Full Paper

Sodium-Calcium Exchangers in Rat Ameloblasts

Reijiro Okumura^{1,2,3}, Yoshiyuki Shibukawa^{1,4,*}, Takashi Muramatsu^{1,3}, Sadamitsu Hashimoto^{1,3}, Kan-Ichi Nakagawa², Masakazu Tazaki^{1,4}, and Masaki Shimono³¹Oral Health Science Center, hrc7, ²Department of Endodontics, Pulp and Periapical Biology, ³Department of Pathology, ⁴Department of Physiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Received September 18, 2009; Accepted December 24, 2009

Abstract. Although the central role of ameloblasts in synthesis and resorption of enamel matrix proteins during amelogenesis is well documented, the Ca^{2+} -transport/extrusion mechanism remains to be fully elucidated. To clarify Ca^{2+} -transport in rat ameloblasts, we investigated expression and localization of Na^{+} - Ca^{2+} exchanger (NCX) isoforms and the functional characteristics of their ion transporting/pharmacological properties. RT-PCR and immunohistochemical analyses revealed expression of NCX1 and NCX3 in ameloblasts, localized in the apical membrane. In patch-clamp recordings, Ca^{2+} efflux by Na^{+} - Ca^{2+} exchange showed dependence on external Na^{+} . Ca^{2+} influx by Na^{+} - Ca^{2+} exchange, measured by fura-2 fluorescence, showed dependence on extracellular Ca^{2+} concentration, and it was blocked by NCX inhibitors KB-R7943, SEA0400, and SN-6. These results showed significant expression of NCX1 and NCX3 in ameloblasts, indicating their involvement in the directional Ca^{2+} extrusion pathway from cells to the enamel mineralizing front.

Keywords: transporter, enamel, mineralization, channel, SLC8 gene family

Introduction

Enamel development and mineralization occurs in at least two stages (secretion and maturation). In the secretion stage, ameloblasts are involved in the synthesis and secretion of extracellular matrix proteins, together with a small amount of mineral ions, which produces a partially mineralized matrix. After the enamel layer has reached full thickness, the ultrastructure of the ameloblasts changes, coinciding with a change in function during the maturation stage. During the maturation stage, the major function of the ameloblasts is the degradation and removal of previously secreted extracellular matrix proteins, which are replaced with water. During this process, mineral ions, mainly calcium and phosphorus, are secreted into the mineralizing extracellular enamel, gradually replacing water from porous maturing tissue. The process of mineralization determines the final hardness of the enamel, and this is achieved predominantly by transport of Ca^{2+} by ameloblasts (1 – 3).

There are two known mechanisms of active Ca^{2+} ef-

flux/extrusion from cells: via plasma membrane Ca^{2+} -ATPase (PMCA) and via plasma membrane Na^{+} - Ca^{2+} exchanger (NCX). Expression of PMCA (PMCA-1 and -4) is involved in enamel mineralization by exclusion of Ca^{2+} to the mineralizing front as a Ca^{2+} -transport pathway (4, 5). PMCA function during enamel formation was investigated using the calmodulin inhibitor trifluoperazine, a PMCA inhibitor. Trifluoperazine yielded partial systemic inhibition of enamel mineralization during the secretion stage, but had no effect during the maturation stage (6). The rate of Ca^{2+} -transport is up to 4-times higher during the maturation stage (1). While early enamel mineralization is dependent upon PMCA in ameloblasts during the secretion stage, further enamel mineralization may be controlled by a different Ca^{2+} -transport mechanism (2).

It has been reported that serum proteins were essentially absent in enamel fluid at the enamel formation site adjacent to the apical membrane in ameloblasts. This indicates that the enamel compartment (mineralizing front with enamel fluid) is a distinctive micro-environment isolated from serum (2). In addition, phosphate and K^{+} were present at higher concentrations and Mg^{2+} and Na^{+} at lower concentrations in enamel fluid than in serum (2). This indicates the necessity of ionic influx/efflux

*Corresponding author. yshibuka@tdc.ac.jp

Published online in J-STAGE on January 30, 2010 (in advance)

doi: 10.1254/jphs.09267FP

between the enamel compartment and apical membrane of the ameloblasts, suggesting expression of membrane ionic transporter(s). Na^+ - Ca^{2+} exchange has been suggested to explain coupling between alteration in enamel fluid composition, characterized by lower Na^+ concentration than in serum, and transcellular Ca^{2+} -transport via Ca^{2+} extrusion from the cell to the enamel fluid, in particular, offering a mechanism for direct transport of Ca^{2+} from ameloblasts (6). However, few pertinent findings have been reported to date (2).

