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HISTOCHEMICAL DEMONSTRATION AND MICROANALYSIS OF POSSIBLE CALCIUM BINDING SITES IN THE ENAMEL ORGAN OF RAT INCISORS

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Abstract

The rat incisors obtained from rats perfused with high-calcium solution containing 30 to 50 mM $CaCl_2$ were processed for rapid freeze/freeze-substitution and embedded in epoxy resin. GBHA staining, a histochemical staining for calcium, of unhydrously prepared sections revealed a large number of granular Ca-GBHA reactions in the enamel organ, most of which being located along the lateral plasma membranes of the ameloblasts. In the ameloblast layer, the reaction was negative in the presecretory stage, became intense in concert with the onset of enamel matrix formation, and remained so by the end of the transitional stage where the reaction gradually diminished. In the maturation stage, similar GBHA reactions emerged only in association with the smooth-ended ameloblasts and were absent in the ruffle-ended ameloblasts. Some granular reactions of much smaller diameter were occasionally noted in other cells of the enamel organ at both the secretory and maturation stages. Electron microscopy confirmed large electron-dense granules, each showing distinct peaks for Ca and P by microanalysis, associated with the cytoplasmic aspect of lateral plasma membranes of secretory, transitional, and smooth-ended ameloblasts.

The present study thus revealed a strong calcium-binding property of the cytoplasmic aspect of lateral plasma membranes of secretory ameloblasts and its fluctuation in the maturation ameloblasts under the given experimental conditions. A necessity for an extensive analysis of membrane constituents of the enamel organ was proposed for better understanding of cellular roles in calcium regulation during amelogenesis.

Key Words: Rat incisor, rapid freezing, freeze-substitution, calcium, microanalysis, vascular perfusion, calcium-binding sites, ameloblast, GBHA.

Introduction

It has been widely accepted that the ameloblasts regulate calcium influx in growing enamel in different manners at different developmental stages. Secretory ameloblasts appear to serve as a selective diffusion barrier to various molecules and restrict calcium acquisition in the newly formed enamel (Bawden and Wennberg, 1977; Crenshaw and Takano, 1982; Bawden, 1989). As regards the maturation stage of amelogenesis, the ruffle-ended ameloblasts actively transport calcium in maturing enamel undergoing extensive mineralization, whereas calcium seems to penetrate to the enamel surface paracellularly at the regions of the smooth-ended ameloblasts (Crenshaw and Takano, 1982; Takano *et al.*, 1987; Bawden, 1989).

Various molecules such as lanthanum (Takano and Crenshaw, 1980; Josephsen, 1984; Takano and Ozawa, 1984), horseradish peroxidase (Kallenbach, 1980a, b; Takano and Ozawa, 1980; Sasaki, 1984; Sasaki *et al.*, 1984; Matsuo *et al.*, 1986), microperoxidase (Takano and Ozawa, 1984), and radio-labeled various molecular-weight proteins (Kinoshita, 1979; McKee *et al.*, 1986) have been used as the tracer to assess the transport pathways of various organic and inorganic materials through the ameloblast layer.

It is obvious that radioactive calcium is the currently-available most reliable tracer for the detection of the transport pathway of calcium only if the isotope can be precisely localized and visualized as a function of time. Recent improvements in, ^{45}Ca autoradiography meet the requirement, and application of this method on anhydrously prepared specimens has in fact enabled demonstration of a concentration gradient of ^{45}Ca and its time-related changes in the secretory ameloblast layer (Kawamoto and Shimizu, 1987; Takano *et al.*, 1990a; Hanawa *et al.*, 1990), supporting transcellular calcium transport through the secretory ameloblasts (Takano *et al.*, 1990a; Hanawa *et al.*, 1990).

As an alternative attempt to trace the practical pathway followed by calcium from the extracellular fluid compartment of the enamel organ to the enamel matrix, efforts have been made to localize calcium in the enamel organ using histo-cytochemical methods. A majority of

investigators employed potassium pyroantimonate (PPA) method (Komnick, 1962; Spicer *et al.*, 1968) which enabled localization of cellular calcium as electron-opaque precipitates of Ca-PPA complex at the electron microscopic resolution. Due to the lack of specificity of PPA to calcium and to the inconsistency in results, the reliability of PPA method has been debated and the necessity for carefully controlled experiments has been suggested. With the PPA method, Ca-PPA deposits occur primarily in association with mitochondria and also along the cytoplasmic surface of disto-lateral membranes of both secretory and maturation ameloblasts (Reith and Boyde, 1985; Kogaya and Furuhashi, 1988; Eisenmann *et al.*, 1990).

Using glyoxal bis(2-hydroxyanil) (GBHA) staining, a histochemical method to localize soluble calcium as red Ca-GBHA complex, Kashiwa and Sigman (1966) demonstrated the first morphological evidence indicating the presence of detectable calcium in the ameloblasts in fresh slices of rat molars. Due to unavoidable deterioration of specimens, their method did not allow correlation of reaction sites with subcellular structures. A marked improvement in demonstrating cellular calcium by GBHA staining has been attained in our previous studies (Takano *et al.*, 1988, 1989, 1990b), allowing a demonstration of clear and reproducible Ca-GBHA reactions in cells of the enamel organ. Despite the improvement of methods, GBHA staining failed to stain plasma membranes of ameloblasts *in vivo* that showed significant calcium precipitates by the PPA method.

The objective of the present study was to introduce an experimental condition by which a distinction between the plasma membranes of ameloblasts with and without calcium-binding property could be made by the GBHA method. A large number of granular Ca-GBHA reactions and distinct electron-dense calcium-loaded deposits occurred in specific regions of the enamel organ at specific developmental stages in our experimental condition.

Materials and Methods

Procedures of vascular perfusion

Fifteen male rats of the Wistar strain (40-100 g), fed *ad libitum* were divided into five groups, each consisting of three animals. The rats were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg body weight) and perfused with 0.25 M sucrose solution containing various concentration of $CaCl_2$ (3, 10, 30 or 50 mM) for 5 to 10 minutes at room temperature by a peristaltic pump (Taiyo, Tokyo, Japan) at the rate of 3 ml/min, through a cannula inserted through the left ventricle to the ascending aorta. The right antrum was cut for drainage.

Rapid freezing and freeze substitution

In each animal, upper and lower incisors were dissected with the intact enamel organ attached and immediately quenched in liquid propane cooled with liquid nitrogen. The frozen specimens were transferred to cold pure acetone (- 80 °C) or cold acetone containing 1% osmium tetroxide, each containing molecular sieves (3A 1/16) (Nakarai, Kyoto, Japan). They were kept for 4 days for freeze substitution. The temperature of the acetone was then slowly brought up to room temperature, and specimens were rinsed in fresh acetone for 30 minutes and embedded in Epon 812 (Taab, Berkshire, U.K.).

