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Gene-expression profile and localization of Na⁺/K⁺-ATPase in rat enamel organ cells

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Abstract

The sodium pump Na⁺/K⁺-ATPase, expressed in virtually all cells of higher organisms, is involved in establishing a resting membrane potential and in creating a sodium gradient to facilitate a number of membrane-associated transport activities. Na⁺/K⁺-ATPase is an oligomer of α , β , and γ subunits. Four unique genes encode each of the α and β subunits. In dental enamel cells, the spatiotemporal expression of Na⁺/K⁺-ATPase is poorly characterized. Using the rat incisor as a model, this study provides a comprehensive expression profile of all four α and all four β Na⁺/K⁺-ATPase subunits throughout all stages of amelogenesis. Real-time PCR, western blot analysis, and immunolocalization revealed that $\alpha 1$, $\beta 1$, and $\beta 3$ are expressed in the enamel organ and that all three are most highly expressed during late-maturation-stage amelogenesis. Expression of $\beta 3$ was significantly higher than expression of $\beta 1$, suggesting that the dominant Na⁺/K⁺-ATPase consists of an $\alpha 1\beta 3$ dimer. Localization of $\alpha 1$, $\beta 1$, and $\beta 3$ subunits in ameloblasts was primarily to the cytoplasm and occasionally along the basolateral membranes. Weaker expression was also noted in papillary layer cells during early maturation. Our data support that Na⁺/K⁺-ATPase is functional in maturation-stage ameloblasts.

Keywords

ameloblast; biomineralization; calcium transport; enamel; sodium pump

Na⁺/K⁺-ATPase is a P-type ATPase composed of three subunits: α , β , and γ (1). The catalytic α subunit contains 10 transmembrane domains; the glycosylated β subunit is a type II membrane protein, responsible for protein folding and membrane integration; and the auxiliary γ subunit (the FXYD protein) is a type I membrane protein, which modulates substrate affinity but is not required for enzymatic activity (2). Na⁺/K⁺-ATPase is widely expressed in many tissues and is responsible for the active transport of Na⁺ and K⁺ across

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plasma membranes. For each unit of ATP consumed, Na^+/K^+ -ATPase exports three Na^+ and imports two K^+ , creating an electrical gradient and a concentration gradient. The electrical gradient is the basis for excitability in nerve and muscle cells, whilst the chemical gradient provides the driving force for several facilitated transporters and creates an osmotic gradient for water absorption (3, 4). Different molecular variants have evolved to fulfill the functional versatility. So far, four α isoforms ($\alpha 1$ –4) and four β isoforms ($\beta 1$ –4) have been identified, each produced respectively from a different gene, *Atp1a1*–4 and *Atp1b1*–4 (1, 5). In addition, seven isoforms of the γ subunit have been identified, which regulate Na^+/K^+ -ATPase in a tissue- and isoform-specific manner (6).

Dental enamel formation can be divided into secretory and maturation stages (7, 8). The secretory-stage enamel organ is composed of four cell layers: outer enamel epithelium; stellate reticulum; stratum intermedium; and the inner ameloblast layer. At this stage, ameloblasts secrete enamel matrix proteins and orchestrate the formation of the final enamel prismatic architecture composed primarily of hydroxyapatite (HA) crystallites. The maturation-stage enamel organ is composed of papillary layer cells and ameloblasts. Maturation-stage ameloblasts are involved in a number of activities, including ion transport, pH regulation, and endocytosis (7–9).

Based on chemically mediated Na^+/K^+ -ATPase inhibition studies, which resulted in delayed enamel maturation in developing teeth, it has been proposed that the enamel organ Na^+/K^+ -ATPase may participate in the net flow (i.e. removal) of the organic matrix components, including water, from the enamel matrix environment during enamel maturation (10). Our earlier array data also indicated that expression of the Na^+/K^+ -ATPase $\alpha 1$ subunit significantly increased in enamel organ cells during enamel maturation (8). These data prompted a more thorough investigation of the Na^+/K^+ -ATPase in amelogenesis. The purpose of our investigation was to complete a systematic study and to obtain a comprehensive profile of the expression of Na^+/K^+ -ATPase in enamel organ cells throughout the stages of amelogenesis.

