A Histochemical Demonstration of Calcium in the Maturation Stage Enamel Organ of Rat Incisors

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Summary. The location of calcium in a rapid-frozen and freeze-substituted maturation stage enamel organ of the rat incisors was demonstrated by means of the glyoxal bis(2-hydroxyanil) (GBHA) staining method, which formed insoluble red precipitates of calcium-GBHA complex. In the ameloblast layer, highly GBHA-reactive tubulo-vesicular structures corresponding to mitochondria and some other membrane-bound structures were localized in both ruffle-ended and smooth-ended ameloblasts, although no significant GBHA reaction was localized in the nucleus, Golgi region, nor along the plasma membrane of these cells. In addition, numerous granular GBHA reactions appeared exclusively in association with the ruffled border of ruffle-ended ameloblasts. GBHA reactions were positive, but were considerably weaker in papillary cells than in the ameloblast. These observations provide a first published histochemical mapping of calcium in the maturation stage enamel organ, and suggest the active participation of mitochondria in maturation stage ameloblasts in calcium regulation.

The process of enamel maturation is characterized by an almost complete removal of water and organic components from the pre-formed young enamel (Reith and Cotty, 1967; Robinson et al., 1979; Robinson and Kirkham, 1985), accompanied by an ingress of massive amounts of calcium via the enamel organ (Engfeldt et al., 1954; Bélanger, 1957; Suga et al., 1970; Hammarström, 1976; Bawden and Wennberg, 1979). However, little is known with regard to the exact mechanism whereby the influx of calcium into the maturing enamel is regulated by the enamel organ.

In rat incisors, maturation ameloblasts undergo cyclical morphological changes during the maturation process (Suga, 1959; Warshawsky and Smith, 1974; Josephsen and Fejerskov, 1977; Takano and Ozawa, 1980; Reith and Boyde, 1981a; Akita et al., 1983). Recent autoradiographic studies have revealed a cyclical uptake of radiocalcium in the maturing enamel of rat incisors (Reith and Boyde, 1981b; Crenshaw and Takano, 1982; Takano et al., 1982; McKee and Warshawsky, 1986; McKee et al., 1987). Takano et al. (1982) pointed out an exact synchronization of the cycle of calcium uptake and that of morphological changes in overlying ameloblasts, and attributed the role of regulation of calcium acquisition in the enamel to the maturation ameloblasts.

In addition to maturation ameloblasts, the cells of the papillary layer (papillary cells), displayed morphological features characteristic of the ion-transporting cells, implicating their role in calcium regulation. The development of extensive gap junctions between the adjacent papillary cells and between them and ameloblasts suggests their possible participation in calcium transport to the enamel as a functional unit with maturation ameloblasts (Kal lenbach, 1966; Elwood and Bernstein, 1968; Garant, 1972; Sasaki and Higashi, 1983; Garant et al., 1984; Sasaki and Garant, 1986).

As is probably due to technical difficulty in retaining cell calcium, previous reports on the localization of calcium in the maturation stage enamel organ have been inconsistent. Reith and Boyde (1985) reported an occasional localization of pyroantimonate reactions along the cytoplasmic side of the plasma membrane of maturation ameloblasts and papillary cells, and proposed a transcellular calcium transport system along the plasma membrane. On the other hand, earlier 45Ca autoradiography (Munhoz and Leblond, 1974) and the electron probe analysis (Boyde and Reith, 1978) of maturation ameloblasts of rat incisors indicated the absence of calcium in both the intracellular and intercellular compartments.
of these cells, and provided support for the absence of positive regulatory roles in maturation ameloblasts with respect to the movement of calcium.

It thus appeared necessary to introduce a reliable tissue preparation method whereby the intracellular calcium may be retained in its original location, and to effect the mapping of calcium in the enamel organ for a better understanding of the role of the enamel organ cells in calcium regulation.