Glyoxal bis(2-hydroxyanil)(GBHA) staining

Semi-thin Epon sections, 2 to 3 μm thick, of the labial aspect of each incisor were cut with glass knives parallel to the longitudinal tooth axis and stained with an alcoholic 5% GBHA solution. The GBHA staining solution consisted of 20 mg GBHA (Fluka, Switzerland) and 0.4 ml 75% ethanol containing 3.4% NaOH. The solution was initially orange in color and turned dark red when it was ready for use. It was poured over the sections collected on clean dry glass slides and left for 3 to 5 minutes at room temperature. The sections were then rinsed with absolute ethanol until the base plastic was decolorized, briefly immersed in xylene, and mounted with Entellan Neu (Merck, Darmstadt, Germany). Observations were made by the light microscope under bright- or dark-field illumination.

Electron microscopy and electron microprobe analysis

For ultrastructural observations, ultrathin sections (pale gold) were cut with a diamond knife, floated on ethylene glycol instead of water in the trough to avoid loss of water-soluble components, and picked up on a collodion-coated copper grid. They were examined unstained under a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Some of the thicker sections (0.1 μm), similarly prepared as described above, were carbon-coated and examined with an analytical electron microscope (Hitachi H-800) equipped with an energy-dispersive X-ray analysis system (KEVEX-7000). The grids were mounted on a carbon holder and point analysis was performed at 75 kV and 30,000x magnification. The spot spectra were stored for 100 second periods at a current of about 4 x 10^{-11} A, and recorded photographically from a TV display.

Results

The overall integrity and cellular morphology of the enamel organ were well preserved in rapidly frozen and freeze-substituted specimens pre-perfused with calcium-containing sucrose solution.

Characteristic granular Ca-GBHA reactions appeared in specimens obtained from rats pre-perfused with high calcium-containing solution, whereas they were negative in those treated with physiological calcium solution (3 mM). The granular reaction was sporadic with 10 mM calcium and became consistent when a perfusate containing 30 mM or higher concentration of calcium was used. The following data were therefore depicted from the specimens perfused with high Ca-sucrose solution containing 30 mM or 50 mM $CaCl_2$.

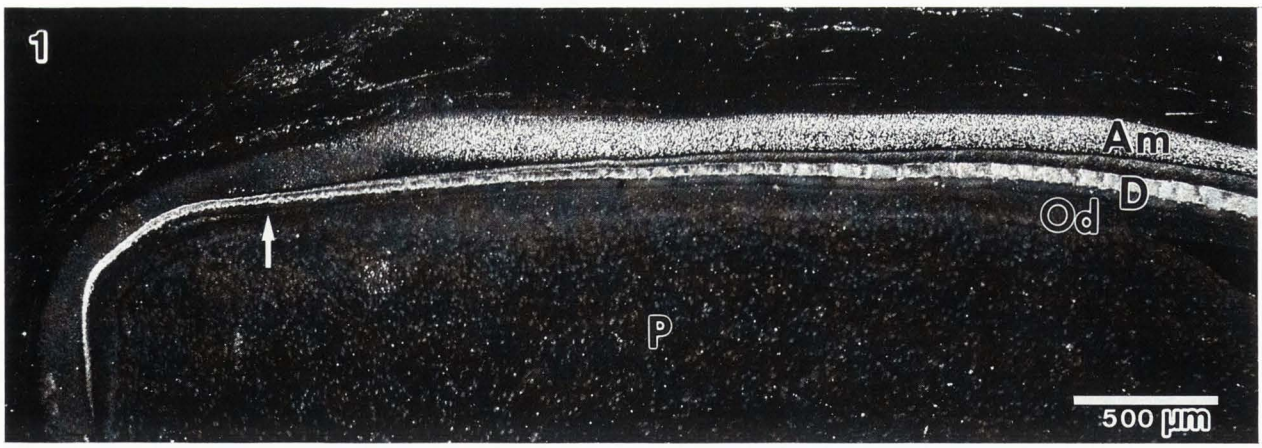
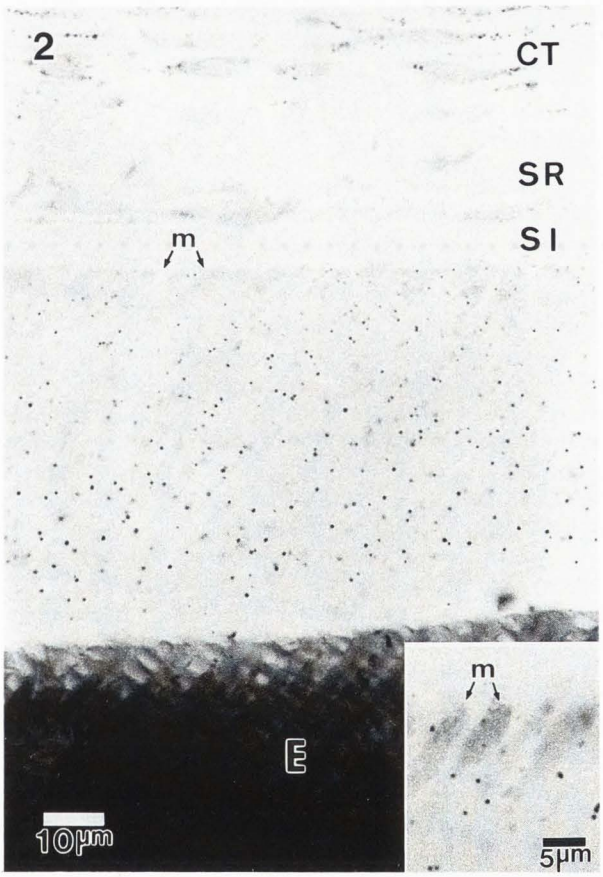


Fig. 1. A panoramic dark-field light microscopic image of granular GBHA reactions in the ameloblast layer (Am) of lower incisor of the rat after vascular perfusion with high Ca-sucrose solution. Granular GBHA reactions (bright dots) first appear at the proximal portion of the ameloblast and extend toward the distal cell end after the onset of enamel matrix secretion (white arrow). Note absence of reactions in the odontoblasts (Od) and pulp tissue (P). D represents dentin.

Fig. 2. A bright-field image of the enamel organ at the stage of inner enamel secretion showing large granular GBHA reactions throughout the ameloblast layer. No comparable reactions are seen in other cells of the enamel organ. Fairly large granular GBHA reactions are recognizable in the cytoplasm of fibroblastic cells of the adjacent connective tissue (CT). Note light GBHA reactions of mitochondria (m) clustered in proximal portions of ameloblasts and its absence in cells of the stratum intermedium (SI). SR stellate reticulum E enamel. **Inset**: Enlarged view of the proximal cytoplasm of ameloblasts. Mitochondria (m) show significant agranular Ca-GBHA reactions.

