so that they remain “packed” inside the vesicles. Upon the release of mucin from the vesicles, bicarbonate binds to Ca^{2+} and H^+ forming $Ca(HCO_3)_2$ and H_2CO_3, respectively. Consequently, negative charges remain unshielded and, due to electrostatic repulsive forces, the mucin molecules open up and become transportable for the mucociliary escalator system (Quinton, 2010). Amelogenins are similarly soluble in acidic environments, and may possibly use a similar system to allow amelogenins to remain soluble intracellularly, and then to develop hydrophobic properties, critical for organizing crystal grown in the secretory stage of amelogenesis.

Another recognized role of bicarbonate is in the control of intracellular cyclic AMP level (cAMP). Cyclic AMP is a second messenger molecule, which plays a pivotal role in the regulation of a variety of cell functions. sAC is a source of cAMP and it is directly regulated by bicarbonate via two mechanisms: 1) it relieves substrate inhibition, and 2) elevates the V_{max} of sAC by facilitating active site closure (Rahman et al., 2013). In fact, because of the regulation of sAC by bicarbonate, it is considered as one of the cell’s pH sensors, which can be achieved by local changes of bicarbonate/CO_2 concentrations as discussed below in this review.

Finally, it is worth noting the antimicrobial properties of bicarbonate (Gutierrez-Huante, Martinez, Bustamante, Puente, & Sanchez, 2015). As an example, the airway surface liquid has a severely reduced bacterial killing capacity in cystic fibrosis (Pezzulo et al., 2012). This defect can be corrected by administration of $NaHCO_3$ suggesting that bicarbonate restores

the activity of antimicrobial peptides, such as lactoferrin and lysozyme, as shown in a piglet cystic fibrosis model (Pezzulo et al., 2012). Additionally, the direct antibiofilm activity of bicarbonate has been established by a number of observations (Gawande et al., 2008). Quorum sensing is a form of communication bacteria use to cooperatively build biofilm communities. Quorum sensing inhibitors can interfere with biofilm formation and increase the susceptibility of biofilms to antibiotics (Sun et al., 2013). Recent data have indicated that bicarbonate interferes with this system by inhibiting the production of the second messenger molecule 3',5'-cyclic diguanylic acid (c-di-GMP) in bacteria by inhibiting the enzyme responsible for its production (Koestler & Waters, 2014).

Present knowledge on the role of bicarbonate in controlling pH in ameloblasts

Bicarbonate ions secreted by ameloblasts neutralize protons that are produced during mineralization of the enamel suggesting a key role of bicarbonate as a buffer in tooth development (Bori et al., 2016; Josephsen et al., 2010; Lacruz, Nanci, Kurtz, et al., 2010; Lyaruu et al., 2008; Smith, 1998; Varga et al., 2015). In mouse incisors, maturation-stage enamel stained with pH indicators show acidic wide bands and neutral narrow stripes (Damkier et al., 2014; Josephsen et al., 2010). The zones correspond to two morphologically distinct ameloblast subtypes: smooth-ended ameloblasts with basal tight junctions are associated with the neutral regions while ruffle-ended ameloblasts with striated apical side and distal tight junctions can be seen at the acidic enamel. Smooth-ended ameloblasts cyclically turn into ruffle-ended ameloblasts and vice versa (Josephsen et al., 2010; Smith, 1998). This modulation process proceeds in a wavelike pattern from the start of the maturation zone towards the edge of the incisor resulting in transverse bands along the enamel. Applying ratiometric fluorometry and colorimetric pH-indicators, the enamel surface pH during maturation was found to cycle between pH 7.2 and 6.2 in smooth-ended and ruffle-ended ameloblasts, respectively (Damkier et al., 2014) The regulation of this cycle, which lasts approximately 8 hours in rats, is obviously critical for ameloblast maturation, is completely unknown.

Maturation-stage ameloblasts have been shown to express the bicarbonate/chloride exchanger, anion exchanger 2 (AE2), and the Cl^- channel, cystic fibrosis transmembrane conductance regulator (CFTR) (Bronckers et al., 2010; Bronckers et al., 2011; Bronckers, Lyaruu, Jansen, et al., 2009; Lacruz, Nanci, Kurtz, et al., 2010; Lacruz, Nanci, White, et al., 2010; Lacruz, Smith, Kurtz, Hubbard, & Paine, 2013; Lyaruu et al., 2008; Smith, Nanci, & Denbesten, 1993). Disruption of these transport proteins impairs ameloblast modulation. This finding suggests a role for extracellular pH in directing ameloblast modulation from smooth ended to ruffle ended cells at the maturation stage. Impaired modulation of ameloblast function may retard completion of the mineralization process and result in hypomineralized enamel, which easily erodes after tooth eruption.

The available data on acid/base control in the forming enamel are contradictory (Bronckers et al., 2012; Damkier et al., 2014; Lacruz, Brookes, et al., 2012). However, it is clear that ameloblasts must have sufficient molecular machinery to secrete bicarbonate ions into the enamel space to buffer the eight moles of protons liberated during one mole of

hydroxyapatite formation. Along with AE2 and CFTR, ameloblasts modulate changes in extracellular and intracellular pH through expression of electrogenic Na^+-HCO_3^- cotransporter 1 (NBCe1), Na^+-H^+ exchanger 1 (NHE1), carbonic anhydrase 2 and 6 (CA2, CA6), pendrin (SLC26A4), DRA (SLC26A3) and Pat1 (SLC26A6) (Damkier et al., 2014; Jalali et al., 2015; Josephsen et al., 2010; Lacruz, Brookes, et al., 2012; Lacruz et al., 2013). Until recently our knowledge about electrolyte transport by ameloblasts was based almost solely on immunohistochemistry, chemical composition analysis and expression studies in wild type and knockout mouse models.