In the present study we employed the rapid-freezing and freeze-substitution method for tissue preparation, and embedded the specimen in epoxy resin. The overall distribution of calcium in the maturation stage enamel organ of rat incisors thus prepared was examined by means of a sensitive histochemical calcium staining method.

MATERIALS AND METHODS

Rapid freezing and freeze substitution
Male Wistar rats weighing approximately 200 g were anesthetized by an intraperitoneal injection of 4% chloral hydrate (400 mg/kg body weight) and were killed by decapitation. The lower incisors were excised with the intact enamel organ attached to the enamel. Pieces of the enamel organ extending from the postsecretory to the mid-stage of enamel maturation were isolated with the underlying enamel using a razor blade. Each specimen was immediately quenched in liquid propane cooled with liquid nitrogen for rapid freezing. The frozen specimens were left in liquid propane for up to 5 min and then transferred to cold acetone (−80°C), where they remained for 4 days for freeze substitution. The temperature of the acetone was then slowly brought up to room temperature (22±2°C) within 8 h. Each specimen was rinsed in acetone for 30 min at room temperature, transferred to propylene oxide and routinely embedded in Epon 812 (Taab, Berkshire, England).

GBHA staining
Semi-thin Epon sections (approximately 3 μm thick) of the freeze-substituted specimen were cut dry with a glass knife along the presumed long axis of the incisor from which the specimen had been isolated. These were transferred to a glass slide using a pair of fine tweezers. A glyoxal bis(2-hydroxyanil) (GBHA) staining solution was prepared as suggested by KASHIWA and HOUSE (1964): 5% GBHA (Fluka, Switzerland) in 75% ethanol containing 3.4% NaOH. The glass slide was flooded with the GBHA solution and was then left for 5–10 min at room temperature. The sections were subsequently rinsed with absolute ethanol for 5 min, briefly immersed in xylene and mounted with New Entellan (Merck, West Germany). Some of the sections stained with GBHA were counterstained with 0.1% methylene blue in absolute ethanol. Care was taken to complete all procedures under an unhydrous condition.

Specificity tests of GBHA reactions
Since GBHA chelates with various divalent cations such as Ba, Sr, Cd, Cu, Co and Ni ions (in addition to calcium) and forms colored precipitates (GOLDSTEIN and STARK-MAYER, 1958), some of the sections already stained with GBHA were immersed in 90% ethanol saturated with Na₂CO₃ and KCN for 15 min at room temperature (KASHIWA and ATKINSON, 1963). It is known that this procedure does not affect calcium-GBHA reactions, but abolishes GBHA reactions with the other cations listed above.

Prior to GBHA staining, some of the semi-thin sections were immersed in one of the following media for 10 h at room temperature: 80% ethanol adjusted at pH 11 with NaOH, 80% ethanol saturated with tetrasicdodium ethylenediaminetetraacetic acid (EDTA) (pH 10), or 80% ethanol saturated with ethylene-glycol bis (β-aminoethyl ether) -N, N', N'-tetraacetic acid (EGTA) (pH 10). All sections were subsequently stained with GBHA and processed as described above.

RESULTS

Intracellular GBHA reactions appeared only in extremely well-frozen portions of each specimen, in which no apparent morphological damage due to ice crystals was noted under the light microscope. On the other hand, the maturing enamel underlying the enamel organ displayed intense GBHA reactions throughout its depth, regardless of the quality of tissue freezing.

In the following, therefore, only the findings of those portions of the specimens where the cell morphology was sufficiently preserved will be described.