Secretory and transitional stages - GBHA staining

Dark-field illumination revealed individual granular Ca-GBHA reactions as distinct bright dots and enabled panoramic observations of reactions at low magnifications (Fig. 1). As shown in Figure 1, granular reactions of Ca-GBHA were almost exclusively localized in the ameloblast layer. In the ameloblast layer, the reaction was negative in the presecretory ameloblasts and appeared almost in concert with the onset of enamel matrix formation. At the initial stage of matrix formation, granular GBHA reactions were restricted to the proximal end of young secretory ameloblasts and gradually extended toward the distal, secretory pole along with the commencement of enamel matrix formation. The granular reaction did not appear beyond the distal junctional complexes of the secretory ameloblasts.



Under bright-field illumination, numerous dark-red granular reactions of Ca-GBHA, approximately 0.7 µm in diameter, appeared primarily in association with lateral cell borders of secretory ameloblasts (Fig. 2). It could not be clarified whether the granular reaction was intracellular or extracellular under the light microscope. Mitochondria packed in the proximal cytoplasmic compartment of secretory ameloblasts showed indistinct but significant agranular Ca-GBHA reactions (Fig. 2, inset). Other cytoplasmic organelles of secretory ameloblasts

did not show reactions for calcium. Other cells of the enamel organ except for those in the stratum intermedium also displayed some GBHA reactions associated with mitochondria (Fig. 2).

Spindle-shaped cells in the connective tissue adjacent to the enamel organ displayed a fairly large number of distinct GBHA reactions in relation to mitochondria (Fig. 2).

At the transitional stage, granular GBHA reactions along the lateral plasma membranes of ameloblasts further extended toward the distal cell ends. In specimens where the maturation stage started off with the ruffle-ended type ameloblasts, GBHA reactions in the transitional ameloblast gradually decreased in number as well as intensity and, disappeared by the onset of the maturation stage. On the other hand, granular GBHA reactions in the transitional ameloblasts did not disappear when the transitional ameloblasts transformed to the smooth-ended type maturation ameloblasts (shown later in Fig. 6). At this stage, GBHA reaction was almost negative in other cells of the enamel organ.

Secretory and transitional stages - Electron microscopy

Electron microscopically, cells of the enamel organ, the distal portion of the ameloblast in particular, suffered from certain morphological damage due to ice crystals formed in the process of rapid freezing and freeze substitution. Nevertheless, it appeared that sucrose in the perfusate dramatically reduced ice crystal damage even at portions of the enamel organ far apart from the frozen surface to such a degree that the electron microscopic distinction of cytoplasmic features could be made. Distinct electron-dense granular deposits appeared exclusively in portions of the enamel organ that showed intense granular reactions in the adjacent thick sections with GBHA staining.

In the secretory ameloblast layer, numerous large electron-dense granules, approximately 0.24 μm in diameter were located along the cytoplasmic aspect of lateral plasma membranes (Figs. 3-5). No correlation between the electron-dense granules and gap junctions was noted. There were no extracellular dense deposits along the outer surface of the cell nor within pericellular spaces. Mitochondria in secretory ameloblasts mostly contained numerous electron-dense granules much smaller in diameter compared with those associated with plasma membranes (Figs. 3, 4). The number and electron density of mitochondrial dense granules fluctuated among the individual cells.

The electron-dense granules associated with the plasma membrane were also noted in cells of the stratum intermedium (Fig. 3). They were generally smaller in size relative to those in the ameloblast layer, but larger than those in ameloblast mitochondria. No electron-dense granules were observed in mitochondria in this cell layer. Similar membrane-associated electron-dense granules also appeared in the stellate reticulum and outer enamel epithelium. The incidence of granular deposits in these cell layers was less frequent than in the stratum intermedium (Fig. 3).

Fig. 3. Electron micrograph of secretory ameloblasts (Am), stratum intermedium (SI), and stellate reticulum (SR) of the lower incisor of the rat perfused with high Ca-sucrose solution. In addition to large electron-dense deposits along lateral cell borders of ameloblasts (Am), many dense granular deposits of much smaller size (arrows) are scattered in association with cells of the stratum intermedium (SI) and stellate reticulum (SR). Mitochondria (m) in the proximal cytoplasm of ameloblasts contain a large number of small electron-dense deposits.

Fig. 4. Enlarged view of one of large granular deposits (arrow) associated with the cytoplasmic aspect of lateral cell membrane of secretory ameloblast. A large number of small granular deposits can be seen in mitochondria (m). N represents nucleus.

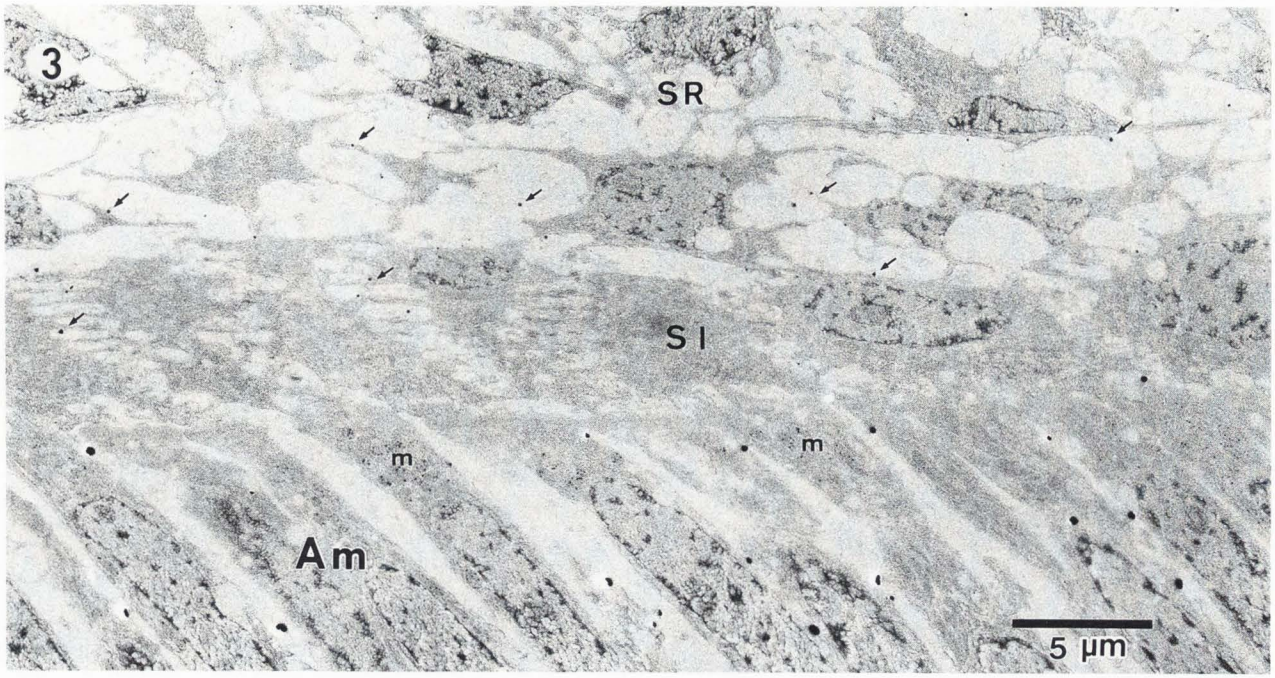
Fig. 5. Scanning-transmission electron micrograph of a 0.1 μm thick section showing numerous electron-dense granules along the lateral cell borders of secretory ameloblasts. Electron-dense granules are not seen beyond the distal intercellular junctions (arrows). N represents nucleus, SI stratum intermedium, and asterisk represents Tomes process.