The plasma membrane NCX is a bi-directional transporter capable of exchanging Na^+ and Ca^{2+} in either the Ca^{2+} efflux (forward exchange) or Ca^{2+} influx (reverse exchange) mode, depending on membrane potential and transmembrane ion gradients. It belongs to a multigene family (SLC8A) comprising NCX1, NCX2, and NCX3 (7–9).

To clarify enamel mineralization by direct Ca^{2+} -transport involving Ca^{2+} extrusion in ameloblasts, we investigated the activity profiles and expression/localization patterns of NCX.

Materials and Methods

All animals were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Council of The Japanese Pharmacological Society, the Physiological Society of Japan, and American Physiological Society, as well as with guidelines laid down by the National Institute of Health in the USA regarding the care and use of animals for experimental procedures. The study was approved by the Ethics Committee of our institute.

Tissue preparation

For reverse transcriptase-polymerase chain reaction (RT-PCR), $[\text{Ca}^{2+}]_i$ measurement, and patch-clamp recordings, ameloblasts were obtained from 7-day-old Wistar rats as described previously (10–13) with modifications. Rat mandibles were dissected under pentobarbital sodium anesthesia (25 mg/kg, i.p.). Hemimandibles embedded in alginate impression material were sectioned transversely through the incisor at a thickness of 500 μm using a standard vibrating tissue slicer (DTK-100; Dosaka EM, Tokyo). Sections were obtained at the midpoint along the length of the incisors. Mandibular sections were sliced so that the dentin and enamel were visible between bone tissue and dental pulp. Surrounding impression material and bone tissue were removed from obtained sections. The ameloblast layer was removed from the surface of the enamel under a stereoscopic microscope. These cells were treated with standard buffered-saline containing 0.17% collagenase and 0.03% trypsin

(type 1, 37°C for 30 min) to obtain acutely isolated ameloblasts. For $[\text{Ca}^{2+}]_i$ measurement and patch-clamp recordings, isolated ameloblasts were plated on a culture dish and bathed in minimum essential medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum and 5% horse serum and maintained at 37°C in a 5%- CO_2 incubator. These primary cultured cells were used for the experiments within 24 h after isolation to avoid changes in cell properties.

RT-PCR

Total RNA was extracted from acutely isolated rat ameloblasts and whole-brain by a modified acid guanidium-phenol-chloroform method. Reverse transcription, cDNA amplification, and PCR were performed by a one-step RT-PCR kit (Qiagen, Valencia, CA, USA) or one-step SYBR primescrypt RT-PCR Kit with Thermal Cycler Dice for semiquantitative real-time RT-PCR (TaKaRa-Bio, Shiga). Results obtained from real-time PCR experiments were quantified using the comparative threshold ($2^{-[\Delta\Delta\text{Ct}]}$) method (Ct is cycle threshold) (14). Products from conventional RT-PCR were visualized by electrophoresis on a 1% agarose gel.

Immunofluorescence and histochemical analysis

Mandibles [and brain tissues as positive control (data not shown)] were dissected from 7-day-old Wistar rats anesthetized with pentobarbital sodium. Mandibles were embedded in OCT-compound (TissueTek, Elkhart, IL, USA), rapidly frozen in isopentane (-80°C), and sagittally sectioned (together with mandibular incisors) to 6 μm in thickness without decalcification. These cryo-sections were mounted on glass slides at -20°C (Matsunami, Osaka). To distinguish maturation and secretion stage ameloblasts, alkaline phosphatase staining for cryo-sections was performed using the StemTAG kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. To identify NCX isoform localization, after blocking in 10% goat or rabbit serum, cryo-sections were incubated with rabbit anti-NCX1 or goat anti-NCX2 or -NCX3 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100) at 4°C overnight. Primary cultured cells were also incubated with rabbit anti-amelogenin polyclonal antibodies (Hokudo, Sapporo; dilution 1:100) at 4°C overnight. Sections and cells were incubated with a secondary antibody (Alexa Fluor 488 goat anti-rabbit or rabbit anti-goat IgG, dilution 1:100; Molecular Probes, Eugene, OR, USA) for fluorescence staining and with 4',6-diamino-2-phenylindole (Molecular Probes) for nuclear staining at room temperature for 5 min. Negative controls were prepared using non-immune IgGs diluted at equivalent concentrations to primary antibodies (see inset in Fig. 1E). Sections

were examined under a conventional fluorescence microscope (Zeiss, Jena, Germany).

Whole-cell recordings

A nystatin-perforated patch-clamp recording for primary cultured ameloblasts was conducted. Cells were arranged on the glass bottom of the recording chamber mounted on the microscope stage (Zeiss). Patch pipettes were pulled from capillary tubes (P80/PC; Sutter Instrument, Novato, CA, USA) and filled with intracellular solution containing nystatin (160 μ g/ml). Whole-cell currents were measured using a patch-clamp amplifier (L/M-EPC-7; List-Medical, Darmstadt, Germany). Current traces were monitored, stored, and analyzed using the pCLAMP (Axon Instruments, Foster City, CA, USA).

