



Multiple Calcium Export Exchangers and Pumps Are a Prominent Feature of Enamel Organ Cells

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OPEN ACCESS

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Specialty section:

This article was submitted to
Craniofacial Biology and Dental
Research,
a section of the journal
Frontiers in Physiology

Received: 13 March 2017

Accepted: 08 May 2017

Published: 23 May 2017

Citation:

Robertson SYT, Wen X, Yin K, Chen J,
Smith CE and Paine ML (2017)
Multiple Calcium Export Exchangers
and Pumps Are a Prominent Feature
of Enamel Organ Cells.
Front. Physiol. 8:336.
doi: 10.3389/fphys.2017.00336

Calcium export is a key function for the enamel organ during all stages of amelogenesis. Expression of a number of ATPase calcium transporting, plasma membrane genes (ATP2B1-4/PMCA1-4), solute carrier SLC8A genes (sodium/calcium exchanger or NCX1-3), and SLC24A gene family members (sodium/potassium/calcium exchanger or NCKX1-6) have been investigated in the developing enamel organ in earlier studies. This paper reviews the calcium export pathways that have been described and adds novel insights to the spatiotemporal expression patterns of PMCA1, PMCA4, and NCKX3 during amelogenesis. New data are presented to show the mRNA expression profiles for the four Atp2b1-4 gene family members (PMCA1-4) in secretory-stage and maturation-stage rat enamel organs. These data are compared to expression profiles for all Slc8a and Slc24a gene family members. PMCA1, PMCA4, and NCKX3 immunolocalization data is also presented. Gene expression profiles quantitated by real time PCR show that: (1) PMCA1, 3, and 4, and NCKX3 are most highly expressed during secretory-stage amelogenesis; (2) NCX1 and 3, and NCKX6 are expressed during secretory and maturation stages; (3) NCKX4 is most highly expressed during maturation-stage amelogenesis; and (4) expression levels of PMCA2, NCX2, NCKX1, NCKX2, and NCKX5 are negligible throughout amelogenesis. In the enamel organ PMCA1 localizes to the basolateral membrane of both secretory and maturation ameloblasts; PMCA4 expression is seen in the basolateral membrane of secretory and maturation ameloblasts, and also cells of the stratum intermedium and papillary layer; while NCKX3 expression is limited to Tomes' processes, and the apical membrane of maturation-stage ameloblasts. These new findings are discussed in the perspective of data already present in the literature, and highlight the multiplicity of calcium export systems in the enamel organ needed to regulate biomineralization.

Keywords: amelogenesis, biomineralization, calcium channels, calcium exchangers, calcium pumps

INTRODUCTION

Enamel is the hardest and most calcified tissue in mammals, and understanding enamel formation is crucial for developing strategies to repair or regenerate it (Smith, 1998; Hubbard, 2000; Lacruz et al., 2013). Amelogenesis, the process of enamel development, can be divided into the secretory and maturation stages with a brief pre-secretory stage before the secretory stage and a transition stage between the secretory and maturation stages. Epithelial-derived enamel-forming cells (ameloblasts) differentiate from the inner enamel epithelium (IEE) during the pre-secretory stage (Orrenius et al., 2015). These ameloblasts are highly polarized with an apical end that faces the enamel area and a basal end that faces the blood circulation. During the secretory stage, ameloblasts migrate away from the dentin while synthesizing and secreting enamel matrix proteins (EMPs) such as amelogenin, ameloblastin, and enamelin into the enamel area from Tomes' processes at their apical ends. These EMPs serve as a scaffold for the orientation and elongation of enamel hydroxyapatite (Hap) crystals (Smith, 1998). Each enamel rod follows a single ameloblast's Tomes' process with the interrod following the border of the cell, giving enamel its characteristic rod-interrod pattern (Skobe, 2006; Hu et al., 2007). There is a massive shift in gene expression during the transition stage, when approximately 25% of ameloblasts undergo apoptosis, after which another 25% undergo apoptosis throughout the following stages of amelogenesis (Tsuchiya et al., 2009). During the maturation stage, the ameloblasts undergo cyclical changes between ruffle-ended (RA) and smooth-ended (SA) morphology (Smith, 1998; Lacruz et al., 2013). Maturation-stage ameloblasts become specialized for ion transport and resorptive activities, which includes the secretion of the protease KLK4 to aid in the degradation of EMPs that are subsequently removed through endocytosis (Smith, 1998; Lacruz et al., 2012a, 2013). The continuously growing incisor of mice makes it a good model for studying the chronological progression of amelogenesis. While general concepts of ion transport throughout amelogenesis have been well-studied and discussed elsewhere (Arquitt et al., 2002; Paine et al., 2007; Lyaru et al., 2008; Bronckers et al., 2010, 2015; Josephsen et al., 2010; Yin et al., 2015), and in particular the transcellular calcium ion (Ca^{2+}) transport (reviewed in Nurbaeva et al., 2015b), in this paper we focus primarily on Ca^{2+} export.

Overview—Calcium Transport

In general high intracellular concentrations of calcium (Ca^{2+}) catalyze cell death signaling cascades, so cells maintain a gradient of $\sim 10^{-3}$ M Ca^{2+} concentration outside the cell, in the mitochondria, and in the endoplasmic reticulum (ER) where Ca^{2+} is stored; while in the cytoplasm, the concentration is $\sim 10^{-7}$ M (Brini and Carafoli, 2011). The plasma membrane contains a variety of Ca^{2+} channels that transiently open to allow Ca^{2+} influx in response to plasma membrane voltage changes, ligand-receptor interaction, or emptying Ca^{2+} stores of the ER and mitochondria (Brini and Carafoli, 2011). Calcium is removed from the cytoplasm through a number of mechanisms including the SERCA pump that replenishes ER stores, the mitochondrial

Ca^{2+} uniporter, that replenishes mitochondrial stores, the plasma membrane low-affinity high capacity $\text{Na}^+/\text{Ca}^{2+}$ exchanger proteins (NCX), the $\text{Na}^+/\text{Ca}^{2+}$ K^+ exchanger proteins (NCKX), and the high-affinity low-capacity plasma membrane Ca^{2+} -ATPase (PMCA) pump proteins (Berridge et al., 2003; Brini and Carafoli, 2011; Hu et al., 2012; Bronckers et al., 2015).

Calcium (Ca^{2+}) transport is crucial to understand the process of amelogenesis because not only is unbound Ca^{2+} a major component of hydroxyapatite (Hap), Ca^{2+} can also act as a major signaling molecule capable of regulating cell processes in eukaryotic cells such as cell division, cell attachment, motility, survival, differentiation as well as gene expression (Hubbard, 1996; Blair et al., 2011).