Material and methods

Sample collection from rat mandibular enamel organ

All vertebrate animal manipulation complied with Institutional and Federal guidelines. This study has been independently reviewed and approved by the Institutional Animal Care and Use Committee of the University of Southern California.

The dissection was performed as previously described (11). Briefly, mandibles were dissected from 100-g (4 wk of age) male Wistar Hannover rats from Taconic (Hudson, NY, USA). The mandibles were then frozen in liquid nitrogen overnight and were subsequently lyophilized for 24 h. Enamel organ cells at three stages of development (secretory, early maturation, and late maturation) were collected (11).

Real-time PCR

Secretory, early-maturation, and late-maturation enamel organ cells from two male rats were pooled, 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 20- 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. Three independent real-time PCR analyses were conducted (a total of six rats) for each gene of interest (the primers are listed in Table 1), and for each of the three stages of amelogenesis. Beta-actin (*Actb* or β -actin) and enamelin (*Enam*) served as control transcripts – *Actb* served as a normalizing control and *Enam* as a gene transcript that was significantly down-regulated during maturation-stage amelogenesis. The Student's *t*-test was used to compare the expression of each gene between the secretory and early-maturation stages, and between the secretory and late-maturation stages.

Western blot analyses

Enamel organ cells from secretory, early-maturation, and late-maturation stages were collected from two 100-g rats, pooled, lysed 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. Antibodies against α 1 (Thermo Scientific, Waltham, MA, USA; catalogue # MA1-16731), β 1 (AbCam, Cambridge, MA, USA; catalogue # AB8344), and β 3 (Epitomics, Burlingame, CA, USA; catalogue # 6713-1) subunits, and *Actb* (Sigma-Aldrich, St Louis, MO, USA; catalogue # A5441), were used at dilutions of 1:3,000; 1:1,000; 1:1,000, and 1:10,000, respectively. Secondary antibodies from Cell Signaling (Danvers, MA, USA; catalogue #7074 and #7076) were applied at a dilution of 1:50,000. Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA; catalogue #32132) was used as the detection system.

Immunohistochemistry

Rats of 100 g in weight were fixed by perfusion (4% paraformaldehyde, 0.1% glutaraldehyde, 0.08 M sodium cacodylate, 0.05% calcium chloride, pH 7.2–7.4), then the maxillae were dissected and decalcified in 4.13% disodium ethylenediaminetetraacetic acid (EDTA) for 2 months at 4°C. The samples were embedded in paraffin and 4- μ m sections were cut using a microtome. The paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by 3% H₂O₂ in methanol for 10 min. The primary antibodies listed above for α 1 and β 3 were used for western blotting, whereas an additional antibody for β 1 (GeneTex, Irvine, CA, USA; catalogue # GTX113390) was used for immunohistochemical (IHC) analyses. Antibodies were diluted in PBS containing 1% BSA and 0.5% Triton X-100 (anti- α 1, 1:100 dilution; anti- β 1, 1:250 dilution; and anti- β 3, 1:100 dilution). ImmPRESS reagent anti-rabbit Ig peroxidase (MP-7401) and ImmPACT 3,3'-diaminobenzidine (DAB) peroxidase substrate (SK-4105) were purchased from Vector

Laboratories (Burlingame, CA, USA) and the recommended protocol methodologies were followed. The wash buffer used throughout staining was PBS containing 0.1% Triton X-100.

Results

Na⁺/K⁺-ATPase α and β subunit gene transcripts in enamel organ cells

Rodent incisors grow continuously, such that regions from the apical end to the incisal end represent progressive developmental stages in enamel development. The enamel organ cells of mandibular incisors were divided into secretory, early-maturation, and late-maturation stages. Real-time PCR was performed on RNA isolated from enamel organ cells for each of the Na⁺/K⁺-ATPase α and β subunits and for *Enam* as a control. As expected, *Enam* expression decreased from secretory to maturation stages (11) (Fig. 1A). Among all eight subunits examined, mRNAs from *Atp1a1*, *Atp1b1*, and *Atp1b3* were expressed at relatively high levels when the data were normalized to *Actb* transcripts (Fig. 1A). The mRNA profiles for *Atp1a1*, *Atp1b1*, and *Atp1b3* were further plotted as fold change (i.e. early-maturation stage vs. secretory stage; and late-maturation stage vs. secretory stage) and an increase in expression of all three isoforms was evident in the transition from secretory to maturation stages (Fig. 1B). These data also indicate that the most highly expressed of all the subunits in late maturation is *Atp1b3* (Fig. 1B).