Fura-2 fluorescence measurement

Primary cultured ameloblasts were incubated for 30 min at 37°C in standard buffered-saline containing 10 μ M fura-2 acetoxymethyl ester (Dojindo, Kumamoto) and rinsed with fresh solution. The dish with fura-2-loaded cells was mounted on the microscope stage (Olympus, Tokyo) incorporated into the Aquacosmos system and software (Hamamatsu Photonic, Shizuoka) equipped with excitation wavelength selector and intensified charge-coupled device camera. Fura-2 fluorescent emission was measured at 510 nm in response to altering excitation wavelengths of 334 and 380 nm. $[Ca^{2+}]_i$ was expressed as fluorescence ratio ($R_{F340/F380}$) at 380 nm and 340 nm.

Solutions

Standard buffered-saline consisted of 136 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Hepes, and 10 mM glucose (pH 7.4). For $[Ca^{2+}]_i$ measurement, extracellular solution consisted of 150 mM NaCl, 0.2 mM CaCl₂, 10 mM sucrose, and 20 mM Hepes (pH 7.4). To activate Ca²⁺ influx by NCX, extracellular Na⁺ ($[Na^+]_o$) was substituted with equimolar Li⁺. To activate currents by Ca²⁺ efflux by NCX, an extracellular solution containing 150 mM LiCl, 10 mM sucrose, 1 mM EDTA, and 20 mM Hepes (pH 7.4) was rapidly alternated with one containing selected concentrations of NaCl (10–150 mM) and 50 μ M EDTA (replacing 1 mM EDTA), maintaining osmotic strength by replacing LiCl with NaCl. Intracellular solution contained 110 mM K-glutamate, 20 mM KCl, 2 mM Mg-ATP, 20 mM TEA-Cl, 10 mM CaCl₂, 10 mM EGTA [free $[Ca^{2+}]$ was 26 μ M (15)], and 20 mM Hepes (pH 7.2). KB-R7943 and SN-6 were obtained from Tocris Cookson (Avonmouth, UK). SEA0400 was a gift from Professors Toshio Matsuda and Akemichi Baba (16). All other reagents were ob-

Table 1. Primer sets used for detection of NCX in conventional and real-time RT-PCR analyses

Sequence (product size)
For conventional RT-PCR
NCX1 (334 bp)
F: 5'-GGATGTGGTTGAAAATGACCCAGT-3'
R: 5'-TATGCCATCTTCCGAGACTTCTGA-3'
NCX2 (324 bp)
F: 5'-GATGGTGCCCCGATGATGAGGAC-3'
R: 5'-AGCATCGCCACACGCAGGTTTCAG-3'
NCX3 (366 bp)
F: 5'-GCATACTGATGAGCCTGAGGACTT-3'
R: 5'-AAAGGAAGACTATTGAGGATGGCG-3'
β -Actin (632 bp)
F: 5'-GATGGTGGGTATGGGTCAGAAGGA-3'
R: 5'-GCTCATTGCCGATAGTGATGACCT-3'
For real-time RT-PCR
NCX1 (109 bp)
F: 5'-GTGTTTGTGCTCTTGGAACTC-3'
R: 5'-CGTTGCTTCCGGTGACATTG-3'
NCX2 (184 bp)
F: 5'-CAGGTCAAGATAGTGGACGACGAA-3'
R: 5'-GAACTGGCTTGCCCATCTCTG-3'
NCX3 (92 bp)
F: 5'-AGCTGTACGGTGTGAGAATGAG-3'
R: 5'-TGACCACAATGATGACCAGATGAA-3'
GAPDH (143 bp)
F: 5'-GGCACAGTCAAGGCTGAGAATG-3'
R: 5'-ATGGTGGTGAAGACGCCAGTA-3'

Conventional RT-PCR amplification consisted of 34 cycles at 94°C for 60 s, 58°C for 60 s, and 72°C for 120 s. Conditions for real-time RT-PCR: 1 cycle of 42°C for 5 min, followed by 1 cycle of 95°C for 10 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s; and for dissociation curve analysis: 1 cycle of 95°C for 15 s, 1 cycle of 60°C for 30 s and 1 cycle of 95°C for 15 s. F: forward primer, R: reverse primer.

tained from Sigma Chemical (St. Louis, MO, USA). All experiments were conducted at room temperature (20°C–22°C).