The HAT-7 cell model to functionally study pH regulation by ameloblasts

To conduct functional studies on pH regulation in ameloblasts, we developed a HAT-7 ameloblast monolayer model (Bori et al., 2016). HAT-7 is a dental epithelial cell line originating from the cervical loop epithelium of a rat incisor (Kawano et al., 2002). Immunocytochemical studies reveal that HAT-7 cells exhibit several ameloblast characteristics, including the expression of ameloblastin and amelogenin (Kawano et al., 2002). Additionally, expression profiling has shown maturation-stage ameloblast marker proteins such as amelotin and kallikrein-4 (Klk4) are also expressed in these cells (Harada et al., 2006; Matsumoto, Harada, Saito, & Taniguchi, 2011; Yoshizaki et al., 2008; Zheng, Linthicum, DenBesten, & Zhang, 2013).

In our recent proof-of-concept work we demonstrated that a 2D *in vitro* model using HAT-7 cells, is suitable for functional investigations of pH regulation, mineral transport and tight-junction formation (Bori et al., 2016). We found that ameloblast-derived HAT-7 cells are able to form polarized confluent monolayers on permeable supports and develop measurable transepithelial electrical resistance. We also found expression of Cldn1, Cldn4, Cldn8 and Tjp1/Zo1, which indicates the presence of mature tight junctions. These are a prerequisite for vectorial electrolyte secretion by restricting free transepithelial ion movements while permitting passage of certain ions between the cells (Hou, 2014; Melvin, Yule, Shuttleworth, & Begenisich, 2005; Steward, Ishiguro, & Case, 2005). The data are also in accordance with previous studies (Amasheh et al., 2009; Colegio, Van Itallie, McCrea, Rahner, & Anderson, 2002; Lal-Nag & Morin, 2009) showing the expression of Cldn1, Cldn4 and Cldn8 in maturation-stage ameloblasts (Hata, Kawamoto, Kawai, & Yamamoto, 2010; Inai, Sengoku, Hirose, Iida, & Shibata, 2008). As previous studies show, ion transporters necessary for intracellular and extracellular pH regulation such as Slc9a1/Nhe1, Slc4a2/Ae2, Slc4a4/Nbce1, Slc26a4/pendrin and CFTR (Bronckers et al., 2011; Jalali et al., 2014; Lacruz et al., 2013) and the cytoplasmic Car2 isoform of carbonic anhydrases (Lacruz, Smith, Moffatt, et al., 2012; Reibring et al., 2014) are expressed by ameloblasts. We identified all of them in HAT-7 cells suggesting that this cell line is suitable as an experimental model for studying ameloblast acid/base transport.

HAT-7 cells are functionally polarized with high apical membrane CO_2 permeability and vigorous basolateral HCO_3^- uptake (Bori et al., 2016). This is similar to other HCO_3^--secreting epithelia, such as guinea-pig pancreatic duct (Ishiguro et al., 2000) and the human CFPAC pancreatic cell line (Rakonczay et al., 2006), and is consistent with the assumption that maturation-stage ameloblasts are equipped with transporters to secrete HCO_3^- to

neutralize the acidity generated during hydroxyapatite formation (Jalali et al., 2014; Lacruz et al., 2013; Smith, 1998). We also detected AE2 exchanger expression in HAT-7 cells by immunocytochemistry, and its function was confirmed in Cl^- substitution experiments by the inhibitory effect of 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) DIDS to block anion exchanger activity (Bori et al., 2016). AE2 is expressed at the basolateral membranes of most epithelial cells (Romero, Fulton, & Boron, 2004), possibly providing an important pathway for the accumulation of intracellular Cl^- against its electrochemical gradient (Demeter, Szucs, et al., 2009; Melvin et al., 2005). The basolateral location in HAT-7 cells is in line with the basolateral expression of AE2 in maturation ameloblasts (Lyaruu et al., 2008; Lyaruu et al., 2014).

The two most likely pathways for basolateral HCO_3^- uptake in ameloblasts are by Na^+-HCO_3^- cotransport, and by CO_2 diffusion into the cells with carbonic anhydrase catalyzing its conversion into HCO_3^- ions and protons (Jalali et al., 2014; Lacruz et al., 2013). In HAT-7 cells in the absence of HCO_3^-/CO_2, we identified a basolateral Na^+ dependent, amiloride sensitive proton extruding entity suggesting the presence of Na^+/H^+ exchanger activity at this membrane (Racz et al., 2017). This is consistent with the observation that the presence of a basolateral Na^+/H^+ exchanger, usually NHE1, is an almost universal feature of the epithelial cells of the gastrointestinal tract (Kiela, Xu, & Ghishan, 2006). We also found that the application of the membrane permeable carbonic anhydrase inhibitor acetazolamide partially inhibited the basolateral base flux in HAT-7 cells suggesting that this mechanism is present, and coupled to H^+ extrusion via a basolateral Na^+-H^+ exchanger such as NHE1 (Bori et al., 2016). Among the large number of different isoenzymes in the carbonic anhydrase gene family the dominant isoform in ameloblasts is Car2 (Lacruz, Smith, Moffatt, et al., 2012; Reibring et al., 2014), the isoform that we found to be expressed in HAT-7 cells (Bori et al., 2016).