Maturation stage - GBHA staining

The cells in the connective tissue adjacent to the enamel organ mostly showed considerable GBHA reactions related primarily to mitochondria (Figs. 6-9). No significant GBHA reaction was detected in the odontoblasts and other pulp cells at all developmental stages examined (see Fig. 1).

The early stage of enamel maturation where the enamel matrix could be sectioned with glass knives was investigated. Generally, each histologic section contained two cycles of alternate ameloblast modulation from the ruffle-ended to smooth-ended and vice versa.

In the ameloblast layer, intense granular Ca-GBHA reactions only appeared in association with the smooth-ended ameloblasts (Figs. 7, 8). The appearance and disappearance of the reaction was basically correlated with the timing of disappearance and re-appearance of the ruffled border at the distal end of ameloblasts (Fig. 7). In some cases, a small group of ruffle-ended ameloblasts located immediately apical to the apical border of the band of smooth-ended ameloblasts showed granular Ca-GBHA reactions. The intensity of the reaction and the extent of reactive ruffle-ended ameloblasts varied according to the smooth-ended regions examined. The most extreme example is shown in Fig. 9. The cells of the papillary layer adjacent to the reactive ruffle-ended ameloblasts also showed variable granular reactions (Fig. 9). In other cells of the enamel organ, no significant intracellular GBHA reaction was noted throughout the entire regions except for some indistinct reactions associated with mitochondria.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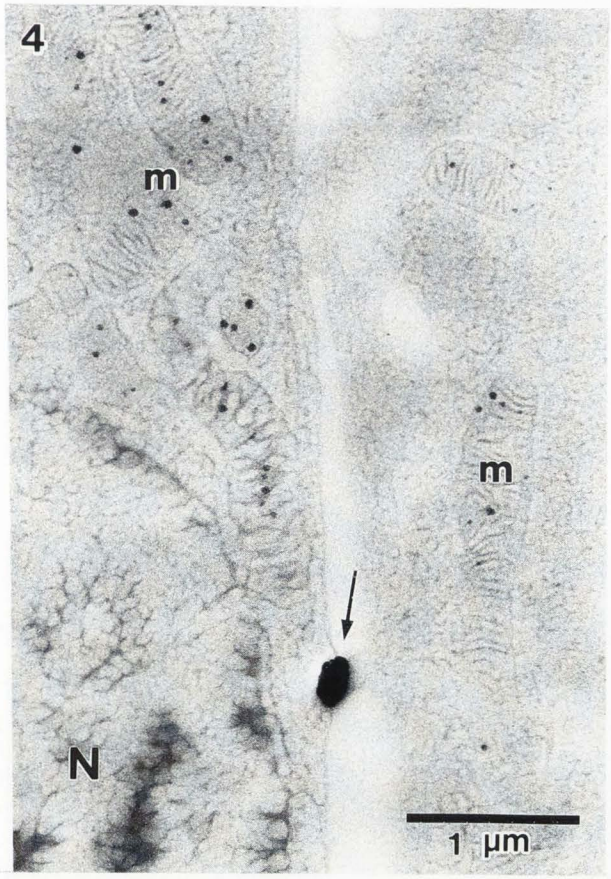
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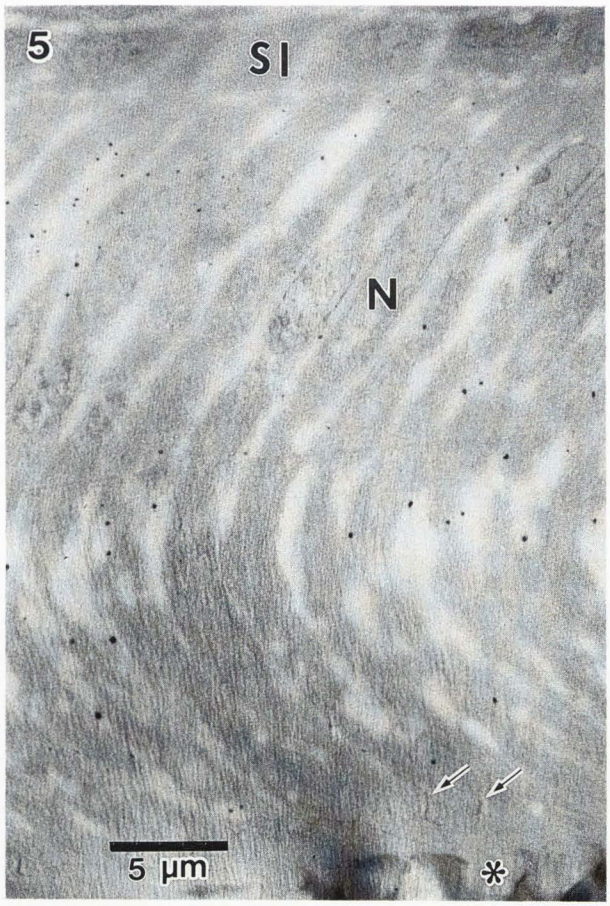
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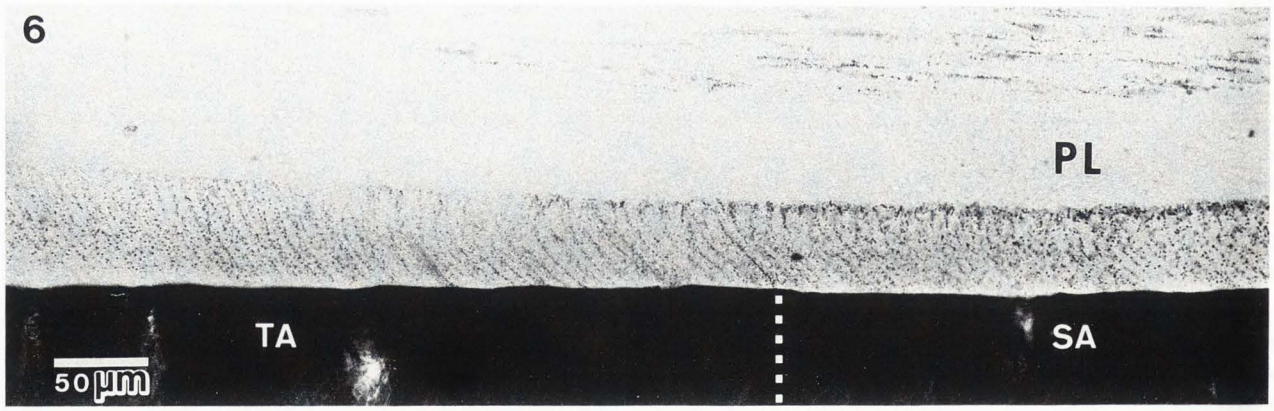
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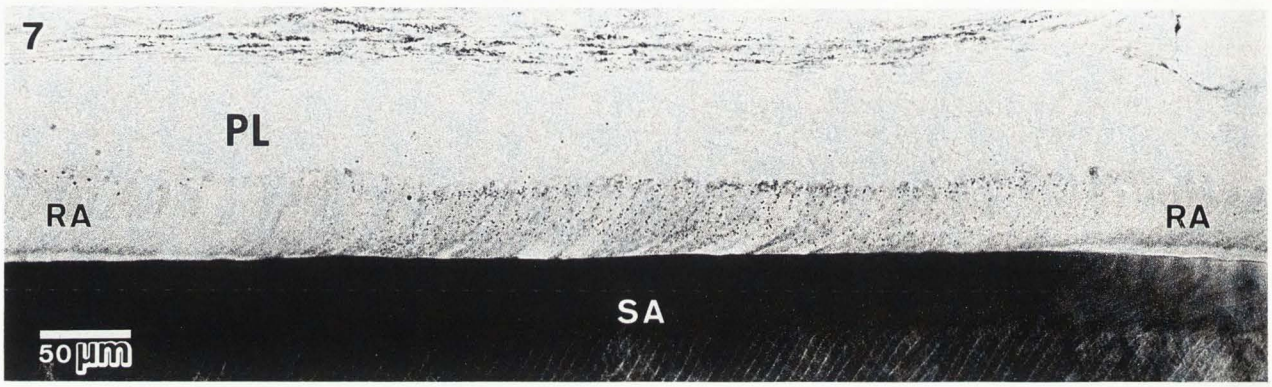
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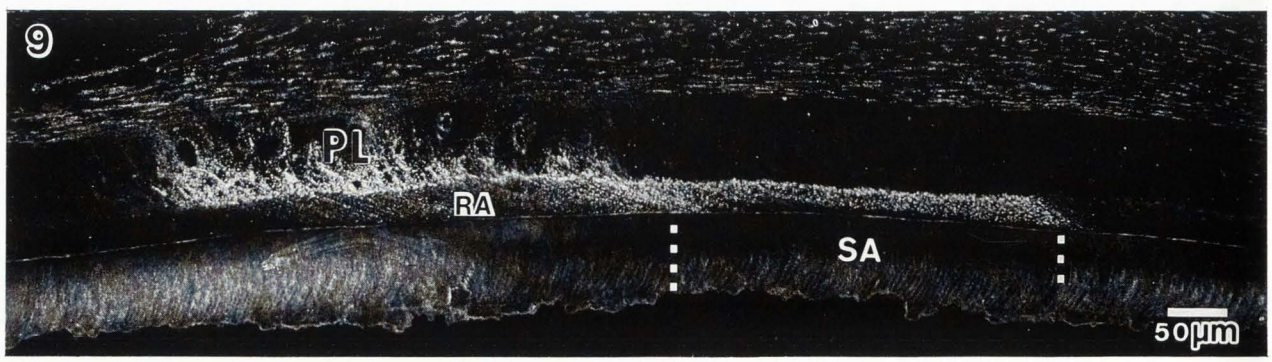
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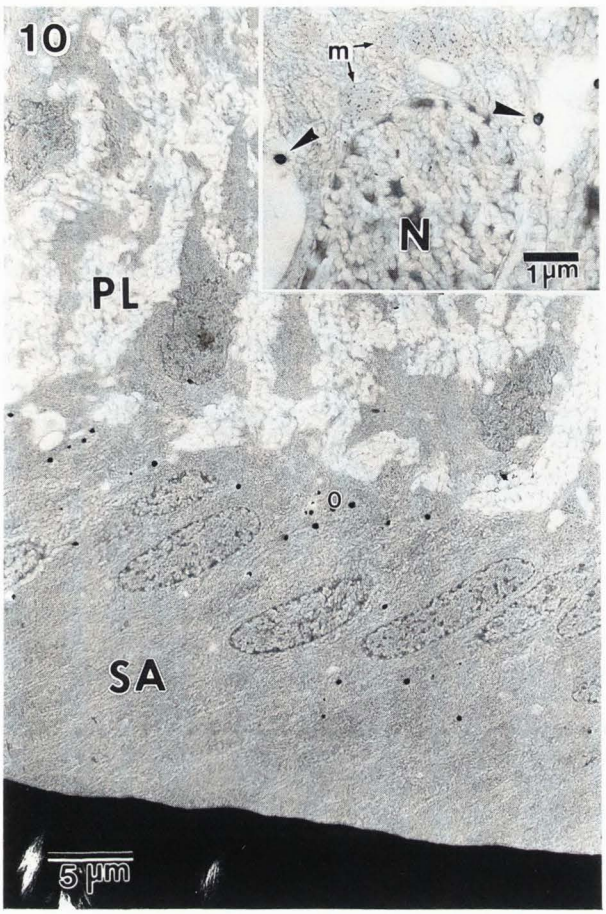