Statistical analyses

Data were expressed as the mean $\pm$ S.E.M. of the tested cells; numbers of cells tested given in the figure legends. A one-way analysis of variance (ANOVA) was used to determine statistical significance. *P*-values of less than 0.05 were considered significant.

Results

Expression and localization of NCX in rat ameloblasts

Gene-specific primers for both conventional and real-time RT-PCR analyses (Table 1) (17) revealed expressions of NCX1 and NCX3 in ameloblasts, but not expression of NCX2 (Fig. 1: A and B). Rat whole-brain cDNA expressing NCX1, NCX2, and NCX3 was used as a positive control (8).

Fig. 1. Detection and localization of NCX in rat ameloblasts. **A** and **B**: NCX isoforms in ameloblasts by conventional (**A**) and real-time (**B**) RT-PCR. For conventional RT-PCR (**A**), β -actin gene expression used as internal control was positive in all samples. Rat whole-brain cDNA expressing NCX1, NCX2, and NCX3 was used as a positive control. PCR products visualized by electrophoresis on 1% agarose gel containing ethidium bromide. For real-time RT-PCR (**B**), PCR products were monitored by increase in fluorescence by binding of SYBR Green dye to double-stranded DNA. Data analyzed by the $2^{-\Delta\Delta Ct}$ method, with brain as calibrator and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control (which was positive in all samples). Data are expressed as fold expression of NCX isoforms in ameloblasts compared with those in brain. Note that the relative mRNA expression level of NCX isoforms does not indicate absolute level of expression. **C** to **J**: Activity of alkaline phosphatase on ameloblast layer indicated in red (**C** and **G**). Secretion stage ameloblasts negative for cytoplasmic alkaline phosphatase (**C**); maturation stage cells positive for cytoplasmic alkaline phosphatase (**G**). Photographs (**C** to **F** and **G** to **J**) obtained from serial sections of incisor. Observation areas in photographs **D** to **F** correspond with the observation area in **C**, meaning that ameloblasts in **D** to **F** were identified as cells in the secretion stage. In the same way, observation areas in photographs **H** to **J** correspond with the area in **G**, indicating that ameloblasts in **H** to **J** were identified as cells in the maturation stage. NCX immunoreaction (green) and nuclei (blue) shown in **D** to **F** and **H** to **J**. Ameloblasts in both stages were tall and columnar. Intense immunoreactivity for NCX1 (**D** and **H**) and NCX3 (**F** and **J**) in ameloblasts in both stages observed; no immunoreaction observed for NCX2 (**E** and **I**). In the negative control (inset of **E**), no fluorescence was detected except for nonspecific labeling of enamel matrix. Nonspecific labeling in enamel was observed throughout, as cryo-sections without decalcification and fixation utilized to avoid reduction in immunoreactivity. Bars in **C** to **J** = 50 μ m. Arrows, ameloblast layer; E, enamel; D, dentin; P, dental pulp.

Alkaline phosphatase-negative ameloblasts were in the secretion stage (Fig. 1C) (18); positive ones were in the maturation stage (Fig. 1G) (19). In the incisor serial sections, both alkaline phosphatase-negative secretion stage [in Fig. 1, C – F: each observation area in D – F corresponds to that in C] and alkaline phosphatase-positive maturation stage [in Fig. 1, G – J: each observation area in H – J corresponds to that in G] ameloblasts showed positive immunoreactivity for NCX1 (Fig. 1: D and H) and NCX3 (Fig. 1: F and J), but not for NCX2 (Fig. 1: E and I). Immunoreaction of NCX3 was strongly

detected at the apical membrane and apicolateral membrane; that of NCX1 was detected at the apical membrane and apicolateral membrane and slightly detected in the basal membrane and infranuclear area. Stratum intermedium cells expressed NCX1 (Figs. 1: D and H) and NCX3 (Fig. 1: F and J).

Ca²⁺ efflux by NCX in ameloblasts

Ca²⁺ efflux by Na⁺-Ca²⁺ exchange was recorded as Na⁺ influx current with a nystatin-perforated patch recording configuration. Primary cultured ameloblasts chosen for