Na^+-HCO_3^- co-transporters (NBCs) may also contribute to HCO_3^- uptake. In our most recent study the presence of a basolateral Na^+-HCO_3^- cotransporter was revealed in acid-loading experiments performed in the presence of HCO_3^-/CO_2 in functional microfluorometric experiments using specific inhibitors (Racz et al., 2017). These data are in line with our RT-PCR evidence for NBCe1 expression in HAT-7 cells (Bori et al., 2016) and also with reports of tissue staining of maturation ameloblasts (Jalali et al., 2015). The basolateral localization of NBCe1 in these cells is similar to that observed in secretory epithelia such as the pancreatic ducts and guinea-pig (Ishiguro et al., 2000) and also the salivary glands (Gresz et al., 2002).

As H_2DIDS and amiloride blocked most of the basolateral uptake of HCO_3^- through NBCe1 and NHE1 inhibition, we were able to apply simple fluorometric methods to test whether HAT-7 cells are able to achieve vectorial HCO_3^- secretion. Figure 2 shows the principle of such experiments. HCO_3^- entry across the basolateral membrane is closely coupled to HCO_3^- efflux across the luminal membrane in various secretory epithelia (Demeter, Hegyesi, et al., 2009; Hegyi, Gray, & Argent, 2003; Ishiguro, Steward, Lindsay, & Case, 1996; Szucs et al., 2006). Thus, blocking HCO_3^- entry by transport inhibitors, the continuing efflux of HCO_3^- across the luminal membrane results in a fall in pH_i. Therefore, the initial rate of fall in pH_i can serve as a measure of instantaneous HCO_3^- efflux across the

apical membrane. Indeed, applying a combination of NHE1/SLC9A1 and NBCe1/SLC4A4 inhibitors to HAT-7 cells, we observed the acidification due to apical HCO_3^- secretion which was accelerated when the cells were stimulated (Bori et al., 2016; Racz et al., 2017) (Figure 2).

When we add all these data together, a clear hypothetical picture emerges regarding the transporters and channels involved in the transport processes leading to neutralization of protons at the luminal (apical) surface of ameloblasts during the maturation phase of amelogenesis (Figure 3). Somewhat controversial is the role of is the vacuolar H^+-ATPase (v- H^+- ATPase at the apical border of ameloblasts. v-H^+-ATPase has been localized at the apical border of ruffle-ended ameloblasts, and an number of authors have suggested that similar to bone, this v-H^+ ATPase functions to pump protons into the forming enamel matrix (Damkier et al., 2014; Josephsen et al., 2010; Lin, Nakamura, Noda, & Ozawa, 1994). However, Bronckers et al used immunostaining to show that v-H^+-ATPase in maturation-stage ameloblasts is not the typical osteoclast-type plasma membrane associated proton pump which acidifies the extracellular space, but rather a v-H^+-ATPase potentially involved in intracellular acidification (Bronckers et al., 2012). Therefore, it is also possible that this v-H^+-ATPase in ameloblasts is involved in endosome formation and protein uptake by ruffle ended ameloblasts. To identify the role of this pump, molecular physiological methods are needed using cellular ameloblast models, such as the HAT-7 model.

Chloride ions are coupled to HCO_3^- transport in multiple ways in secretory epithelia (Demeter, Hegyesi, et al., 2009), and they are most likely also essential in pH modulation during enamel formation (Bronckers, 2017). Additionally, a strong positive correlation between calcium and chloride contents are observed during enamel maturation, as suboptimal Cl^- content results in hypomineralisation (Bronckers, Lyaruu, Guo, et al., 2015). Both in CFTR-null and in AE2-null mice the enamel is severely hypomineralized (Bronckers, Lyaruu, Guo, et al., 2015; Sui, Boyd, & Wright, 2003). Very little is known about Cl^- accumulation, although ameloblasts must accrue Cl^- intracellularly in order to secrete it through the luminal membrane. This assumption indicates that Na^+-K^+-Cl^- cotransporters (NKCCs) may also be required for amelogenesis, although no direct evidence of NKCC expression in ameloblasts was available (Bronckers, Lyaruu, Jalali, et al., 2015) until our studies. NKCCs are electroneutral symporters that move Na^+, K^+ and Cl^- ions into the cell by secondary active transport, driven by the Na^+-K^+-ATPase.

In our most recent study in HAT-7 cells, we observed a NKCC1 inhibitor bumetanide-sensitive decrease in pH_i during NH_4Cl exposure, a standard microfluorometric protocol to identify NKCC in living cells (Paulais & Turner, 1992b). According to these data, the NKCC1 cotransporter is an important contributor to Cl^- uptake across the basolateral membrane of HAT-7 ameloblast cells (Racz et al., 2017), similar to other secretory epithelial models including the pancreatic ductal cell lines Capan-1 and HPAF (Demeter, Hegyesi, et al., 2009; Szucs et al., 2006) and salivary acinar cell line Par-C10 (Demeter, Szucs, et al., 2009), in which Cl^- secretion is largely dependent on basolateral NKCC1 activity (Melvin et al., 2005; Paulais & Turner, 1992a). Our study represents the first functional evidence that NKCC1 could have a role in Cl^- accumulation in ameloblasts.

Taken together, these functional studies show an intricate pattern of bicarbonate regulation in ameloblasts. The function of bicarbonate transport likely includes neutralizing the acidified mineralizing enamel matrix, and coordinating with other co-transporters to direct calcium and phosphate across the apical boundary of ameloblasts to form the mineralized enamel matrix.