Fig. 6. Bright-field light micrograph of GBHA reactions in the ameloblasts layer at the transitional (left hand side of vertical dots) and initial phase of enamel maturation (right hand side of vertical dots). In this particular specimen where granular GBHA reactions in transitional ameloblasts persist, the maturation phase starts off with smooth-ended ameloblasts (SA) that display distinct granular reactions. PL represents papillary layer without reaction.

Fig. 7. Bright-field light micrograph showing distinct granular GBHA reactions associated with smooth-ended ameloblasts (SA) and its absence in adjacent ruffle-ended ameloblasts (RA). Note absence of reactions in the papillary layer (PL) and intense reactions in cells of adjacent connective tissue.

Fig. 8. Dark-field image of similar region as shown in Fig. 7 showing connective tissue (CT), papillary layer (PL), ameloblasts (Am), and enamel (E).

Fig. 9. Dark-field view of smooth-ended ameloblasts (SA) and apically situated ruffle-ended ameloblasts (RA) and adjacent cells of the papillary layer (PL), all showing distinct granular GBHA reactions. Vertical dots indicate approximate border between smooth-ended and ruffle-ended ameloblasts.

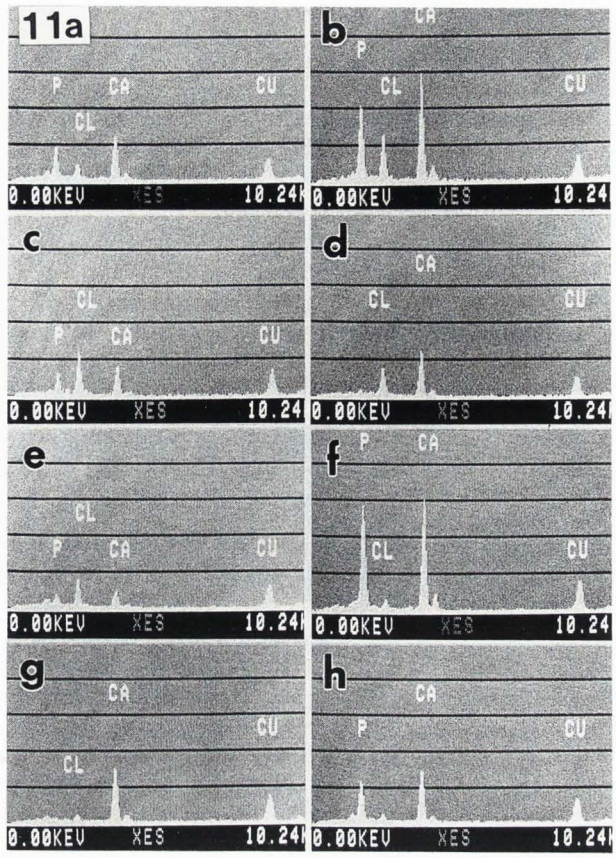

Fig. 10. Electron micrograph of the region of the smooth-ended ameloblasts (SA). Large electron-dense granular deposits are only seen in the ameloblast layer. PL represents papillary layer. **Inset**: Proximal end of a smooth-ended ameloblast. Electron-dense granules are associated with the cytoplasmic surface of lateral plasma membranes (arrowheads). m represents mitochondria containing small granular deposits, and N nucleus.

Fig. 11. A comparison of X-ray energy spectra generated from electron-dense granular deposits at various portions of the enamel organ of anhydrously prepared ultrathin sections. **a**: Mitochondria of secretory ameloblasts. **b**: Large granular deposit associated with lateral plasma membrane of secretory ameloblast. **c, d**: Stratum intermedium. **e**: Stellate reticulum. **f**: Smooth-ended ameloblasts. **g, h**: Papillary layer. Note the presence of peaks of calcium (CA) and chloride (CL) in all the deposits examined and lack of peaks of phosphorus (P) in some of those in the stratum intermedium (d) and papillary layer (g). Relative proportion of major constituents of the individual deposits (Ca, P, CL) differs markedly among each other.

Maturation stage - Electron microscopy

Large electron-dense granular deposits were located along the cytoplasmic aspect of all the smooth-ended ameloblasts examined (Fig. 10). Some of the

ameloblasts undergoing a smooth-ended to ruffle-ended morphologic transition, and their neighboring cells of the papillary layer also showed similar granular deposits. Electron-dense granular deposits in the latter cells were generally smaller in diameter than those associated with the smooth-ended ameloblasts. Some of the mitochondria in both types of ameloblasts (ruffle-ended and smooth-ended) contained electron-dense granules of much smaller diameter (Fig. 10, inset). Mitochondria in papillary cells displayed no such granular deposits.

Electron microprobe analysis

Since the X-ray spectrum for phosphorus and osmium overlap, specimens freeze-substituted with acetone alone were chosen for electron microprobe analysis.

Microprobe analysis of the electron-dense granules associated with secretory ameloblasts, cells of the stratum intermedium, stellate reticulum, papillary layer, and smooth-ended ameloblasts respectively demonstrated considerable presence of calcium in X-ray spectra (Fig. 11). There was a positive correlation between the size of the granular deposit and its calcium content: the larger the granule, the more the calcium. Phosphorus and chlorine were also major constituent of the granular deposit. Relative proportion of these three elements was consistent among the electron-dense granules located in the ameloblast layer in both the secretory and maturation stages. It fluctuated to a great extent in some of the granular deposits in other regions of the enamel organ. In the stratum intermedium and the papillary layer, the phosphorus was almost missing in X-ray spectrum for some of the electron-dense granules and, occasionally, in the stellate reticulum, chlorine showed the largest peak. Calcium or phosphorus were undetectable in X-ray spectrum from any areas of the enamel organ which was devoid of electron-dense granular deposits.

Discussion

Calcium along the plasma membrane

The present data have provided firm evidence that indicate a strong tendency of lateral plasma membranes of secretory, transitional, and smooth-ended ameloblasts of rat incisors to bind calcium and form coarse granular calcium-rich deposits along its cytoplasmic aspects, when perfused with high calcium-containing solution before freezing.

The presence of similar GBHA-stainable, calcium-rich electron-dense granules was demonstrated in the duodenal mucous membrane of the rat where the granular deposits were shown to be associated with basolateral membranes of absorptive epithelial cells (Takano and Akai, 1988). Since the calcium-rich granular deposits were constantly seen in the duodenal epithelium in the rat grown under normal diet and the granular deposits fluctuated drastically in number according to the luminal calcium concentration, the authors speculated that the granular deposits might represent calcium in transit through the absorptive epithelial cell layer *in vivo*. The X-ray peaks for calcium and phosphorus generated from dense granular deposits in the duodenal tissue were comparable to those detected in the enamel organ in the present study. It should be noted that despite the similarity to calcium transporting epithelia, the physiological significance of the granular deposits in both tissues should not simply be correlated. In the present study, vascular perfusion of the enamel organ was undertaken deliberately in such a way that no physiological cellular events could be expected. Calcium-rich membrane-associated large granular deposits never appear in untreated enamel organ (Takano *et al.*, 1988, 1989) or those specimens pretreated with a perfusate containing physiological calcium (3 mM). The membrane-associated granules depicted in our system in the ameloblast and some other cells of the enamel organ may not represent biological cellular activity related to calcium. We assume that the deposits may be the consequence of physico-chemical interactions between high doses of calcium ions in pericellular fluids and certain constituents of the plasma membrane. The location of granular deposits in the enamel organ appears to represent possible calcium-binding sites that have been undetectable by regular methods. Whether the putative calcium-binding sites contribute to the translocation of calcium or to other cellular events is a subject to be explored later in the discussion.