Fig. 2. Na⁺ dependence on NCX expressed in rat ameloblasts. **A:** Amelogenin immunoreaction (green) with nuclei (blue) observed in primary cultured ameloblasts used for patch-clamp recordings and [Ca²⁺]_i measurement (see Fig. 3). These cells showed elongated morphology (tall columnar shape) when isolated from incisor. Antibody was raised against bovine amelogenin and it was reactive to rat tissues. For patch-clamp recordings and [Ca²⁺]_i measurements, these primary cultured amelogenin-positive cells were used within 24 h after isolation to avoid changes in cell properties. Bar = 50 μm. **B:** Typical inward Na⁺ current via Ca²⁺ efflux by NCX in ameloblasts. A nystatin-perforated patch recording configuration, which can be performed without disturbing intracellular conditions, was utilized. These cells were voltage-clamped at a holding potential of 0 mV. Boxes in the upper panel indicate protocol of solution switches applied. Ca²⁺ efflux by NCX was activated by substituting Li⁺-containing external solution with Na⁺-containing solution, maintaining osmotic strength by replacing LiCl with NaCl. Amplitudes of inward current increased with increase in [Na⁺]_o, ranging from 0 – 150 mM. Current signals were digitized at 0.5 kHz and low-pass filtered at 10 Hz. **C:** Concentration–response relationship for [Na⁺]_o. Data points illustrate current densities as a function of applied concentrations of extracellular Na⁺. Changes in current (current densities) were normalized with respect to cell size, as judged by cell capacitance. Data points represent the mean ± S.E.M. of the tested cells (numbers in parentheses). Curve (solid line) was fitted to the following function: $A = A_{\max} / [1 + (K_D / [\text{Ion}]_o)^P] + A_{\min}$ (equation 1), where K_D is the apparent dissociation constant, P is the Hill coefficient, $A_{\max}$ is the maximal current densities (2.4 pA/pF), $A_{\min}$ is the minimal current densities (0 pA/pF), and $[\text{Ion}]_o$ is applied extracellular ionic concentration of Na⁺. The 95% confidence interval for K_D was 56.5 – 69.9 mM for [Na⁺]_o. * $P < 0.05$: significant differences in current densities recorded between each combination of 10 – 150 and 0 mM [Na⁺]_o.

patch-clamp recordings and [Ca²⁺]_i measurement exhibited amelogenin-positive staining (Fig. 2A) (20 – 22). Inward current at a membrane potential of 0 mV increased with increasing [Na⁺]_o, ranging from 0 to 150 mM (Fig. 2B). The plot (Fig. 2C) illustrates inward current densities as a function of applied [Na⁺]_o, with an apparent dissociation constant (K_D) of 63.2 mM and Hill coefficient of 2.6 (Fig. 2C, solid line).

Ca²⁺ influx by NCX and its pharmacological properties in ameloblasts

Further studies were carried out to investigate Ca²⁺ influx by Na⁺-Ca²⁺ exchange activity. Response of [Ca²⁺]_i was expressed as F/F₀ units, with R_{F340/F380} values (F) normalized to resting value (F₀). Increases in [Ca²⁺]_i caused by Ca²⁺ influx by NCX were clearly extracellular Ca²⁺ concentration ([Ca²⁺]_o)-dependent (Fig. 3A). Increases in [Ca²⁺]_i in the ameloblasts were induced by addition of four different concentrations of [Ca²⁺]_o (0.02 – 2.0 mM). The semilogarithmic plot in Fig. 3B illustrates F/F₀ values as a function of applied [Ca²⁺]_o, with an equilibrium binding constant of 0.31 mM (Fig. 3B, solid line).

Application of KB-R7943 (C and D), SEA0400 (E and F), and SN-6 (G and H) clearly blocked the increase in [Ca²⁺]_i (Fig. 3). The concentration producing 50% blockage (IC₅₀) was 0.66 μM for KB-R7943, 0.22 nM for SEA0400, and 0.54 μM for SN-6.

Discussion

Our biophysical/pharmacological, RT-PCR, and immunohistochemical analyses indicated that ameloblasts expressed NCX1 and NCX3 from the secretion to the maturation stages. Earlier studies found that benzyloxy-phenyl derivatives KB-R7943, SEA0400, and SN-6 specifically inhibited the NCX, and they have been noted to inhibit Ca²⁺ influx more effectively than Ca²⁺ efflux by NCX (7, 8). Values of IC₅₀ for SN-6 and KB-R7943 were consistent with the general characteristics of those for CCL39 cells transfected with NCX1, NCX2, and NCX3 (23). However, the ameloblast NCX was highly sensitive to SEA0400. SEA0400 predominantly blocked NCX1, only mildly blocked NCX2, and exerted almost no influence on NCX3. In the literature, although KB-R7943 and SN-6 inhibited all NCX isoforms, SN-6 was 3- to 5-times