Protein expression levels of Na⁺/K⁺-ATPase α 1, β 1, and β 3 subunits

To confirm the expression of Na⁺/K⁺-ATPase α 1, β 1, and β 3 subunits in enamel organ cells, total protein was extracted from secretory, early-maturation, and late-maturation stages and western blot analysis was performed (Fig. 2). Consistent with what was observed in real-time PCR, α 1, β 1, and β 3 were all expressed in enamel organ cells and the level of expression clearly increased from secretory stage to early-maturation stage (Fig. 2). The multiple bands seen for β 1 and β 3 isoforms are typical of protein glycosylation, as previously described (12). Of note was that the protein expression level of all three subunits examined plateaued at the early-maturation stage (Fig. 2) as opposed to the levels of mRNA for *Atp1b1* and *Atp1b3*, which increased gradually from secretory stage to late-maturation stage (Fig. 1B). This discrepancy between *Atp1b1* and *Atp1b3* mRNA and *Atp1b1* and *Atp1b3* protein profiles could be a result of differences in mRNA or protein stabilities, such as increased mRNA degradation during late-stage maturation.

Immunolocalization of Na⁺/K⁺-ATPase α 1, β 1, and β 3 subunits during amelogenesis

Immunohistochemistry was performed to examine the expression profiles in different enamel organ cell types. Our data show that expression of the α 1 subunit was undetectable in secretory stage (Fig. 3A1) and was observed in ameloblasts, and to a lesser extent in papillary layer cells, during early maturation (Fig. 3A2, A3). In late maturation, α 1 was expressed primarily in ameloblasts (Fig. 3A4). The expression patterns for β 1 and β 3 showed a similar distribution with weak expression noted in the entire enamel epithelial organ during the secretory stage and a noticeable increase in papillary layer cells in early maturation stage (Fig. 3B1,B2, C1,C2). The strongest expression levels for the β 1 and β 3 subunits were in ameloblasts in the late-maturation stage (Fig. 3B3,B4,C3,C4).

In summary, the expression of $\alpha 1$, $\beta 1$, and $\beta 3$ subunits in ameloblasts showed a gradual increase from the secretory stage to the late-maturation stage, whereas in the outer layer cells, stratum intermedium and stellate reticulum in the secretory stage and papillary layer in the maturation stage, the expression of these three subunits seemed to increase from secretory to maturation, peak at early maturation, and then decrease gradually in late maturation. This was consistent with the results of western blotting, which compared the three stages of combined enamel organ cells, showing that expression of protein for each of the three subunits plateaued at the early-maturation stage.

Within ameloblasts, the localization of all three subunits was primarily cytoplasmic and occasionally concentrated along the basolateral membranes, and this distribution is consistent with cytoplasmic localization to the endoplasmic reticulum (ER) of kidney cells (13, 14). To highlight this epitope distribution, an enlarged image for the $\beta 1$ subunit is shown (Fig. 3D). No consistent staining was detected at the apical membrane of ameloblasts, although some fields suggested the presence of α and β subunits in the ruffled border of ameloblasts (Fig. 3A3,B3).

Discussion

We have shown that the Na^+/K^+ -ATPase $\alpha 1$, $\beta 1$, and $\beta 3$ subunits are expressed in enamel organ, most notably in the basolateral membranes, and/or in the basal portion of the cytoplasm in maturation-stage ameloblasts. In an early study by GARANT & SASAKI (15), where an enzyme-based histochemical methodology was used to locate Na^+/K^+ -ATPase pump activity in enamel organ cells, this pump activity was sensitive to ouabain and was most noticeable in the cells of the stratum intermedium and in papillary layer cells of both secretory-stage and transition-stage ameloblasts, with little expression evident in ameloblasts. However, since that study was published (15), it has been shown that rats have both ouabain-sensitive ($\alpha 2$ and $\alpha 3$) and ouabain-resistant ($\alpha 1$) forms of Na^+/K^+ -ATPase (16, 17). Thus, the rat enamel-organ Na^+/K^+ -ATPase activity identified previously in this enzymatic-based study (15) may have excluded the ouabain-resistant $\alpha 1$ subunit profile, which is the focus of our report in rat ameloblasts using isotope-specific antibody. A more recent study, by JOSEPHSEN *et al.* (18), showed that one of the α subunits of Na^+/K^+ -ATPase localized almost exclusively to the papillary layer cells of the enamel organ, which clearly contrasts with the data obtained here, although it was unclear which one of the α subunits was recognized by the antibody used in this earlier study (18).