Calcium Extrusion

The SLC8A (sodium/calcium exchangers or NCX), SLC24A (potassium-dependent sodium/calcium exchangers or NCKX), and ATP2B (ATPase plasma membrane Ca^{2+} transporting pumps or PMCA pumps) gene families of Ca^{2+} transporters mediate Ca^{2+} extrusion in most cell types (Brini and Carafoli, 2011), and proteins from all three of these families have been reported in enamel organ cells (Sasaki and Garant, 1986; Borke et al., 1995; Zaki et al., 1996; Okumura et al., 2010; Hu et al., 2012; Wang et al., 2014). The SLC8A gene family has 3 members (NCX1-3) and all have a generally accepted stoichiometry of the extrusion of 1 Ca^{2+} in exchange for the intrusion of 3 Na^+ (Brini and Carafoli, 2011), while the SLC24A gene family has 5 members (NCKX1-5) and extrudes 1 Ca^{2+} and 1 K^+ in exchange for 4 Na^+ , typically against the Ca^{2+} gradient; however, the directionality both NCX and NCKX exchangers can be reversed depending on the Na^+ and Ca^{2+} gradients (Jalloul et al., 2016b; Zhekova et al., 2016). SLC8A and SLC24A gene families are electrogenic because there is a translocation of net charge across the plasma membrane, have a low Ca^{2+} affinity, and are capable of transporting Ca^{2+} in bulk rapidly across the plasma membrane (Brini, 2009). They are reversible but in ameloblasts they likely operate in extruding Ca^{2+} from the cytoplasm facilitated by transport of Na^+ and K^+ down their gradients (Brini and Carafoli, 2011; Hu et al., 2012). PMCA pumps/proteins have 4 members (PMCA1-4, coded by genes ATP2B1-4) and are part of a larger family of genes, called P-type primary ion transport ATPases, that catalyze the auto-phosphorylation of a conserved aspartyl residue within the pump from ATP (Palmgren and Nissen, 2011).

Calcium Extrusion—SLC8A and SLC24A Gene Products

The SLC8A and SLC24A families are primarily expressed in excitable tissues such as muscle and heart, as their rapid bulk transport of Ca^{2+} is important in, for example, muscle and heart contraction (Brini and Carafoli, 2011). The SLC8A/NCX and SLC24A/NCKX transporters are $\text{Na}^+/\text{Ca}^{2+}$ exchangers and can be either K^+ -dependent (NCKX) or K^+ -independent (NCX) (Shumilina et al., 2010).

NCX1 is expressed in heart, brain, bladder, kidney, and cells of the enamel organ; NCX2 is expressed in brain and skeletal muscle; and NCX3 is expressed in brain, skeletal muscle and

cells of the enamel organ (Lytton, 2007; Okumura et al., 2010; Lacruz et al., 2012b; Sharma and O'halloran, 2014). Okumura et al. demonstrated NCX1 and NCX3 expression at the apical pole of both secretory and maturation ameloblasts, and expression of NCX1 was also observed in cells of the stratum intermedium and papillary layer (Okumura et al., 2010). In addition, protein levels of NCX1 and NCX3 throughout amelogenesis remained relatively unchanged (Okumura et al., 2010). Using real-time PCR, Lacruz et al. confirmed that the mRNA levels of both NCX1 and NCX3 did not significantly change from secretory- to maturation-stage enamel organ cells (Lacruz et al., 2012b).

NCKX1 is expressed primarily in retinal rod photoreceptors and platelets (Schnetkamp, 2004; Lytton, 2007). NCKX2 is expressed in cone photoreceptors and is involved in mouse motor learning and memory (Schnetkamp, 2004; Lee et al., 2009, 2013), and NCKX3 is expressed in the brain and the kidneys (Schnetkamp, 2004; Lee et al., 2009) though it is expressed in the kidneys at higher levels in female mice than in male mice (Lee et al., 2009). NCKX3 is also highly expressed in the human endometrium during the menstrual cycle, where its expression is partially regulated by the steroid hormone 17 β -estradiol (Yang et al., 2011). NCKX4 is expressed in olfactory neurons (Stephan et al., 2011), and also in the maturation-stage ameloblasts (Hu et al., 2012). NCKX5 is expressed in skin melanocytes, retinal epithelium, and brain (Schnetkamp, 2004; Lytton, 2007; Sharma and O'halloran, 2014; Jalloul et al., 2016a,b). NCKX6/NCLX was originally considered a member of the NCKX family but is now considered part of the Ca²⁺ cation (CCX) exchanger branch (Cai and Lytton, 2004; Sharma and O'halloran, 2014) as a mitochondrial membrane Ca²⁺, Li⁺/Na⁺ exchanger with a wide tissue distribution (Schnetkamp, 2004; Lytton, 2007; Sharma and O'halloran, 2014).

Calcium Extrusion—ATP2B Gene Products

The ATPase plasma membrane Ca²⁺ transporting (or PMCA) gene family is postulated to be involved in Ca²⁺ homeostasis, as it has a high affinity for Ca²⁺ but cannot transport Ca²⁺ as rapidly as either the NCX or NCKX transporters (Brini and Carafoli, 2011). The PMCA family is part of the superfamily of P-type ATPase pumps that form a stable phosphorylated intermediate as it hydrolyzes one molecule of ATP for each Ca²⁺ transported (Strehler and Zacharias, 2001; Cai and Lytton, 2004). The phosphorylated enzyme intermediate of the P-type ATPases, which include the SERCA family of transporters in the ER membrane (Giacomello et al., 2013), occurs between γ -phosphate of a hydrolyzed ATP with a D-residue in a highly conserved region of the ATPase pump (Brini and Carafoli, 2011).

PMCA1 is expressed in most tissues throughout development. Its expression is highest in the nervous system, heart, skeletal muscle, and intestine (Zacharias and Kappen, 1999), and regulated by growth factors such as glucocorticoids and Vitamin D (Zacharias and Kappen, 1999; Giacomello et al., 2013). PMCA2 is expressed mainly in the brain, heart, mammary glands and ear, and decreased expression of PMCA2 causes increased apoptosis in breast cancer cells (Curry et al., 2012; Giacomello et al., 2013). PMCA3 has the highest calmodulin affinity and is detected primarily in the brain and skeletal muscles

(Krebs, 2009; Giacomello et al., 2013). PMCA4 is involved in the fertilization process and cardiac function, and has been found to associate with lipid rafts, which often function to aggregate protein complexes important in signaling pathways (Giacomello et al., 2013). It has been suggested that PMCA4 is more involved in cell-specific Ca²⁺ signaling than as a pump for bulk Ca²⁺ export (Strehler, 2013; Brini et al., 2017). PMCA4 interacts with nitric oxide synthase and with calcineurin, which regulates NFAT signaling (Brini, 2009; Kim et al., 2012; Strehler, 2013).