Fig. 3. Ca^{2+} dependence and pharmacological characteristics of NCX expressed in ameloblasts. Ca^{2+} -dependent activation (A and B) and dose-dependent inhibition of NCX by KB-R7943 (C and D), SEA0400 (E and F), and SN-6 (G and H). A: Ca^{2+} influx by NCX in ameloblasts activated by substituting Na^+ -containing external solution with Li^+ -containing solution, maintaining osmotic strength by replacing NaCl with LiCl. Increases in Ca^{2+} influx by NCX induced by addition of four different concentrations of $[Ca^{2+}]_o$ (0.02 – 2.0 mM). Applied solution switch protocol for $[Na^+]_o$ and extracellular Li^+ concentration ($[Li^+]_o$) is shown in the upper panel; protocol for $[Ca^{2+}]_o$ is shown in the lower panel. B: Concentration–response relationship for $[Ca^{2+}]_o$. Data points illustrate F/F_0 values as a function of the applied concentrations of extracellular Ca^{2+} . Data points represent the mean $\pm$ S.E.M. of the tested cells (numbers in parentheses). Curve (solid line) on semilogarithmic scale was fitted to the following function: $A = A_{max} / [1 + (K / [Ion]_o)] + A_{min}$ (equation 2), where K is the applied extracellular Ca^{2+} concentration providing half response (equilibrium binding constant), A_{max} is maximal F/F_0 (1.3), A_{min} is minimal F/F_0 (1.05), and $[Ion]_o$ is applied extracellular Ca^{2+} concentration. The 95% confidence interval for K was 0.21 – 0.40 mM for $[Ca^{2+}]_o$. * $P < 0.05$: significant differences in F/F_0 values recorded between each combination of 0.02 – 2.0 and 0 mM $[Ca^{2+}]_o$. C, E, and G: Increases in $[Ca^{2+}]_i$ evoked by Ca influx by NCX reversibly (not shown) inhibited by KB-R7943 (C), SEA0400 (E), and SN-6 (G) in concentration-dependent manner. External solution change protocols for $[Na^+]_o$ alone and $[Li^+]_o$ with 0.2 mM $[Ca^{2+}]_o$ are shown in upper panels of each figure. Black bars in each figure indicate times of addition of these inhibitors to the external solution. D, F, and H: Dose–response relationship for inhibitory effects of KB-R7943 (D), SEA0400 (F), and SN-6 (H) on Ca^{2+} influx by NCX. Data points in each figure illustrate F/F_0 values as a function of the applied concentrations of inhibitors and represent the mean $\pm$ S.E.M. of the tested cells (numbers in parentheses). Curves (solid lines) were fitted to the following function: $A = A_{max} / [1 + 10^{([x]_o - log[IC_{50}])}] + A_{min}$ (equation 3), where IC_{50} is 50% inhibitory concentration; A_{max} is maximal F/F_0 ; A_{min} is minimal F/F_0 ; and $[x]_o$ is applied concentration of inhibitors KB-R7943, SEA0400, and SN-6. These experiments were performed after basal value (which shows reduction to resting value) appeared to be steady-state [as expressed as F_0 (see text)]. * $P < 0.05$: significant differences in F/F_0 values between before and after application of each concentration of inhibitors.

more inhibitory against NCX1 than against NCX2 or NCX3, and KB-R7943 was 3-times more effective against NCX3 than against NCX1 or NCX2 (7, 23–25). These three inhibitors partially blocked Ca²⁺ influx by NCX. Thus, our pharmacological results indicate functional expression of NCX isoforms in the primary cultured ameloblasts, which is consistent with the results of the RT-PCR in the acutely isolated cells and immunohistochemical analyses *in vivo*.

Cells selected for functional analysis of Na⁺-Ca²⁺ exchange activities were cytoplasmic amelogenin-positive. Amelogenin expression was identified in secretory ameloblasts and stratum intermedium cells at the apical bud of the incisor (21, 22). Both maturation stage ameloblasts and stratum intermedium cells associated with maturation and secretion stage ameloblasts are amelogenin-negative (20). We isolated ameloblasts attached to the enamel surface, but not those in the apical bud. These amelogenin expression patterns suggest that the results of our study were mainly obtained from a population of secretory ameloblasts, not stratum intermedium cells. However, it is possible that the cell culture included both maturation and transition stage ameloblasts. Furthermore, although stratum intermedium cells also expressed NCX1 and NCX3, further study is needed to clarify the functional significance of exchangers in these cells.

Differential expression of NCX was observed in ameloblasts: NCX3 was localized in apical/apicolateral membrane; NCX1 was detected in apical/apicolateral/basal membrane and the infranuclear area. Basal membrane NCX1 may play an important role in regulation of subcellular [Ca²⁺]_i by Ca²⁺ extrusion (2, 8). In brain tissue, mitochondria express different levels of NCX1 to NCX3 (26). The infranuclear area of ameloblasts contains many mitochondria. Infranuclear NCX1 may contribute to mitochondrial/intracellular Ca²⁺ homeostasis.