The unknown regulatory factors affecting ameloblast function to be studied

The hormonal and neuronal regulatory factors guiding ameloblast function and differentiation are largely unknown. The only exception is the control of steroid hormone receptors. When the expression pattern of mRNAs extracted from microdissected enamel organs of adult rats were studied at different stages of ameloblast differentiation, the level of retinoid receptor RXRα was found to be the highest, similar to the vitamin D receptor (VDR), but other steroid receptors such as androgen receptor (AR), estrogen receptor (ER)-α and progesterone receptor were also detected in expression in maturation-stage ameloblasts (Houari, Loiodice, Jedeon, Berdal, & Babajko, 2016). Immunofluorescent analysis of VDR, ERα and AR confirmed their presence mainly in maturation- stage ameloblasts. These data provide evidence that ameloblasts express a specific combination of hormonal receptors depending on their developmental stage (Houari et al., 2016). A number of studies have been conducted not only *in vivo* in experimental animals, but also *in vitro* using the HAT-7 cell line, to investigate the effect of endocrine-disrupting chemicals such as Bisphenol A (BPA) that interfere with the steroid axis, which can affect amelogenesis (Jedeon et al., 2014). This leads to enamel hypomineralization, similar to that of human molar incisor hypomineralization (MIH), a recently described enamel disease (Babajko, Jedeon, Houari, Loiodice, & Berdal, 2017). Rats exposed to daily BPA developed enamel hypomineralization similar to (MIH) (Jedeon et al., 2014). Both androgenic, estrogenic and BPA exposure modulate ameloblast cell proliferation in vivo in rats and in vitro in HAT-7 cells, and affect expression of the ion transporter SLC26A4/pendrin and KLK4, both of which are regarded as important for enamel maturation (Babajko et al., 2017; Jedeon et al., 2014; Jedeon et al., 2016). Clearly there are many unanswered questions as to the role of steroid receptors in enamel formation. The use of the HAT-7 cell model allows the study of the effects of these agents at the cellular level.

Though these proof-of-concept studies clearly indicate the significance of hormonal regulation in ameloblast differentiation and enamel formation, obviously steroid hormones cannot regulate the short term, approximately 8 hour switches in maturation ameloblasts cycling (Damkier et al., 2014; Josephsen et al., 2010; Smith, 1998), because their action is long-term, primarily affecting the expression of functional proteins, but not on their activity. Most likely candidates for such a regulatory role are various G-protein coupled receptors and pH sensors, which are known to have similar functions in various secretory and absorbing epithelia.

One of these potential candidate pathways is controlled by extracellular ATP, a bioactive molecule acting through purinergic receptors to raise intracellular Ca^{2+} (Novak, 2011; Schwiebert & Zsembery, 2003). ATP stimulating purinergic receptors (P2X inotropic and/or P2Y metabotropic) increase cytosolic Ca^{2+} concentrations, which in turn activate Ca^{2+-}

dependent Cl^- channels. Chloride efflux lowers its intracellular concentrations establishing a higher driving force for bicarbonate secretion through the Cl^-/HCO_3^- exchanger (Zsembery, Strazzabosco, & Graf, 2000). Furthermore, Ca^{2+}-dependent Cl^- channels become highly permeable to bicarbonate at high cytosolic Ca^{2+} concentrations (Jung et al., 2013), providing a direct efflux pathway for HCO3-. Therefore, one could speculate that extracellular ATP and Ca^{2+}-dependent Cl^- channels could be involved in pH sensing and/or regulation. In HAT-7 cells ATP stimulated bicarbonate transport when applied to the basolateral but not to the apical side (Bori et al., 2016). In other secretory epithelia the divergent actions of apical and basolateral ATP on bicarbonate secretion are well established (Baggaley, McLarnon, Demeter, Varga, & Bruce, 2007; Demeter, Hegyesi, et al., 2009; Schwiebert & Zsembery, 2003; Szucs et al., 2006). Our studies with HAT-7 cells raises the possibility that ATP could be an important extracellular regulator of ameloblast function, acting via calcium-activated chloride channels, which are expressed in maturation-stage ameloblasts (Lacruz, Smith, Bringas, et al., 2012). Indeed, a similar function has already been well established in other exocrine organs such as the salivary glands and the pancreas (Kordas et al., 2004; Novak, 2011; Szucs et al., 2006).

The extracellular calcium sensing receptor (CaR) plays a key role in the calcium homeostatic system and it is widely expressed in tissues involved in calcium metabolism (Riccardi & Valenti, 2016). It has been identified in many tissues, including the kidney (Riccardi & Valenti, 2016; Ward & Riccardi, 2002) and the pancreas (Racz et al., 2002). As calcium is also a critical component of tooth enamel, dentin, and the surrounding extracellular matrix, Ca^{2+} also may regulate tooth formation through its receptor by similar G-protein coupled receptor activation (Mathias et al., 2001). Indeed, CaR has been shown to be expressed in developing teeth, particularly in ameloblasts (Mathias et al., 2001). Furthermore, in an immortalized ameloblast-like cell line (PABSo-E) CaR was shown to be expressed, and increases in extracellular Ca^{2+} concentration stimulated intracellular Ca^{2+} signals. These early study suggests that the CaR is expressed in developing teeth and regulate intracellular signal pathways (Mathias et al., 2001). However, the resulting changes in intracellular and transcellular ion movements, particularly the regulation of Ca^{2+} and bicarbonate transports, have to be addressed by future investigations.

Even less is known about the potential function of other G-protein receptor activators in amelogenesis, although some pieces of evidence support such a role of cholecystokinin (CCK), gastrin releasing peptide (GRP), pituitary adenylate cyclase activating polypeptide (PACAP) and neuropeptide Y (NPY).