PMCA1 and PMCA4 are important in osteoclast differentiation, maturity, and survival, and *Atp2b1*^{+/-} and *Atp2b4*^{-/-} mice have decreased bone density due to an increased number of mature osteoclasts and increased osteoclast apoptosis (Kim et al., 2012). The PMCA family members can also influence IP₃-mediated calcium signaling by binding to phosphatidylinositol-4,5-bisphosphate (PIP₂) on the plasma membrane as well as removing Ca²⁺ necessary for phospholipase C (PLC) activity, which prevents cleaving by PLC and thereby prevents Ca²⁺ release from the ER (Penniston et al., 2014). Altered PMCA expression is a characteristic of many cancers (Curry et al., 2011) and many other human diseases (Brini et al., 2013), but the diversity in isoforms and splicing and lack of specificity of small molecules to target PMCAs present challenges in therapeutic agent development (Strehler, 2013).

Summary—Calcium Export Exchangers and Pumps in Amelogenesis

There now are a number of reports that show expression and localization data for NCX1 and NCX3 (Okumura et al., 2010; Lacruz et al., 2012b), and NCKX4 (Hu et al., 2012; Wang et al., 2014) in the enamel organ. Reports on PMCA expression and activities throughout amelogenesis are scant (Sasaki and Garant, 1986c; Borke et al., 1995; Zaki et al., 1996), and to the authors' knowledge, the only investigations into the role of PMCA proteins in amelogenesis date back decades. Data presented here better defines the mRNA profiles of all SLC8A, SLC24A, and ATP2B gene family members, and adds additional insight into the protein and spatiotemporal expression profiles of NCKX3, PMCA1, and PMCA4.

Calcium Export Exchangers and Pumps and Disease

A number of the ATP2B, SLC8A, and SLC24A gene family members are linked to mammalian disease, but notably mutations to *SLC24A4* are associated with non-syndromic amelogenesis imperfecta (AI) (Parry et al., 2013; Seymen et al., 2014; Wang et al., 2014; Herzog et al., 2015). A comprehensive list of the PMCA, NCX, and NCKX pumps and exchangers, their links to human pathologies, and mouse models of each gene is found in **Table 1**.

MATERIALS AND METHODS

Animals

All vertebrate animal manipulation was carried out in accordance with Institutional and Federal guidelines. The animal protocols were approved by the Institutional Animal Care and Use

TABLE 1 | Pathologies associated with genes ATP2B1-4, SLC8A1-3 and SLC24A1-6.

Gene symbol	Protein name	Predominant substrates	Link to human disease	Animal models	References
ATP2B1	PMCA1	Ca ²⁺		Embryonic lethal	Okunade et al., 2004
ATP2B2	PMCA2	Ca ²⁺		Vestibular/motor imbalance, Deafness	Street et al., 1998; Bortolozzi et al., 2010
ATP2B3	PMCA3	Ca ²⁺	Spinocerebellar ataxia		Bertini et al., 2000; Zanni et al., 2012
ATP2B4	PMCA4	Ca ²⁺	Familial spastic paraplegia	No overt phenotype in <i>Atp2b4</i> null mice, Male mice are infertile	Okunade et al., 2004; Ho et al., 2015
SLC8A1	NCX1	Na ⁺ , Ca ²⁺		Embryonic lethal	Wakimoto et al., 2000
SLC8A2	NCX2	Na ⁺ , Ca ²⁺			
SLC8A3	NCX3	Na ⁺ , Ca ²⁺		Skeletal muscle fiber necrosis, Defective neuromuscular transmission	Sokolow et al., 2004
SLC8B1*	NCLX	Na ⁺ , Li ⁺ , Ca ²⁺			Khananashvili, 2013
SLC24A1	NCKX1	Na ⁺ , Ca ²⁺ , K ⁺	Congenital stationary night blindness	Night blindness	Riazuddin et al., 2010; Vinberg et al., 2015
SLC24A2	NCKX2	Na ⁺ , Ca ²⁺ , K ⁺			
SLC24A3	NCKX3	Na ⁺ , Ca ²⁺ , K ⁺			
SLC24A4	NCKX4	Na ⁺ , Ca ²⁺ , K ⁺	Amelogenesis imperfecta	Amelogenesis imperfecta	Parry et al., 2013; Seymen et al., 2014; Wang et al., 2014; Herzog et al., 2015
SLC24A5	NCKX5	Na ⁺ , Ca ²⁺ , K ⁺	Hypopigmentation, Oculocutaneous albinism		Mondal et al., 2012; Wei et al., 2013
SLC24A6	NCKX6	Na ⁺ , Ca ²⁺ , K ⁺			

Note that *SLC8B1* is found in the mitochondria of mammalian skeletal and heart muscle, neurons and a few other cell types.

Committee at the University of Southern California (Protocol #20461).

Quantitative PCR Analysis

Secretory-stage and maturation-stage enamel organ cells from mandibular incisors of 4-week old Wistar Hanover rats were collected as previously described (Lacruz et al., 2012b; Wen et al., 2014), and RNA extraction was performed using a QIAshredder, an RNeasy Protect Mini Kit, and DNase I solution from Qiagen (Valencia, CA, USA). Reverse transcription and real-time PCR were performed using the iScript cDNA Synthesis kit and SYBR Green Supermix from BioRad, respectively. Real-time PCR was performed on the CFX96 system (BioRad Laboratories, Hercules, CA, USA) in 10 μ l volumes with a final primer concentration of 100 nm, for 40 cycles at 95°C for 10 s and 58°C for 45 s. Six independent real-time PCR analyses were conducted using samples from a total of 6 rats, 3 males, and 3 females, for each gene of interest (primers are listed in **Table 2**), and for both stages of amelogenesis. The male and female data were analyzed separately and no significant differences were noted between the sexes, so the data presented in the graph were generated from all 6 animals ($n = 6$). Rat enamel organ is preferred to mouse enamel organ for real-time PCR and western blot studies because separating secretory and maturation stage from adult mouse incisors is technically difficult and yields less RNA and protein per animal.