The rate of Ca²⁺-transport is much higher in ameloblasts during maturation (1). Although PMCA is responsible for early enamel mineralization during secretion, it is not the only transmembrane protein involved in enamel maturation (27). During the maturation stage, up-regulation of protein related to intracellular Ca²⁺-stores, including Ca²⁺-binding proteins (calreticulin and endoplasmic reticulum Ca²⁺-ATPases, and inositol 1,4,5-trisphosphate receptors have been reported (28). In the present study, expression of the NCX was observed throughout differentiation, indicating that the NCX is involved in enamel mineralization from the secretion to the maturation stages. The route by which Ca²⁺ from extracellular medium enters cells remains to be determined. Detection of Ca²⁺ signaling event(s)/protein(s) in ameloblasts is of immediate interest.

The value of the equilibrium binding constant for Ca²⁺

dependence on Ca²⁺ influx by NCX fell within the range of that for Ca²⁺ concentration in enamel fluid (0.25 mM). The value of K_D for Na⁺ dependence on Ca²⁺ efflux by NCX also fell within the range of that for Na⁺ concentration in enamel fluid, as well as serum. These results indicate that the ameloblast NCX is potentially active under normal physiological conditions. In addition, Na⁺ concentrations are 10% lower in secretory enamel fluid (140 mM) at the enamel formation site adjacent to the apical membrane in ameloblasts than in serum (2, 29). Radioactive sodium was localized on the basal side of the ameloblasts during maturation (30). This clearly indicates a coupling mechanism between incorporation of Na⁺ and extrusion of Ca²⁺ via Na⁺-Ca²⁺ exchange in ameloblasts. At a [Na⁺]_o level the same as that in enamel fluid, maximum rate of Ca²⁺ efflux reached a current density of 2.4 pA/pF. Current density for the NCX was reported to be 0.2–1.0 pA/pF in rat ventricular myocytes (31, 32), which express it at high density (250–400 exchanger molecules/μm²) (33). This indicates that high density expression of NCX in ameloblasts plays a key role in the Ca²⁺-transport pathway.

In conclusion, expression of NCX1 and NCX3 in apical/apicolateral membrane in ameloblasts acts as a Ca²⁺ extrusion system, using the transmembrane Na⁺ gradient to pump Ca²⁺ out of the cell. This serves as a directional Ca²⁺-transport pathway to the enamel mineralizing front.

Acknowledgments

This work was supported by HRC7/6A03 [MEXT HAITEKU (2004, 2006)], Grant-in-Aid (18592050/20592187) for Scientific Research, and grant for special expenses for graduate schools from MEXT of Japan; and a grant from the Dean of our school. We thank Professors Toshio Matsuda and Akemichi Baba for their kind gift of SEA0400 and Jeremy Williams, for his assistance with the English of this manuscript.

References

- 1 Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med*. 1998;9:128–161.
- 2 Hubbard MJ. Calcium transport across the dental enamel epithelium. *Crit Rev Oral Biol Med*. 2000;11:437–466.
- 3 Franklin IK, Winz RA, Hubbard MJ. Endoplasmic reticulum Ca²⁺-ATPase pump is up-regulated in calcium-transporting dental enamel cells: a non-housekeeping role for SERCA2b. *Biochem J*. 2001;358:217–224.
- 4 Salama AH, Zaki AE, Eisenmann DR. Cytochemical localization of Ca²⁺-Mg²⁺ adenosine triphosphatase in rat incisor ameloblasts during enamel secretion and maturation. *J Histochem Cytochem*. 1987;35:471–482.
- 5 Borke JL, Zaki Ae-M, Eisenmann DR, Mednieks MI. Localization of plasma membrane Ca²⁺ pump mRNA and protein in human ameloblasts by *in situ* hybridization and immunohistochem-