CCK is an important regulatory peptide both in the gastrointestinal tract and the brain. Its signalling is coupled to Gq activation leading to phospholipase C and calcium signal increases, resulting in PKC-induced ERK activations (Racz et al., 2006). This causes an increase of a wide range of activities including pancreatic secretion (Szalmay et al., 2001) and intestinal motor function (Varga, Balint, Burghardt, & D'Amato, 2004). In gene expression, microdissection studies the most highly up-regulated gene transcript in the genome-wide analysis in ameloblasts was CCK. Furthermore, a twenty-fold increase was found in its expression between early and late-maturation, as measured by qPCR (Lacruz et al., 2011) without any functional data.

GRP is considered an important gastrointestinal regulatory peptide and also a cancer-promoting growth factor. Its receptor is a G protein-coupled receptor as well. GRP regulates the mobilization of calcium from intracellular stores (Burghardt et al., 2001), regulating a number of secretory and motor functions in the gut (Milusheva et al., 1998; Szalmay et al., 2001). A recent study to identify the function of GRP during incisor development by a gain-of-function analysis was done by overexpressing GRP by renal capsule transplantation, and resulted in increased enamel and dentin thickness (Lee, Jin, Kim, Lee, & Jung, 2015). Additionally, in GRP-overexpressed incisor organ cultures morphological changes were also detected but no molecular or cell physiological experiments were performed (Lee et al., 2015).

PACAP is a pleiotropic neuropeptide with widespread distribution. PACAP-immunoreactive fibers have been found in the tooth-related structures. In PACAP-deficient mice serious structural changes have been observed in both the dentin and the enamel of the molars and incisors (Sandor et al., 2014; Sandor et al., 2016). Again, no functional studies have been performed to seek for the mechanism of action of this peptide. Likewise, the expression of NPY, another neuropeptide was identified in ameloblasts with down-regulation in late maturation relative to early maturation in rat incisors. The significance of this is not known (Lacruz et al., 2011). Some other regulatory peptides such as somatostatin, PYY, galanin, VIP, CGRP and substance P, which also function by activating G-protein coupled receptors, are also possible candidates as regulators of the epithelial function process of amelogenesis (Monteiro & Batterham, 2017), but no data are available about them. Very clearly, the development functional cellular systems are needed to identify their function in ion transport processes leading to proper enamel formation.

The unknown pH sensors of ameloblasts to be studied

It is not known how ameloblasts sense the changes in pH in the extracellular matrix; an essential function required to direct the ameloblasts to neutralize the eight moles of protons liberated by the formation of a single hydroxyapatite molecule. Over the past few years, a number of candidate molecular pH sensors have been identified primarily in research related to renal function. The list of these sensors include acid/alkali-sensing receptors (GPR4, InsR-RR), kinases (Pyk2, ErbB1/2), pH-sensitive ion channels (ASICs, TASK, ROMK), and the bicarbonate-stimulated adenylyl cyclase (sAC) (Brown & Wagner, 2012). Some acid-sensing mechanisms in other tissues, such as CAII-PDK2L1 in taste buds, might also have similar roles to play in the pH regulation (Brown & Wagner, 2012). The function of a variety of additional membrane channels and transporters may also be altered by pH changes both within and outside the cell, and the expression and function of cellular metabolic enzymes may also modify acid-base control (Brown & Wagner, 2012). Thus, it is possible that there is no single master pH sensor to control pH cycling during amelogenesis, rather, the ameloblasts might be equipped with a battery of molecules that scan the cell environment to regulate and maintain acid-base homeostasis both within and around the cells.

However, recent data indicate that there is one very strong candidate as a master pH sensor in ameloblasts. GPR68 encodes a proton-sensing G-protein-coupled receptor with sensitivity

in the pH range that is relevant to the developing enamel matrix during amelogenesis. It is a recognized pH sensor in osteoblasts and osteocytes sensing pH between 7.8 (completely inactive) and 6.8 (fully active) (Ludwig et al., 2003). Its activation results in generation of inositol trisphosphate formation and calcium release from intracellular stores (Ludwig et al., 2003). Importantly, homozygous variants in the GPR68 protein were identified with amelogenesis imperfecta in three independent human families. Each of these homozygous variants was predicted to result in loss of function phenotype. Immunohistochemical studies of rat mandibles confirmed localization of GPR68 in the enamel organ at all stages of amelogenesis. In Caco-2 intestinal cells the overexpression of GPR68 in Caco-2 cells leads increased barrier formation upon acidification of the environment (de Valliere et al., 2015), a function that might be related to tight junctional changes during maturation ameloblast cycling. Additionally, GPR68 signalling regulates the expression of Na^+/H^+ antiporters and H^+-ATPase transporters in epithelial cells (Mohebbi et al., 2012). Collectively, these data suggest that GPR68 may act as a proton sensor in enamel formation, but obviously, further functional studies are needed using ameloblasts and ameloblast originated cells, such as HAT-7, to confirm the exact molecular mechanisms of its pH regulation.