Western Blot Analysis

Secretory (S) and maturation (M) enamel organ cells from mandibular incisors of 4-week old Wistar Hanover rats were collected. Brain (B) and heart (H) tissues were also collected

as control tissues. Total protein extraction was performed with RIPA buffer (1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris, pH 8.0) and protease inhibitor cocktail, complete mini (Roche Applied Sciences, Indianapolis, IN, USA). Samples were homogenized manually with a pestle six times, then sonicated with a BRANSON digital sonifier Model 450 (All-Spec Industries, Wilmington, NC, USA; 10% intensity, 10 s on and 10 s off). Samples were then cleared by centrifugation (15,000 g, 15 min, 4°C). Proteins were quantified using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and equal quantities were loaded (15 μ g per lane) onto 4–12% SDS–PAGE resolving gels. Protein was transferred to a PVDF membrane, then blocked with 5% milk in TBST. Antibodies against PMCA1 (AbCam, Cambridge, MA, USA; catalog #ab190355), PMCA2 (ab3529), PMCA3 (ab3530), PMCA4 (ab2783), NCKX3 (St. John's Laboratory, London, UK, catalog #STJ94358), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalog #sc-32233), amelogenin (ThermoFisher Scientific, catalog #PA5-31286), and cardiac muscle actin (ACTC1) (GeneTex Inc., Irvine, CA, catalog #GTX101876) were used at dilutions of 1:500, 1:2,000, 1:300, 1:5,000, 1:500, 1:500, 1:3,000, and 1:500, respectively in 5% milk in TBST. Secondary antibody for PMCA1-4 from Cell Signaling (Danvers, MA, USA; catalog #7074 and #7076) was applied at a dilution of 1:10,000. Secondary antibodies for NCKX3, amelogenin, and ACTC1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA; catalog #sc-2004 and sc-2418) were applied at a dilution of 1:7500. Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA; catalog #32132) was used as the detection system for all antibodies. TBST (tris-buffered saline with 1% Tween-20) was used as a wash buffer.

Immunofluorescence

Mandibular incisors were dissected from 9-day-old wild type mice and placed in 4% paraformaldehyde in PBS overnight. Mouse incisors were preferred to rat incisors in the immunofluorescence studies because mouse incisors decalcify more rapidly and all stages of amelogenesis are visible in one sagittal section. The incisors were then washed in PBS and decalcified in 10% EDTA in PBS pH 7.4 for 4 weeks at 4°C. The sample was embedded in paraffin and 4 μm sections were cut with a microtome. The sections were deparaffinized and rehydrated. The primary antibodies for PMCA1 and PMCA4 (AbCam, Cambridge, MA, USA; catalog #ab3528 and #ab2783, respectively) were used at dilutions of 1:40 and 1:200 in 1% BSA in PBS, respectively. The primary antibody for NCKX3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog #sc-50129) was used at a dilution of 1:50. The secondary antibodies (Vector Laboratories, Burlingame, CA, catalog #DI-1088, DI-2488, DI-2594, DI-3094) were used at a dilution of 1:300 in 1% BSA in PBST. Sections were mounted with mounting medium with DAPI (Vector Laboratories, Burlingame, CA, catalog #H-1200) and imaged on a Leica TCS SP8 confocal microscope (Leica Biosystems). PBST (0.1% Tween-20) was used as a wash buffer for the experiments. Negative control sections, using secondary antibody only, under identical conditions, were included and showed negligible auto fluorescence—see Supplemental Figure 1.

RESULTS

Messenger RNA Expression Profiles

Quantitative PCR (qPCR) comparing mRNA expression levels in secretory and maturation enamel organ cells for all PMCA (*Atp2b*), NCX (*Slc8*) and NCKX (*Slc24*) gene family members indicate that: (1) PMCA1 (*Atp2b1*), 3 (*Atp2b3*), and 4 (*Atp2b4*), and NCKX3 (*Slc24a3*) expression is highest during secretory-stage amelogenesis; (2) NCX1 (*Slc8a1*) and 3 (*Slc8a3*),

and NCKX6 (*Slc24a6*) were expressed during secretory and maturation stages; and (3) NCKX4 (*Slc24a4*) is most highly expressed during maturation-stage amelogenesis (Figure 1). The expression levels of PMCA2 (*Atp2b2*), NCX2 (*Slc8a2*), NCKX1

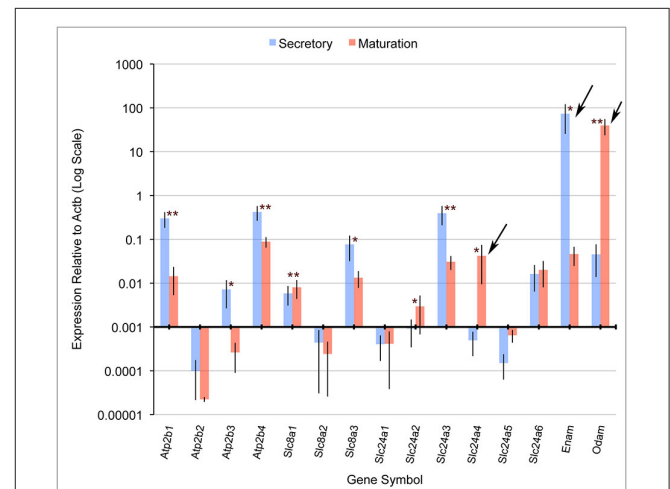


FIGURE 1 | Real-time PCR for rat *Atp2b*, *Slc8a*, and *Slc24a* gene family members. *Atp2b1*, *Atp2b3*, *Atp2b4*, *Slc8a3*, and *Slc24a3* have significantly higher expression in secretory stage than maturation stage, while *Slc8a1*, *Slc24a2*, and *Slc24a4* were significantly more highly expressed in maturation stage compared to secretory stage. β -actin (*Actb*) served as a normalizing control, and enamel (*Enam*) and Odam as control transcripts that were significantly down-regulated and up-regulated, respectively (as expected), during maturation-stage amelogenesis. *Slc24a4*, *Enam* and *Odam* (arrows) have all been linked to non-syndromic cases or amelogenesis imperfecta. The x-axis is placed at the 0.001 expression level relative to *Actb*, and below this "cut-off" figure is arbitrarily considered non-significant. The Student's *t*-test (paired two-tail) was used to compare the expression of each gene between the secretory and maturation stages (* $p < 0.05$, and ** $p < 0.01$). Standard deviations are also included.

TABLE 2 | Primers used for real-time PCR.