The real-time PCR and western blot data in the present study are highly supportive of our IHC data and suggest that of the four Na^+/K^+ -ATPase α subunits, only the $\alpha 1$ subunit is expressed at any appreciable level in the enamel organ. We showed that Na^+/K^+ -ATPase $\alpha 1$, $\beta 1$, and $\beta 3$ subunits were expressed primarily in ameloblasts in the late-maturation stage. We hypothesize that just like the enterocytes in intestine and the epithelium lining in kidney tubules (19), ameloblasts form a continuous sheet of cells capable of performing a classical transepithelial transport function. Na^+ is pumped out of the ameloblasts, establishing a high osmolarity in the intercellular spaces between adjacent ameloblasts, thus allowing water to diffuse into this intercellular space from the enamel matrix and subsequently enter the intercellular tissue fluid circulating within the papillary layer. This

could help to explain, in part, water removal from the enamel matrix during enamel maturation. There may also be a role for membrane-bound aquaporins, such as AQP4 and AQP5, known to be expressed in earlier stages of amelogenesis (20). The role of individual aquaporins in amelogenesis warrants further investigation. This published report on aquaporins in dental hard-tissue development examined only secretory-stage amelogenesis, and the authors noted that because of the limited specimens examined, no conclusions could be made relative to the process of enamel (or dentin) biomineralization (20).

The export of Na^+ and the coupled import of K^+ by the Na^+/K^+ -ATPase can also create an Na^+ gradient that can support secondary active transport, which couples the kinetic energy of Na^+ moving down its concentration gradient to the movement of other cations in an opposing direction. In ameloblasts this could be a working relationship with Na^+ and Ca^{2+} import and export activities. Yet, in the maturation stage of amelogenesis, Ca^{2+} is required to support continued volumetric growth of HA crystallites. The presence of sodium–calcium exchangers (NCX1 and NCX3), and also the sodium/calcium/potassium exchanger, NCKX4, at the apical membrane of maturation ameloblasts, probably mediates Ca^{2+} export (21, 22). All three calcium transporters require a transmembrane sodium gradient (23). Our data show a dramatic increase in expression of the Na^+/K^+ -ATPase in a-meloblasts from secretory stage to maturation stage, consistent with the Ca^{2+} -export profiles of enamel cells that are up to four times higher in maturation stage, again when compared with secretory-stage amelogenesis (7, 24). It is important to point out that Na^+/K^+ -ATPase is expressed at the basolateral membrane, whereas NCX1, NCX3, and NCKX4 are primarily expressed at apical and/or apicolateral membranes. This arrangement, for example with Na^+/K^+ -ATPase and NCKX4 at opposing poles, would allow for the transcellular movements of both Na^+ (import at the apical pole and export at the basolateral membrane) and K^+ (export at the apical and import at the basolateral), which could partially drive the net extrusion of Ca^{2+} into the maturing enamel matrix (25). On the other hand, Ca^{2+} pumped out into the intercellular spaces between ameloblasts is able to access the enamel in smooth-ended ameloblasts (SA) because tight junctions at the distal (enamel-facing) end of the ruffle-ended ameloblasts (RA) are relaxed in SA (26). Also, as a consequence of removing the tight junction seal in SA, Na^+ can diffuse to the extracellular space adjacent to the apical membrane to fuel the $\text{Na}^+/\text{Ca}^{2+}$ exchangers there.

Moreover, the Na^+/K^+ -ATPase, through its α subunit, interacts with the inositol 1,4,5-triphosphate (IP_3) receptor (ITPR1), a receptor that mediates calcium release from the ER. The binding of IP_3 to the IP_3 receptor leads to Ca^{2+} release through the opening of calcium channels on the ER membrane (27, 28). It could be hypothesized that the interaction with the Na^+/K^+ -ATPase may potentially change the gating property of IP_3 receptors, affecting Ca^{2+} release. Considering the huge demand for calcium during enamel maturation and the high level of Na^+/K^+ -ATPase expression, regulation of the interaction between Na^+/K^+ -ATPase and ITPR1 may represent a fundamental mechanism for release of Ca^{2+} during amelogenesis.