Finally, another strong potential candidate as pH sensor is the sAC that we have already introduced above when discussing the non-buffering functions of bicarbonate. Forskolin, which activates the cAMP/protein kinase A pathway, strongly potentiated the effect of ATP on vectorial bicarbonate secretion in HAT-7 cells (Bori et al., 2016). Activation of CFTR requires phosphorylation by protein kinase A. Because channel phosphorylation by PKA is mandatory for channel activity, the CFTR channel is frequently defined as a cAMP-activated channel (Moran, 2017). This channel was highly expressed in maturation-stage ameloblasts (Bronckers, Lyaruu, Guo, et al., 2015; Lacruz et al., 2013). As previously mentioned, sAC is a source of cAMP and it is directly regulated by bicarbonate (Rahman et al., 2013). In fact, sAC is considered as one of the pH sensors, which can be achieved by local changes of bicarbonate/CO2 concentrations. The sAC was first identified in kidney intercalated cells where it colocalizes extensively with the V-ATPase (Clague, Urbe, Aniento, & Gruenberg, 1994). It generates cAMP in response to an increase in intracellular bicarbonate, entering the cell either luminally or basolaterally. This cAMP signal leads to an accumulation of V-ATPase at the apical surface of intercalated cells, possibly by phosphorylation of the V-ATPase (Brown & Wagner, 2012). Increased intracellular bicarbonate could lead the increased activity of apical V-ATPase, while acidification may lead to the opposite effect (Schmid, Meili, & Salathe, 2014). Whether such a pH regulatory mechanism exists in ameloblasts is presently unknown but it seems to be a worthwhile direction to explore as a mechanism to regulate intracellular and luminal pH regulation during amelogenesis.

Significance of and potential applications of gained knowledge by molecular physiology studies to understand amelogenesis

The authors trust that all of the above described research and hypotheses could serve much more than simply lead to a better understanding of the molecular physiology of

amelogenesis. An example, for possible applications, is to model molecular events during dental fluorosis in ameloblast-originated cells.

Enamel formation can be altered in response to genetic alterations, as is seen in amelogenesis imperfecta, or epigenetics, such as occurs in the presence of high concentrations of fluoride to result in enamel fluorosis (Bronckers, Lyaruu, & DenBesten, 2009; P. K. Denbesten, Crenshaw, & Wilson, 1985; Smith et al., 1993). In the case of fluoride, the exact mechanism(s) is (are) still not fully understood, and multiple factors might contribute to this phenomenon. For example, fluoride may affect ion secretion by ameloblasts, which could alter pH regulation by ameloblasts. To test this possibility, we investigated whether fluoride exposure modulates transcellular HCO_3^- secretion in HAT-7 cells. Our data clearly showed that acute exposure to a wide range of fluoride concentrations caused no change in the rate of acidification of the cells when basolateral HCO_3^- uptake was blocked (Racz et al., 2017), indicating that fluoride has no direct acute inhibitory effect on HCO_3^- secretion, which we consider to be a crucial requirement for mineralization (Varga et al., 2015). Interestingly, we found that instead tight junction formation was reduced in the presence of 1 mM fluoride, a concentration that resulted in no, or very little, change in HAT-7 cell viability. These observations of a minimal effect of 1mM fluoride on cell viability are in line with recent reports on HAT-7 cells by other investigators (Zhang et al., 2016) and with studies of the mouse LS8 ameloblast cell line (Kubota et al., 2005; Sharma, Tsuchiya, & Bartlett, 2008; Zhang, Li, Chi, Chen, & Denbesten, 2007; Zhang, Yan, Li, & DenBesten, 2006) showing that ameloblast survival is not seriously affected up to millimolar fluoride concentrations. However, further increases in the fluoride dose results in rapid deterioration over a very narrow concentration range. Tight junctions are critical for bicarbonate transport by ameloblasts to the extracellular matrix, so it is possible fluoride related changes in tight junction formation could also indirectly alter bicarbonate transport by ameloblasts.

In conclusion, amelogenesis, which results in the formation of the highly mineralized enamel matrix, is a unique system, unlike any other in the human body, but showing many similarities to other epithelial secretory organs. The authors believe that there is an essential need to develop functional models to advance studies of amelogenesis at the molecular physiology and pathophysiology levels. Our recently developed model using HAT-7 rat ameloblast cells enables us to functionally study key elements of enamel formation. We suggest that in the future the HAT-7 2D system, and other similar cellular models, will be used not only to study the ion transport processes during amelogenesis, but also the regulation of the cycling processes in maturation ameloblasts, and the pH sensory mechanisms required for normal enamel formation.