Symbol	Accession	Size	Region	Forward	Reverse	Temp
<i>Atp2b1</i>	NM_053311	219	1,387–1,605	AAAGCAGGTCTGCTGATGTC	GACGGAGTAAGCCAGTGAGA	58
<i>Atp2b2</i>	NM_012508	168	4,129–4,296	GAGACGTCGCTTTAGCTGAG	AAAGGGTCTGTGTGTGGAAA	58
<i>Atp2b3</i>	NM_133288	207	4,073–4,279	GCTCCATGACGTAACCAATC	GCGGAATATTGTGGGTGTAG	58
<i>Atp2b4</i>	NM_001005871	184	3,698–3,881	AATCCAAGAACCAGGTCTCC	ACGGCATTGTTATTCGTGTT	58
<i>Slc8a1</i>	NM_019268	150	2,430–2,579	CCTGCTTCATTGTCTCCATC	CAAATGTGTCTGGCACTGAG	58
<i>Slc8a2</i>	NM_078619	166	377–542	AAACGGTGTCCAACCTTACA	ACACACACAGCAATGACCAC	58
<i>Slc8a3</i>	NM_078620	206	4,310–4,515	TGGTGGAAAGCCATTCTATGT	AATATGGCCCACTCCCTTAG	58
<i>Slc24a1</i>	NM_004727	155	3,238–3,392	TTCTGACCTCATCACCAGT	TGGAAGTGGCTGTAATCCAT	58
<i>Slc24a2</i>	NM_031743	214	1,280–1,493	GGGAGGTTCCAGAGAAAAGC	CGATGCTGTGAGAGAGGTTT	58
<i>Slc24a3</i>	NM_053505	150	2,941–3,090	TGACATGTGCTCTTGTGTTGCT	AATTGGGACTTCATTGACGA	58
<i>Slc24a4</i>	NM_001108051	235	401–635	AAAGTTGATGGCACCGATAA	AGGGATGGGACAAAGAAGTC	58
<i>Slc24a5</i>	NM_001107769	161	371–531	AACATGGTTTCAACGCTCTC	CACAGCAGCAGGACATACAG	58
<i>Slc24a6</i>	NM_001017488	160	613–772	TTCTCAGACCCTCGTACTGC	ACACGGCCACCATATAGAAA	58
<i>Enam</i>	NM_001106001	169	1,139–1,307	ATGCTGGGAACAACCTACTACA	GTGGTTTGCCATTGTCTTTTC	58
<i>Odam</i>	NM_001044274	206	658–863	TTGACAGCTTTGTAGGCACA	GACCTTCTGTTCTGGAAGCAG	58
<i>Actb</i>	NM_031144	272	559–830	CACACTGTGCCATCTATGA	CCGATAGTGATGACCTGACC	58

(*Slc24a1*), NCKX2 (*Slc24a2*), and NCKX5 (*Slc24a5*) are negligible throughout amelogenesis (Figure 1). These data for NCX (*Slc8*) and NCKX (*Slc24*) gene family members are consistent with previously published gene expression data (Okumura et al., 2010; Hu et al., 2012), and add novel information suggesting that PMCA1, PMCA4, and to a lesser extent PMCA3 (which is expressed in secretory enamel organ cells at a level an order of magnitude lower than seen for PMCA1 and PMCA4), play an important role in secretory-stage amelogenesis.

Western Blot Analysis Confirms Expression of PMCA Proteins in Enamel Organ Cells

Western blot data indicate that PMCA1 and PMCA4 are more highly expressed in secretory stage than in maturation stage, and PMCA2 is not expressed at any appreciable level in amelogenesis, consistent with the qPCR data (Figures 2Ai,Aiv,Aii respectively). Contrary to the qPCR data, PMCA3 and NCKX3 appear to be expressed at similar levels during both secretory stage and maturation stage (Figures 2Aiii,B respectively). Rat brain and heart protein samples were used as control tissues and analyzed with the secretory- and maturation-stage protein samples. The expected molecular weights for PMCA1-4 are ~130, 133, 123, and 129 kDa respectively, and relate to the single bands seen at approximately the 150 kDa molecular weight mark (as indicated by an arrow, Figure 2A). The expected molecular weight of NCKX3 is ~60 kDa (Figure 2B). Gapdh has been included as a loading control for all samples, and additional controls include Western analysis for both amelogenin (Amelx) and cardiac muscle alpha actin (Actc) (Figure 2B).

PMCA1 and PMCA4 Localization by Immunofluorescence

In the enamel organ, PMCA1 expression is seen primarily on the basolateral membrane of both secretory- and maturation-stage ameloblasts, with stronger signals seen in secretory ameloblasts (green; Figures 3A–C). These data complement both the qPCR (Figure 1) and Western blot data (Figure 2) on the spatiotemporal expression of PMCA1 in enamel organ cells. When compared to ameloblasts, a weaker signal of PMCA1 is seen in the stratum intermedium (Figure 3A) and papillary layer cells of the enamel organ (Figure 3B); as reflected by the orange color observed in the merged images (Figures 3G–I). In the enamel organ, PMCA4 expression is also seen on the basolateral membrane of secretory- and maturation-stage ameloblasts, and also cells of the stratum intermedium and papillary layer cells (red; Figures 3D–F). Similar to the PMCA1 data, these PMCA4 immunolocalization data complement the qPCR and Western blot data (Figures 1, 2). The co-localization of both PMCA1 and PMCA4 in polarized ameloblasts can be appreciated in the merged image (yellow; Figures 3G–I), while PMCA4 (but not PMCA1) is also expressed in the stratum intermedium and papillary layer cells of the enamel organ (red; Figures 3G–I).

NCKX3 Localization by Immunofluorescence

NCKX3 expression is highest in the Tomes' processes (Figure 3M) and the apical membrane of transition- and maturation-stage ameloblasts (Figures 3N,O), while some minor ameloblast-specific intracellular granular immune-reaction is also apparent (Figures 3M–O). We compared the expression