The association of detectable calcium with the cytoplasmic aspect of the plasma membrane of the ameloblast *in vivo* has been demonstrated by a number of investigators by means of a cytochemical method in which potassium pyroantimonate (PPA) has been used as calcium precipitant (Eisenmann *et al.*, 1979, 1982, 1990; Reith and Boyde, 1985; Chen *et al.*, 1986; Ashrafi *et al.*, 1987; Lyaruu *et al.*, 1985; Kogaya and Furuhashi, 1988). Although the results from preceding studies and current investigation both indicate the tendency of calcium to precipitate along the plasma membranes of ameloblasts, the significance of the individual data appears totally different. Cytochemical calcium-PPA reactions have been considered as representing physiological, detectable calcium and have been shown to occur along the inner aspect of the plasma membranes of both the secretory ameloblasts and maturation ameloblasts (Reith and Boyde, 1985; Kogaya and Furuhashi, 1988; Eisenmann *et al.*, 1990). In fact, contrary to our observations, some of the authors revealed distinct membrane-bound Ca-PPA reactions in the ruffle-ended ameloblasts and much less in the smooth-ended ameloblasts, implicating less involvement of the latter ameloblasts in calcium uptake in adjacent enamel (Kogaya and Furuhashi, 1988; Eisenmann *et al.*, 1990).

As regards the presence or absence of calcium-rich deposits in maturation ameloblasts, the discrepancy between previous Ca-PPA cytochemistry and our data may be attributed both to the differences in the sensitivity of the methods used and the concentration of calcium ions in the microenvironment in the respective studies. In our experimental system, membrane-bound GBHA reactions and calcium-rich electron-dense granules are lacking in the enamel organ unless high doses of calcium are preloaded in the tissue (Takano *et al.*, 1988, 1989, 1990b). The results indicate a tendency of lateral plasma mem-

branes of smooth-ended ameloblasts to bind to large amounts of calcium when exposed to high doses of calcium ions, whereas they display no calcium precipitate detectable by our methods under biological conditions. It is also indicated that the plasma membrane of ruffle-ended ameloblasts that is assumed to be associated with cytochemically detectable calcium *in vivo* fails to induce calcium-rich granular precipitates when exposed to large doses of calcium.

Extensive microchemical and/or histochemical analyses of the membrane constituents of cells of the enamel organ will be needed to disclose factors causing the distinct precipitation of membrane-bound calcium to occur or not to occur in relation to cellular differentiation and function.

Calcium-binding proteins

Vitamin D-dependent calcium-binding proteins (Ca-BPs) have been implicated to play important roles in calcium transport processes through the intestinal absorptive epithelium although the exact mechanism how Ca-BPs contribute to the transport or absorption of calcium by these cells remains to be established (see discussion by Taylor, 1984).

The presence of two types of intestinal-type vitamin D-dependent Ca-BPs associated primarily with ameloblasts undergoing matrix formation and/or maturation has been confirmed immunohistochemically in the enamel organ of rat incisors (Taylor, 1984; Taylor *et al.*, 1984; Berdal *et al.*, 1989, 1991). Although the findings implicate the role of these proteins in calcium regulation by the ameloblasts, there is no spatial correlation between the Ca-BP immunoreactivity and GBHA-reactive, electron-dense granular deposits observed in our study. Moreover, Taylor *et al.* (1984) clearly showed immunoreactivity for Ca-BP10, a calcium-binding protein located specifically in maturation ameloblasts, in both ruffle-ended and smooth-ended ameloblasts.

Calmodulin, a heat stable calcium-binding protein that activates calcium pumping ATPase (Ca^{2+}-ATPase), has been known to exist in developing tooth germs (Hubbard *et al.*, 1981) and, has been localized immunohistochemically in the secretory ameloblasts of rat incisors (Sasaki and Garant, 1987a). Experimental evidence by the latter authors suggested the role of calmodulin as the modulator of calcium extrusion pumps situated in the plasma membrane of secretory ameloblasts (Sasaki and Garant, 1986, 1987b). In a preliminary experiment, we injected some of the rats with trifluoperazine, a calmodulin blocker, according to Sasaki and Garant (1987a) 2 hours prior to vascular perfusion with high Ca-solution. As a result we found no differences as to the distribution and intensity of granular GBHA reactions in the enamel organ between the injected and uninjected animals (unpublished data). Taking all these and preceding experimental evidence into consideration, it is proposed that Ca-BPs known to be distributed in the ameloblast layer are unrelated to the formation of GBHA-stainable, electron-dense granular deposits in the ameloblast of perfused animals.

Heterogeneity of mitochondria in relation to calcium deposit

In previous histochemical studies we have demonstrated Ca-GBHA reactions in mitochondria of secretory as well as maturation ameloblasts, and lack or little staining of it in other cells of the enamel organ in rapidly frozen and freeze substituted rat incisors (Takano *et al.*, 1988, 1989).

Based on the results from calcium preincubation studies of rat molar tooth germs, Reith and Boyde (1978) have drawn a conclusion that calcium appears in ameloblast mitochondria if the plasma membrane of the cell is disrupted and/or if excess calcium gains entry to the cytosol from the surrounding extracellular space. They attributed the difference in staining among the cells of the enamel organ to differences in the magnitude of membrane disruption among the individual cells in tissue processing which, thereby allow an influx of various amounts of excess calcium from the extracellular fluid. The present observations do not support the conclusion made by these authors because of the lack of difference in mitochondrial calcium deposits between the experimental animals pretreated with physiological- or high-calcium containing solutions. Judging from numerous calcium-loaded precipitates along the cytoplasmic aspect of the plasma membrane, it is obvious that influx of vast amount of calcium in the cytosol of the ameloblast has occurred during vascular perfusion. These and our previous studies (Takano *et al.*, 1988, 1989) all seem to support that mitochondria in ameloblasts may contain higher amounts of calcium relative to those in other cells of the enamel organ under physiological conditions.

The heterogeneity of mitochondria in cells of the enamel organ in relation to calcium is supported by the experimental evidence that a drastic migration of mitochondria takes place only in secretory ameloblasts exposed to low calcium environment (Sasaki and Garant, 1987c; Takano and Wakita, 1991), but it was not the case in maturation ameloblasts (Takano and Wakita, 1991).

Ca-GBHA granules versus electron-dense granules

The average diameter of membrane-bound Ca-GBHA granular deposits in the ameloblast layer was approximately three times as large as that of the electron-dense granular deposits revealed by electron microscopy.

A question may be raised as to whether each granular Ca-GBHA reaction in fact corresponds to a single electron-dense granular deposit. Due to high alkalinity of the alcoholic GBHA staining solution (pH 13.5-14.0) that contains 3.4% NaOH, a marked softening and some deterioration of Epon sections occur in the staining process. This makes it extremely difficult to make GBHA-stained ultrathin sections observable with the electron microscope and, hence, no firm evidence supporting or contradicting the correlation between the granular Ca-GBHA deposit and calcium-rich electron-dense granule has been provided. Nevertheless, a careful examination of serial semithin and ultrathin sections by the respective methods supported an intimate correlation of the location, size, and density of the deposits that appeared in each histologic

section. The large diameter of granular deposits in GBHA-stained sections relative to the electron microscopic images may be attributed to the diffusion of Ca-GBHA complex occurring while staining within the sections.