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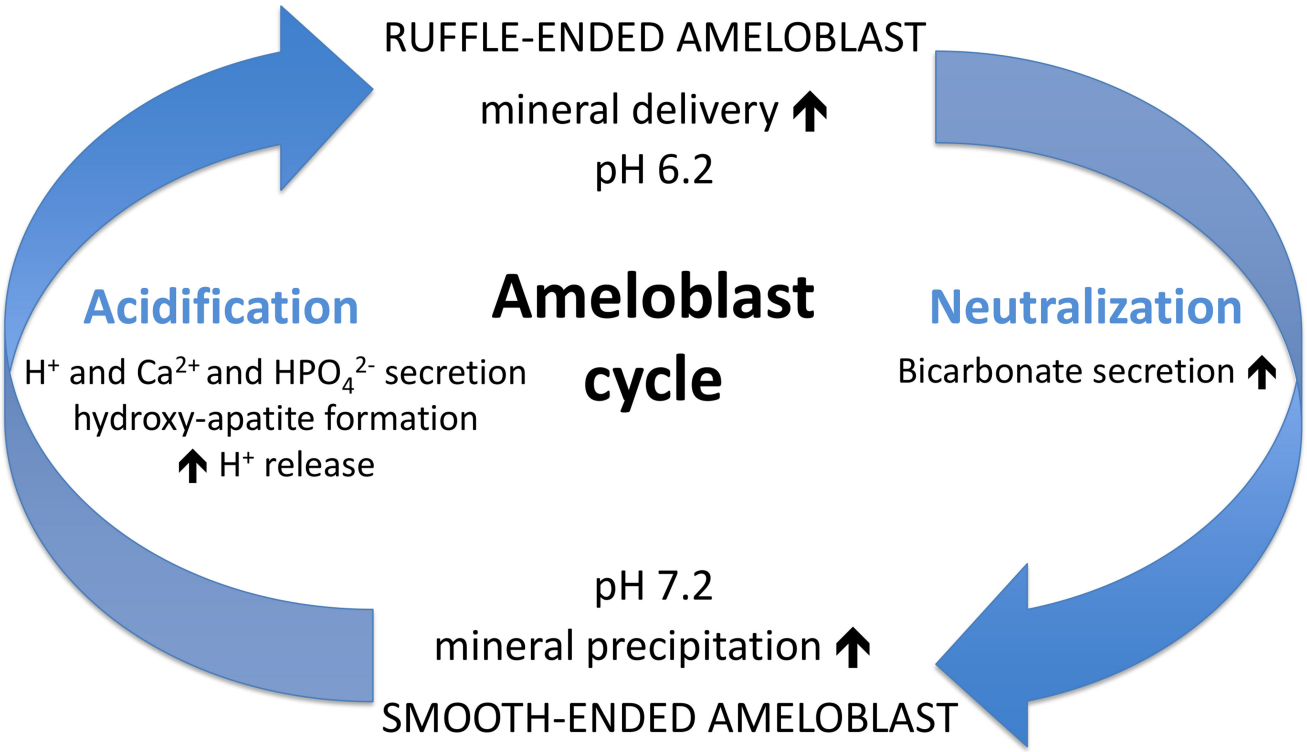

Figure 1. pH cycle in the luminal surface of ameloblasts during the maturation phase of amelogenesis

Cells cycle between smooth-ended and ruffle-ended stages multiple times. This cycling is accompanied by luminal pH changes between approximately 6.2 and 7.2. The primary driver of acidification is the release of protons liberated during hydroxyl-apatite formation. Acidification is counterbalanced then by the buffering effect of bicarbonate.

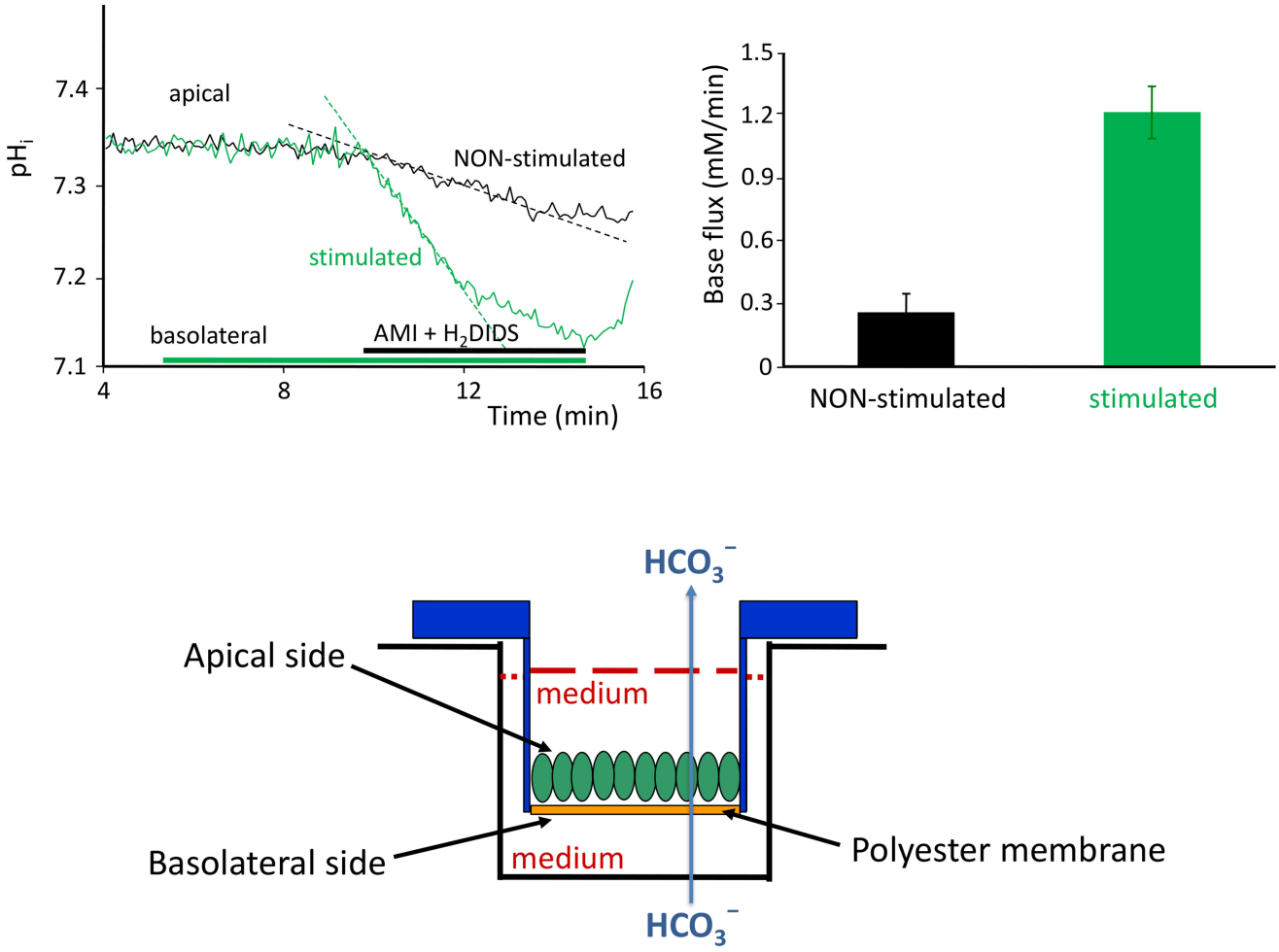

Figure 2. Schematic representation of the 2D HAT-7 cellular model, and the method for assessing vectorial, basolateral-to-apical bicarbonate transport in this system