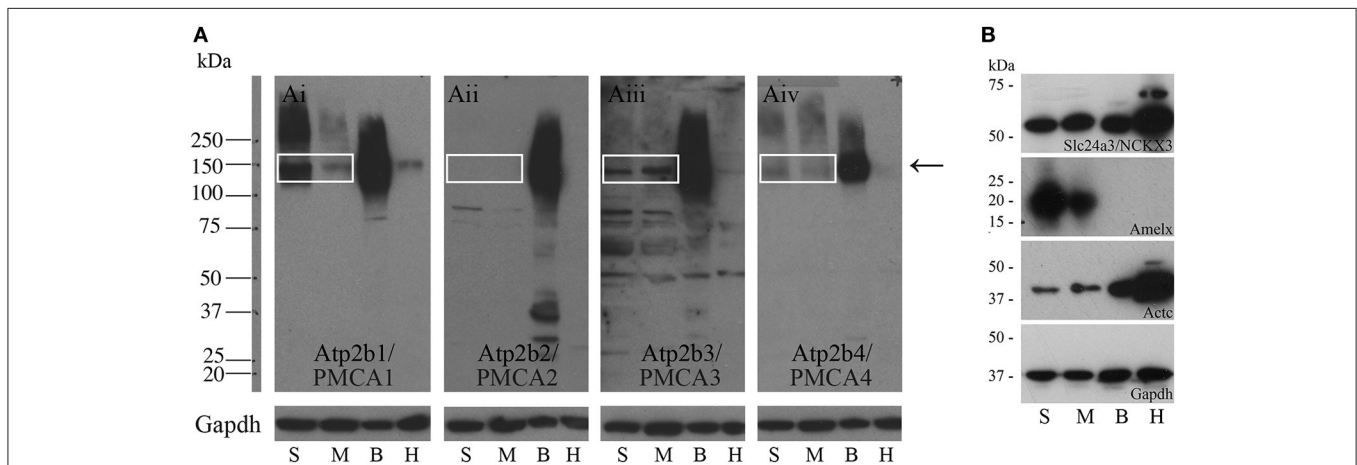
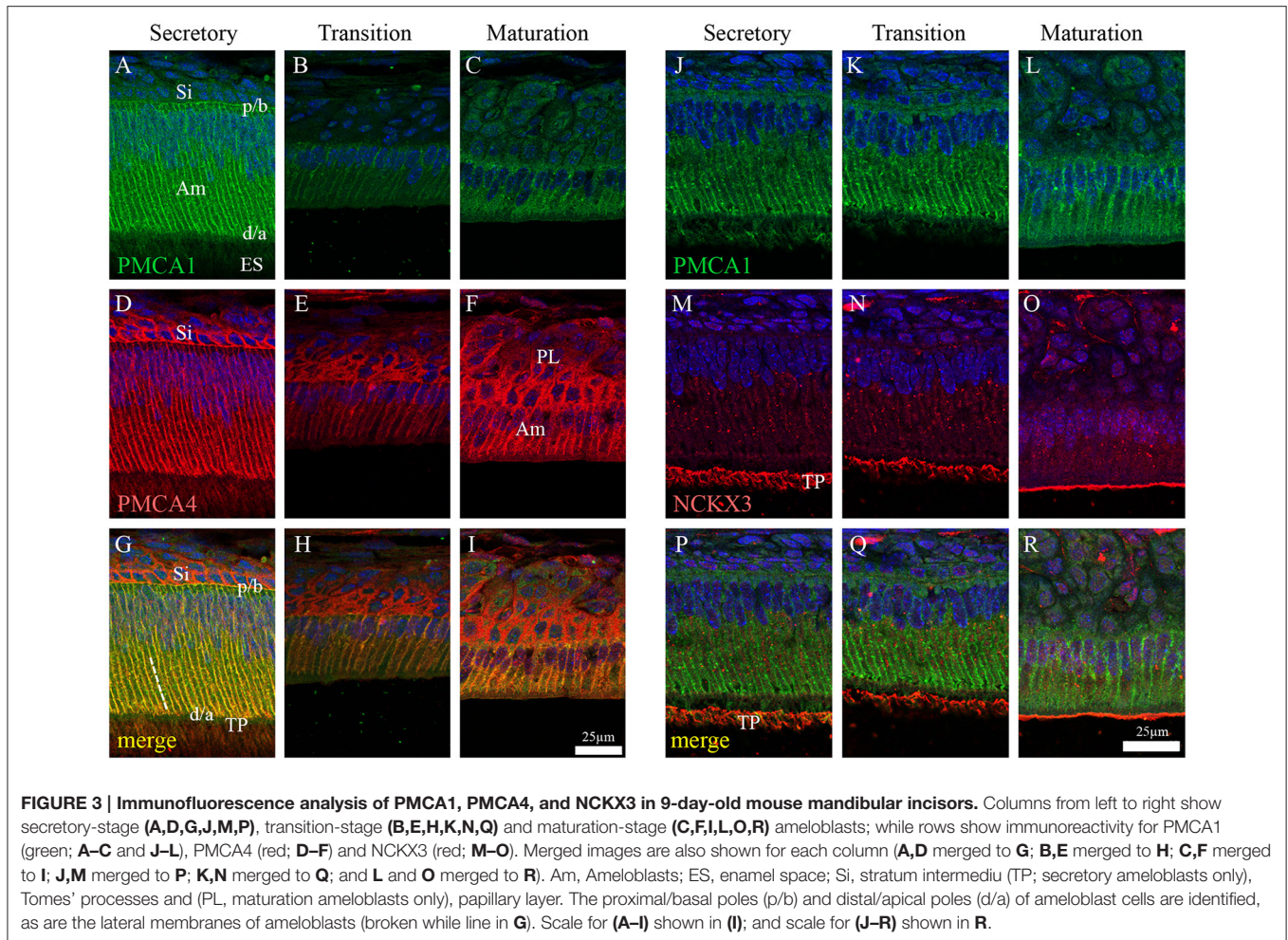


FIGURE 2 | Western blot analyses of PMCA1-4, and NCKX3 in secretory-stage and maturation-stage rat enamel organs. (A) Western blot analysis for PMCA1 (Ai), PMCA2 (Aii), PMCA3 (Aiii) and PMCA4 (Aiv). Samples are secretory-stage enamel organ cells (S), maturation-stage enamel organ cells (M), brain tissue (B) and heart tissue (H). Brain and heart samples are shown for comparison, as all PMCA are highly expressed in brain and at lower levels in the heart (Brini and Carafoli, 2011; Brini et al., 2017). Molecular weight markers are indicated at left. The expected molecular weights for PMCA1, PMCA2, PMCA3, and PMCA4 are ~130, 133, 123, and 129 kDa, respectively. The bands are seen for PMCA1, PMCA3, and PMCA4 (boxed and arrow). No expression of PMCA2 is evident. GAPDH is used here as a loading control. **(B)** Western blot analysis of NCKX3. The expected molecular weight for NCKX3 is ~60 kDa. NCKX3 is expressed in all 4 tissue samples tested, with similar expression noted in both secretory-stage and maturation-stage enamel organ cells, and brain tissue. Relatively higher levels of NCKX3 expression can be appreciated in heart tissue. Amelx and Actc are used as controls as expression is highest in secretory-stage ameloblasts (Lacruz et al., 2012a,b) and heart tissue (Hamada et al., 1982) respectively. GAPDH is used here as a loading control.