- istry. *Connect Tissue Res.* 1995;33:139–144.
- 6 Sasaki T, Colflesh DE, Garant PR. Calcium transport by a calmodulin-regulated Ca-ATPase in the enamel organ. *Adv Dent Res.* 1987;1:213–226.
- 7 Iwamoto T. Forefront of Na⁺/Ca²⁺ exchanger studies: molecular pharmacology of Na⁺/Ca²⁺ exchange inhibitors. *J Pharmacol Sci.* 2004;96:27–32.
- 8 Quednau BD, Nicoll DA, Philipson KD. The sodium/calcium exchanger family-SLC8. *Pflugers Arch.* 2004;447:543–548.
- 9 Schnetkamp PP. The SLC24 Na⁺/Ca²⁺-K⁺ exchanger family: vision and beyond. *Pflugers Arch.* 2004;447:683–688.
- 10 Shibukawa Y, Suzuki T. Measurements of cytosolic free Ca²⁺ concentrations in odontoblasts. *Bull Tokyo Dent Coll.* 1997;38:177–185.
- 11 Shibukawa Y, Suzuki T. A voltage-dependent transient K⁺ current in rat dental pulp cells. *Jpn J Physiol.* 2001;51:345–353.
- 12 Shibukawa Y, Suzuki T. A small-conductance Ca²⁺-activated K⁺ current and Cl⁻ current in rat dental pulp cells. *Bull Tokyo Dent Coll.* 2000;41:35–42.
- 13 Shibukawa Y, Suzuki T. Ca²⁺ signaling mediated by IP₃-dependent Ca²⁺ releasing and store-operated Ca²⁺ channels in rat odontoblasts. *J Bone Miner Res.* 2003;18:30–38.
- 14 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods.* 2001;25:402–408.
- 15 Bers DM, Patton CW, Nuccitelli R. A practical guide to the preparation of Ca²⁺ buffers. *Methods Cell Biol.* 1994;40:3–29.
- 16 Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, et al. SEA0400, a novel and selective inhibitor of the Na⁺-Ca²⁺ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther.* 2001;298:249–256.
- 17 Nagano T, Kawasaki Y, Baba A, Takemura M, Matsuda T. Up-regulation of Na⁺-Ca²⁺ exchange activity by interferon-gamma in cultured rat microglia. *J Neurochem.* 2004;90:784–791.
- 18 Wöltgens JH, Lyaruu DM, Bronckers AL, Bervoets TJ, Van Duin M. Biomineralization during early stages of the developing tooth in vitro with special reference to secretory stage of amelogenesis. *Int J Dev Biol.* 1995;39:203–212.
- 19 Takano Y, Ozawa H. Ultrastructural and cytochemical observations on the alternating morphologic changes of the ameloblasts at the stage of enamel maturation. *Arch Histol Jpn.* 1980;43:385–399.
- 20 Karg HA, Burger EH, Lyaruu DM, Wöltgens JH, Bronckers AL. Gene expression and immunolocalisation of amelogenins in developing embryonic and neonatal hamster teeth. *Cell Tissue Res.* 1997;288:545–555.
- 21 Nagano T, Oida S, Ando H, Gomi K, Arai T, Fukae M. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J Dent Res.* 2003;82:982–986.
- 22 Papagerakis P, Ibarra JM, Inozentseva N, DenBesten P, MacDougall M. Mouse amelogenin exons 8 and 9: sequence analysis and protein distribution. *J Dent Res.* 2005;84:613–617.
- 23 Iwamoto T, Shigekawa M. Differential inhibition of Na⁺/Ca²⁺ exchanger isoforms by divalent cations and isothiourea derivative. *Am J Physiol.* 1998;275:C423–C430.
- 24 Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T. The exchanger inhibitory peptide region-dependent inhibition of Na⁺/Ca²⁺ exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol.* 2004;66:45–55.
- 25 Iwamoto T, Kita S, Uehara A, Imanaga I, Matsuda T, Baba A, et al. Molecular determinants of Na⁺/Ca²⁺ exchange (NCX1) inhibition by SEA0400. *J Biol Chem.* 2004;279:7544–7553.
- 26 Gobbi P, Castaldo P, Minelli A, Salucci S, Magi S, Corcione E, et al. Mitochondrial localization of Na⁺/Ca²⁺ exchangers NCX1-3 in neurons and astrocytes of adult rat brain in situ. *Pharmacol Res.* 2007;56:556–565.
- 27 Takano Y, Akai M. Demonstration of Ca²⁺-ATPase activity in the maturation ameloblast of rat incisor after vascular perfusion. *J Electron Microscop.* (Tokyo). 1987;36:196–203.
- 28 Hubbard MJ. Abundant calcium homeostasis machinery in rat dental enamel cells. Up-regulation of calcium store proteins during enamel mineralization implicates the endoplasmic reticulum in calcium transcytosis. *Eur J Biochem.* 1996;239:611–623.
- 29 Aoba T, Moreno EC. The enamel fluid in the early secretory stage of porcine amelogenesis: chemical composition and saturation with respect to enamel mineral. *Calcif Tissue Int.* 1987;41:86–94.
- 30 Kawamoto T, Shimizu M. Changes in the mode of calcium and phosphate transport during rat incisal enamel formation. *Calcif Tissue Int.* 1990;46:406–414.
- 31 Despa S, Brette F, Orchard CH, Bers DM. Na/Ca exchange and Na/K-ATPase function are equally concentrated in transverse tubules of rat ventricular myocytes. *Biophys J.* 2003;85:3388–3396.
- 32 Ricci E, Smallwood S, Chouabe C, Mertani HC, Raccurt M, Morel G, et al. Electrophysiological characterization of left ventricular myocytes from obese Sprague-Dawley rat. *Obesity.* 2006;14:778–786.
- 33 Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev.* 1999;79:763–854.