HAT-7 cells, grown on Transwell support, form a tight epithelium, and are polarized to show distinct apical and basolateral surfaces. Vectorial bicarbonate transport is tested in this system by the pH drop method using microfluorometry. As transporters involved at the basolateral side involved in bicarbonate uptake and proton extrusion are blocked by specific inhibitors H_2DIDS and amiloride, respectively, and apical bicarbonate loss is accelerated by secretagogue stimulation, a sudden decrease of intracellular pH can be detected. By appropriate calibrations, the apical base flux, ie bicarbonate extrusion, can be quantitatively estimated.

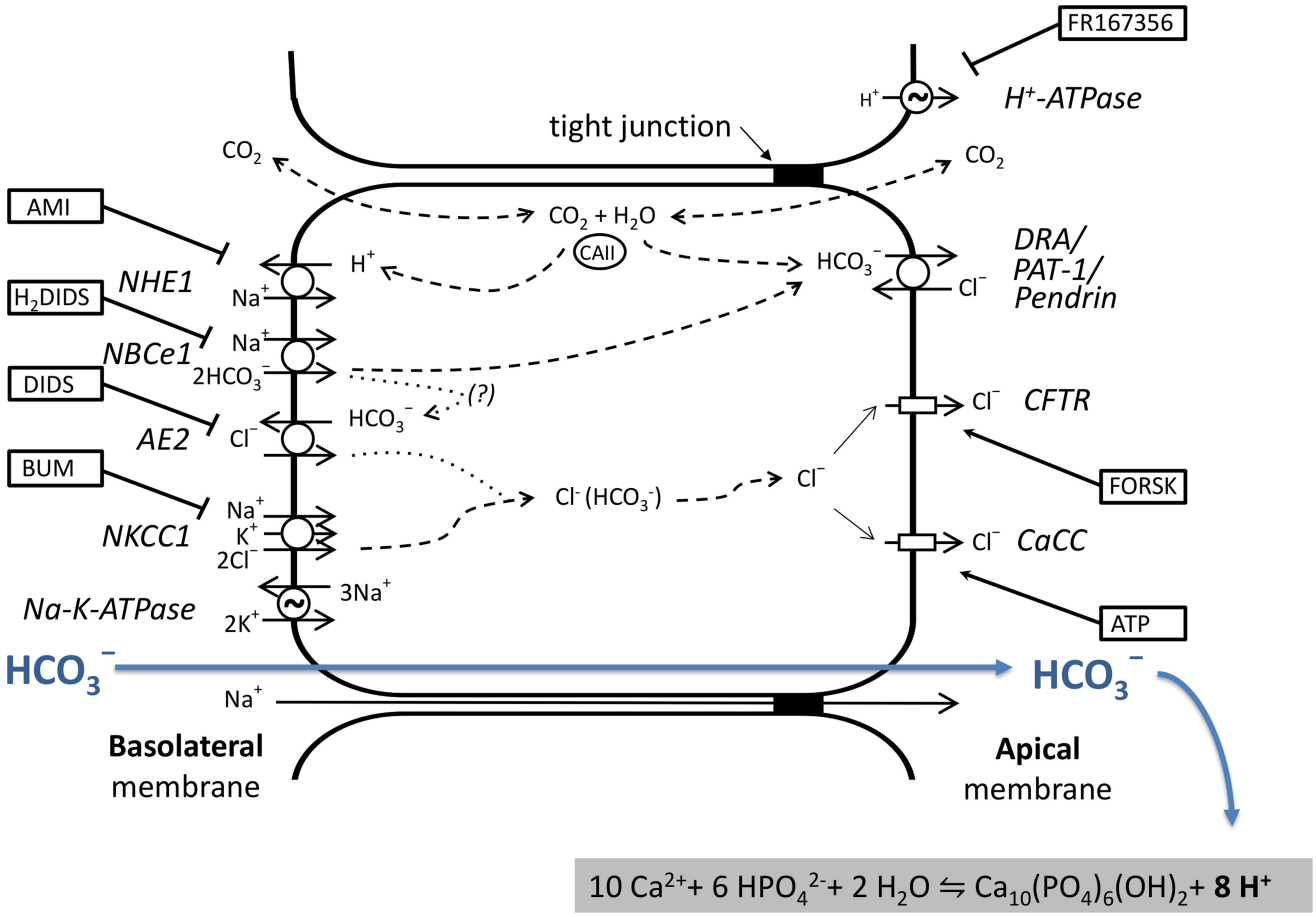

Figure 3. Schematic drawing of the proposed mechanism of vectorial bicarbonate transport of HAT-7 cells, showing the major ion channels and transporters involved

For most transporters, specific inhibitors or activators were used, as shown on the figure, to test their involvement in intracellular pH control, bicarbonate and Cl- secretion. Note that data about the H^+-ATPase are shown based on in vivo studies using its specific inhibitor FR167356, but no molecular physiology studies were applied to test its direct involvement in maturation-associated ameloblast cell transport processes.