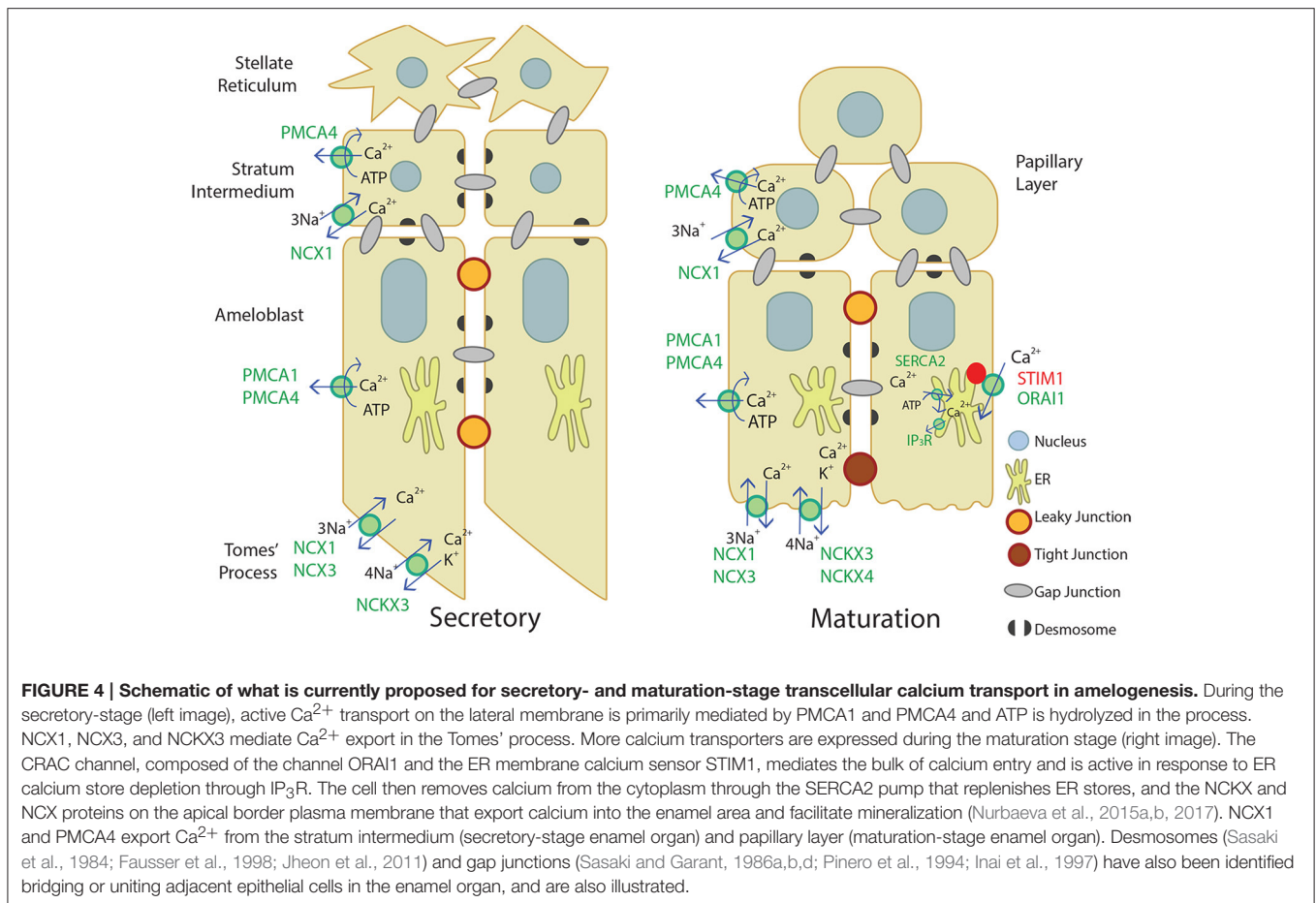


profile for NCKX3 (red; **Figures 3M–O**) to the expression profile of the control PMCA1 (green; **Figures 3J–L**). As can be appreciated from the images (**Figures 3M–R**), the expression profile for NCKX3 in the enamel organ is highest at the distal/apical pole, and this is distinct from the expression profiles seen for PMCA1 and PMCA4 where expression is seen on the lateral membranes of polarized ameloblasts (for both PMCA1 and PMCA4) and stratum intermedium and papillary layer cells (only PMCA4) (**Figures 3A–L**).

DISCUSSION

From data presented here and prior studies, it is possible to make the following generalizations. First, of the four unique genes coding the PCAs, PMCA1, and PMCA4 are highly expressed on the basolateral membranes of polarized ameloblasts; and both are expressed during secretory- and maturation-stage amelogenesis. These data somewhat contradicts previously published data suggesting PMCA1 and PMCA4 are localized primarily to Tomes' processes of secretory ameloblasts (Sasaki and Garant, 1986c; Borke et al., 1995). These differences likely result from the different specificities of antibodies used to carry

out these studies, and as noted previously, protein localization differences may also result from the different chemical and processing techniques used by the various laboratories (Takano, 1995). While expression of PMCA1 is primarily in the basolateral membrane of ameloblasts, there are also lower expression levels noted in the cells of stratum intermedium and papillary layer. Similarly, while expression of PMCA4 is seen in the basolateral membrane of ameloblasts, expression of PMCA4 is also recognized as a feature of the cells of the stratum intermedium and papillary layer cells. Of the three unique genes coding for the NCXs, NCX1, and NCX3 are highly expressed at the apical pole of both secretory- and maturation-stage ameloblasts (Okumura et al., 2010). Finally, of the six unique genes coding for NCKXs, NCKX3 (data reported here; **Figures 1–3**) and NCKX4 (Hu et al., 2012; Wang et al., 2014) are both highly expressed at the apical pole of polarized ameloblasts. A similar level of expression of NCKX3 is noted in both secretory- and maturation-stage ameloblasts (**Figures 2, 3**). While expression of NCKX4 is negligible in secretory-stage ameloblasts, it is highly expressed in maturation-stage ameloblasts (Hu et al., 2012; Wang et al., 2014). All six proteins expressed in ameloblasts (PMCA1, PMCA4, NCX1, NCX3, NCKX3, and NCKX4) export Ca^{2+} from the



cytoplasm to the extracellular space, thus ameloblasts may be one of the more complicated epithelial cell types when it comes to understanding ion movements related to Ca^{2+} transport as they relate to a mineralizing dental enamel.

The data suggest that there are likely redundancies amongst similarly functioning proteins from these gene families. For example, from this list of six Ca^{2+} export proteins expressed in ameloblasts, only mutations to SLC24A4/NCKX4 have been linked to enamel pathologies (Parry et al., 2013; Seymen et al., 2014; Wang et al., 2014; Herzog et al., 2015). NCKX4 exports Ca^{2+} from the apical pole of maturation-stage ameloblasts at the developmental stage where enamel mineralization is at its greatest; thus, NCKX4 may play a greater role in enamel formation than either NCX1 or NCX3, which have expression localized to the apical pole throughout the entire process of amelogenesis. It is conceivable that if the function of either NCX1 or NCX3 is less than optimal, the other may compensate such that no overt enamel phenotype results. Future studies may be able to address whether NCX1 and NCX3 are equivalent in enamel formation.