Heterogeneity of electron-dense granular deposits

Although most of the electron-dense granular deposits in the enamel organ of perfused animals display distinct X-ray peaks for calcium and phosphorus by microanalysis, there are some granular deposits from which no significant X-ray peak for phosphorus is generated in our system. The latter case has not been encountered in the ameloblast layer, but in some of the cells of the stratum intermedium and the papillary layer. Such heterogeneity in elemental composition of the individual electron-dense granular deposits among cells of the enamel organ may be related to differences in the membrane constituents they are associated with. The peaks for chlorine may, to a large extent represent exogenous chlorine from the resin and from the chloride administered as $CaCl_2$.

It is likely that, at least in the ameloblast layer, the granular deposits associated with the plasma membrane represent calcium bound to membrane phospholipids, presumably phosphatidyl serine in particular, which has a strong and specific tendency to bind to calcium (Cullis and DeKruijff, 1979). Hauser *et al.* (1975) proposed a model for calcium binding to monolayer of phosphatidyl serine in that calcium is bound to adjacent phospholipid molecules through a two-point electrostatic attachment and the bound ions form a Stern layer instead of penetrating into the plane of the phosphate groups. Since this model of binding would allow the calcium atoms to interact with inorganic phosphate ions present in solutions, phosphorus detected in our dense granules might represent cytosolic phosphate coupled with membrane-bound calcium. In this context, phosphorus-free deposits in certain cells of the enamel organ may implicate the lack of available phosphate in the microenvironment of the respective cells. The absence of granular deposits in preameloblasts and ruffle-ended ameloblasts may indicate the lack or absence of membrane constituents having a high affinity for calcium ions.

Biological significance of membrane-bound calcium

The distribution of Ca-GBHA reactions in the ameloblast layer correlates intimately with the pattern of progressive differentiation of the ameloblast. As mentioned earlier, our findings do not represent physiological cellular activities related to calcium but only depict the sites of plasma membrane showing high tendency to bind to calcium when exposed to high-calcium containing solution. Accordingly, our data should not simply be correlated with the hypotheses raised by Reith and Boyde (1985) in that they proposed a calcium translocation within or along the cytoplasmic surface of the plasma membrane of both the secretory and maturation ameloblasts (not specified whether ruffle-ended or smooth-ended). In fact, in our experiments, calcium-loaded granular deposits in the layer of maturation ameloblasts occur almost specifically in association with the smooth-ended ameloblasts and are completely abolished in the ruffle-ended ameloblasts, the latter being regarded as sites for active calcium transport to adjacent enamel (Takano *et al.*, 1987).

The strong tendency of lateral plasma membranes of secretory, transitional, and smooth-ended ameloblasts to bind to excess amount of calcium may indicate the differences in mechanisms whereby cytosolic calcium level is regulated by these and non-reactive cells. It is of interest to note that the occasional appearance of Ca-GBHA deposits in portions of the ruffle-ended ameloblasts and papillary layer (Fig. 9) roughly corresponds with the regions of the enamel where significant uptake of radioactive calcium does not occur in ^{45}Ca autoradiography (Takano *et al.*, 1982). Extensive analyses of the membrane constituents of ameloblasts and other cells of the enamel organ throughout the amelogenesis are awaited to explore the biological significance of intriguing calcium-membrane interactions.

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Discussion with Reviewers

D.M. Lyaruu: The calcium concentration in the perfusate needed to produce GBHA reaction product in any of the cells studied was 30 mM Ca or higher, which is at least ten times higher than the physiological concentration of calcium in the body fluids; and secondly, the experiment was deliberately performed in such a way that no physiological events could be expected. In addition, it is assumed that the observed GBHA reaction product may be the consequence of physicochemical interactions between high doses of calcium ions in pericellular fluids and certain constituents of the plasma membrane. Taking the above-mentioned facts into consideration, is it possible that the phenomenon reported in the paper is in fact due to the initial phases of phospholipid mineralization rather than due to intrinsic calcium-binding activity of the plasma membranes?

Author: The author understands that the observed phenomena in this study do not represent cellular activity *in vivo* but reflect a physico-chemical property of the cell membranes. The major point I wish to raise in this paper is that there is a clear-cut difference in the calcium-related property of the plasma membrane of ameloblasts at different stages of enamel development, the evidence that has been overlooked in experiments undertaken under "physiological experimental conditions". The use of deliberately non-physiological high-calcium containing solution was in fact found to be an efficient mean to disclose differences in calcium-membrane interactions among the enamel organ cells. As you suggest, the deposition of electron-dense granules in the ameloblast layer may be comparable with so-called initial phase of phospholipid mineralization if one may wish. However, similar correlation may not be applicable to the phosphate-free, calcium-rich electron-dense granular deposits in some of the non-ameloblastic cells in the enamel organ. The significance of the deposition of calcium-loaded electron-dense granules will be further explored in future.

Reviewer I: It is stated in the discussion that the absence of granular GBHA deposits in preameloblasts and ruffle-ended ameloblasts may indicate lack or absence of membrane constituents having a high affinity for calcium. How can this be reconciled with the previous observations (under physiological conditions) of abundant pyroantimonate-bound calcium deposits in association with the membranes of these cells?

D.M. Lyaruu: The sites of the GBHA reaction product described in this paper are suggested to represent the calcium-binding domains. One question arises: Why does this phenomenon manifest itself only when abnormally high calcium concentrations are used and not at low (physiological) levels where one would expect the maximum calcium-binding activity of these sites?

Author: No firm evidence to explain the points raised by both reviewers is currently available. I would assume that one of the reasons is related to the sensitivity of the methods used in the respective experiments. An exposure to high-calcium solution may have exaggerated the minor differences in Ca-binding properties of the respective membranes in perfused specimens. It is important to emphasize that no calcium precipitant was used in current electron microscopy, and that GBHA only stains what would have appeared as calcium-loaded electron-dense granules if observed unstained under the electron microscope (sections stained with GBHA cannot be subjected for electron microscopic observations). An introduction of proper calcium precipitant or other novel calcium-detecting methods may allow distinction of similar variations in calcium-binding property of cell membranes without pre-exposing the tissue to such high doses of calcium as used in this study. Potassium pyroantimonate staining is worth trying on perfused animals.

Reviewer I: A substantial amount of GBHA precipitate is present on ruffle-ended ameloblasts and adjacent papillary cells in Fig. 9. How frequently was this observed and can it be explained?

Author: Among the eight SA regions examined, five were associated proximally with small groups of ruffle-ended ameloblasts showing some granular GBHA reactions; Fig. 9 is an extreme example. The bands of SA are known to move rather quickly toward the incisal direction. This means that the ruffle-ended ameloblasts apically adjacent to the band of SA have just transformed from smooth-ended ameloblasts and, hence, temporarily retain the membrane property of smooth-ended ameloblasts. The GBHA reaction in the papillary layer adjacent to the reactive ruffle-ended ameloblasts is unexplained.