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Fig. 1.

Expression of Na⁺/K⁺-ATPase subunits at successive stages of enamel organ development. (A) Results of real-time PCR analyses for four α subunits (*Atp1a1*, *Atp1a2*, *Atp1a3*, and *Atp1a4*) and four β subunits (*Atp1b1*, *Atp1b2*, *Atp1b3*, and *Atp1b4*) of the Na⁺/K⁺-ATPase. The expression of each gene is compared within three stages of development (secretory, early maturation, and late maturation) in rat enamel organ epithelial cells, and normalized to β -actin. Enamelin (*Enam*), the expression of which is known to be high in secretory stage and low in the maturation stage, is used here as an mRNA quality indicator. For primer sequences, see Table 1. The averages and standard error of deviation were based on three independent real-time PCR amplifications using RNA from three different preparations. (B) The fold change relative to secretory stage was plotted for

Atp1a1, *Atp1b1*, and *Atp1b3*. The Student's *t*-test was used to compare the expression of each gene between secretory and early-maturation stages, and between secretory and late maturation-stages. *, significance at $p < 0.05$.

Fig. 2.

Expression of $\alpha 1$, $\beta 1$ and $\beta 3$ protein subunits of Na^+/K^+ -ATPase in enamel organ of rat incisors. Western blot analyses are shown using protein extracts from secretory (S), early maturation (EM), and late maturation (LM) stages of rat enamel organ epithelial cells and antibodies recognizing $\alpha 1$, $\beta 1$, $\beta 3$, and Actb (β -actin; a loading control). Protein weight marker in kDa is indicated on the left.

Fig. 3.

Immunolocalization of $\alpha 1$, $\beta 1$, and $\beta 3$ subunits in enamel organ of rat incisors. Sagittal sections of maxillary incisors from 4-wk-old rats were stained with antibodies against subunit $\alpha 1$ (panels A1–A4), $\beta 1$ (panels B1–B4), and $\beta 3$ (panels C1–C4) and were counterstained with haematoxylin. Differential localization is shown at secretory (panels A1, B1, and C1), early-maturation (panels A2–3, B2–3, and C2–3) and late-maturation (panels A4, B4, and C4) stages of enamel organ development. Enamel organ cells, ameloblasts (am), stratum intermedium (si, and white arrowhead), stellate reticulum (sr), and papillary layer

cells (p1) are labelled. (D) Magnified image of late-maturation-stage ameloblasts illustrating the basolateral (black arrow) and cytoplasm expression, possibly associated with the endoplasmic reticulum, of the $\beta 1$ subunit. Scale bars = 50 μm .

Table 1

Rat primers used in this study

Gene symbol	Accession no.	Primer sequences (forward)	Primers sequences (reverse)
<i>Atp1a1</i>	NM_012504	AGAAGGAAAGGGACATGGAC	ATGGCTCCAATCCACAGTAA
<i>Atp1a2</i>	NM_012505	ATGTGCTGGTGATGAAAGGT	TCTGTGGGAAAGTTCAGCTC
<i>Atp1a3</i>	NM_012506	GCTTTGCCTTTGACTGTGAT	GATGATGCCTACACCTTTGG
<i>Atp1a4</i>	NM_022848	GCGCAAGGATGTAAGGTAGA	GCCCACTTACTGTGTGGTCTC
<i>Atp1b1</i>	NM_013113	TGGAGACTTACCCTCTGACG	GGATTTTCAGTGTCCAAGGTG
<i>Atp1b2</i>	NM_012507	CAGCAGTTCTGTCCTCACCT	GGAGTTGTTTGGATGACAGG
<i>Atp1b3</i>	NM_012913	AAAATGGTACCTTGCCAACA	AGAGCATACGACAGGCACTC
<i>Atp1b4</i>	NM_053381	GCCAAGTTGCTTTGGTTTTA	AAGTTTCCCCATCAGTCCTC
<i>Enam</i>	NM_001106001	ATGCTGGGAACAATCCTACA	GTGGTTTGCCATTGTCTTTC
<i>Actb</i>	NM_031144	CACACTGTGCCCATCTATGA	CCGATAGTGATGACCTGACC