Similar to NCX1 and NCX3 in ameloblasts, PMCA1 and PMCA4 or other calcium handling proteins may be able to overcome the effects of *Atp2b1* or *Atp2b4* mutations, or gene silencing. No human pathologies have yet been linked to *ATP2B1*

mutations (as noted in the Online Mendelian Inheritance in Man; <http://omim.org/entry/108731>), however *Atp2b1*-null mice are embryonic lethal (Okunade et al., 2004). Only recently a case of familial spastic paraplegia has been linked to an *ATP2B4* mutation (Ho et al., 2015; <http://omim.org/entry/108732>), and while *Atp2b4*-null mice have no overt phenotype, the male mice are infertile due to reduced sperm motility (Okunade et al., 2004; Schuh et al., 2004; Kim et al., 2012). The similar expression profiles of PMCA1 and PMCA4 in ameloblasts (that being to the basolateral membrane) may suggest that loss of PMCA4 function in ameloblasts may be compensated by PMCA1, while the loss of PMCA1 function remains embryonic lethal. If this is correct, studying PMCA1 activities in *in vivo* enamel formation would, in the future, be limited to conditional knockout or heterozygote animal models. The localization of PMCA1 and PMCA4 on the ameloblast basolateral membrane may suggest that these Ca^{2+} pumps are unlikely to have a critical role in enamel mineralization; i.e., Ca^{2+} removed by the PMCA pumps may not directly be incorporated into the Hap mineral phase. Instead, the PMCA pumps may be indirectly involved in amelogenesis by maintaining ameloblast Ca^{2+} homeostasis; or being a part of ameloblast cell signaling pathways. As calcium is transported through the stratum intermedium and papillary layer to the ameloblasts and the enamel organ, the PMCA

TABLE 3 | Major and most highly expressed calcium export pumps and exchangers in enamel organ cells.

Gene/protein	Secretory ameloblasts	Stratum intermedium	Stellate reticulum	Maturation ameloblasts	Papillary layer cells	References
Atp2b1/PMCA1	Basolateral	✓ (weak)	✗	Basolateral	✓ (Weak)	
Atp2b4/PMCA4	Basolateral	✓	✗	Basolateral	✓	
Slc8a1/NCX1	Apical	✓	✗	Apical	✓	Okumura et al., 2010
Slc8a3/NCX3	Apical	✗	✗	Apical	✗	Okumura et al., 2010
Slc24a3/NCKX3	Apical	✗	✗	Apical	✗	
Slc24a4/NCKX4	✗	✗	✗	Apical	✗	Hu et al., 2012; Wang et al., 2014

family may additionally be valuable in shuttling the calcium from circulation to the ameloblasts. The PMCA family can be involved in IP₃-mediated calcium signaling (Penniston et al., 2014), and PMCA1 and PMCA4 are involved in RANKL signaling and regulate osteoclast differentiation (Kim et al., 2012). In cultured osteoclasts, the knockdown and/or silencing of both PMCA1 and PMCA4 increased protein expression of SERCA2 and TRPV5 (Kim et al., 2012). Indeed, the PMCA transporters may have evolved to fine-tune intracellular calcium concentration as they have higher calcium affinity and lower capability for bulk Ca²⁺ transport than the NCX/NCKX exchangers (Brini and Carafoli, 2011), and are therefore more likely to play a housekeeping role by removal of intracellular Ca²⁺ during maturation stage enamel mineralization which may also prevent calcium overload and possibly ameloblasts apoptosis. The PMCA transporters have multiple known expressed isoforms in other tissues but this has not yet been studied in the enamel organ.

Our novel data adds to the current understanding of Ca²⁺ transport in secretory and maturation stage enamel organ, described in **Figure 4**. Ca²⁺ import by the CRAC channel, ER Ca²⁺ export by IP₃R, and ER Ca²⁺ import by the SERCA2 pump have been well-described elsewhere (Nurbaeva et al., 2015a,b, 2017). In summary, when Ca²⁺ is released from the ER through IP₃R, STIM1 associates with ORAI1 and forms the CRAC channel that allows Ca²⁺ to flow into the cell. Ca²⁺ is then removed from the cytoplasm through PMCA1 and PMCA4 on the basal and lateral membranes, NCX1, NCX3, NCKX3, and NCKX4 on the apical membrane, and SERCA2 on the ER membrane. NCX1 and PMCA4 are also involved in Ca²⁺ export in the stratum intermedium and papillary layer. This process of Ca²⁺ cycling occurs more during the maturation stage, when large amounts of Ca²⁺ are necessary for enamel mineralization. During the secretory stage, PMCA1, PMCA4, NCX1, NCX3, and NCKX3 are the known Ca²⁺ exporters expressed, but further studies are necessary to understand Ca²⁺ import and other mechanisms of Ca²⁺ export during enamel secretion.

CONCLUSION

Based on the available data, we have reviewed and summarized the expression profiles of the major Ca²⁺ export pumps and

exchangers in enamel organ cells (**Table 3**; Okumura et al., 2010; Hu et al., 2012; Wang et al., 2014), and illustrated these Ca²⁺ export proteins along with the current model for Ca²⁺ import in enamel organ cells as proposed by Nurbaeva et al. (2015a,b, 2017) (**Figure 4**). As future studies continue to better define Ca²⁺ export (and also Ca²⁺ import—see Nurbaeva et al., 2015a,b, 2017) during amelogenesis, the information in **Table 3** will undoubtedly expand and become more precisely defined. Ultimately, elucidating the multitude of mechanisms involved in transcellular Ca²⁺ movements in the enamel-forming cells will result in a better understanding of the physiology and formation of enamel, the hardest and most calcified tissue in mammals.

AUTHOR CONTRIBUTIONS

SR, XW, CS, and MP designed the experiments and wrote the manuscript; SR and XW performed the experiments; SR, XW, KY, JC, CS, and MP analyzed the data; SR, XW, and MP prepared the figures and tables; All listed authors critically read, edited, and approved the final manuscript. MP accepts full responsibility for the integrity of the data analysis.

ACKNOWLEDGMENTS

The authors would like to thank Bridget Samuels for her help in the preparation of this manuscript, and Dr. Rodrigo S. Lacruz for his helpful comments and edits of the final version. This work was supported by grants DE019629 (MP), DE021982 (SR), and DE022528 (KY) from the National Institute of Dental and Craniofacial Research/National Institutes of Health. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fphys.2017.00336/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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