Ruffle-ended ameloblasts
Numerous GBHA-reactive, tubulo-vesicular structures were localized in the ruffle-ended type of ameloblasts, including those at the morphological transition from or to the smooth-ended ameloblasts. The reactive structures were located exclusively at both the distal and proximal ends of the cytoplasm. Practically no GBHA reaction was shown in the nucleus,
supranuclear (Golgi) region, or along the plasma membranes (Figs. 1, 2). The reactive structures in the distal cell compartment were much greater in number, and were more tightly packed than those in the infranuclear cytoplasm. The former appeared round or oval in shape while the latter showed long, tubular profiles (Fig. 2). Occasionally, a considerable number of tubular structures showing less intense GBHA reaction were observed extending through the mid portion of the cytoplasm primarily along the lateral plasma membrane (Fig. 4). Careful examinations of sections at different focusing depths revealed a close association of such tubular structures with the tubulovesicular structures located at both ends of the cell, showing intense GBHA reactions. No GBHA-reactive, tubulo-vesicular structures were localized in the

Fig. 1. A panoramic view of the ruffle-ended ameloblasts (Am) and adjacent papillary layer (P). Numerous GBHA-reactive, tubulo-vesicular structures are localized in the ruffle-ended ameloblasts, while most of the cells in the papillary layer lack GBHA reactions. Arrows indicate papillary cells located adjacent to the ameloblast and/or capillaries (c), showing slight GBHA reactions. Note numerous granular GBHA reactions in the adjacent connective tissue (CT). Enamel (E) is evenly stained. ×1,100

Fig. 2. Typical ruffle-ended ameloblasts showing numerous GBHA-reactive, tubulo-vesicular structures located exclusively at the distal and basal cell compartments. GBHA reactions in the distal compartment (asterisk) are vesicular in profile and are much greater in number than those at the basal pole, displaying long, tubular profiles. The GBHA reaction is practically absent in the nucleus (N), Golgi region (G), and along the plasma membrane. RB ruffled border, E enamel. ×2,600
Fig. 3. GBHA reactions in the maturation ameloblasts at the transitional phase from the ruffle-ended (RA) to smooth-ended (SA) type. In the latter cell type, numerous tubulo-vesicular structures showing intense GBHA reactions extend to the very end of the distal cytoplasm. Arrow indicates the incisal direction. Note the abrupt disappearance of the ruffled border as indicated by the vertical bar. P papillary layer. ×910

Fig. 4. Ruffle-ended ameloblasts showing numerous granular GBHA reactions in the ruffled border region (arrows) adjacent to the enamel (E). Arrowheads indicate GBHA-reactive, tubular structures in the mid portion of the cell. They appear to extend along the long axis of the ameloblast, primarily at the periphery of the cytoplasm. Weak granular GBHA reactions are seen in the papillary cell (P) adjacent to the ameloblast. N nuclei of ameloblasts. ×2,200

Fig. 5. Ruffle-ended ameloblasts comparable to those in Figure 4, stained with GBHA after EDTA treatment. Note the complete absence of GBHA reactions. N nucleus of the ruffle-ended ameloblast, E enamel. ×2,200
ruffled border region at the distal end of the cell. However, numerous granular GBHA reactions were occasionally seen in this region at a high magnification (Fig. 4).

**Smooth-ended ameloblasts**
Numerous GBHA-reactive, tubulo-vesicular structures were also localized in this type of ameloblast. The intensity as well as dimension of the GBHA-reactive structures were comparable to those of the ruffle-ended ameloblasts. They were located throughout the cytoplasm and extended to the very end of the distal cytoplasm. Only a few reactive structures were shown in the Golgi region. The nucleus and plasma membranes of these cells did not show any reaction. Figure 3 shows the ruffle-ended type of ameloblasts and their abrupt transition to the smooth-ended ameloblasts.

**Papillary cells and adjacent connective tissues**
A majority of the papillary cells did not show GBHA reactions. However, a significant albeit weak GBHA reaction was localized in a limited number of papillary cells located adjacent to the ameloblasts and/or the capillaries indented deep in the papillary layer (Fig. 1). The reactions in each cell appeared granular in profile and resembled the distribution of mitochondria (Figs. 1, 4).

Numerous granular GBHA reactions were localized in the cells of the adjacent connective tissue (Fig. 1). The granular reactions were much stronger than those in the papillary cells. At a higher magnification, the cells with granular reactions showed a spindle-shaped profile typical of the fibroblast (data not depicted).

**Specificity tests of GBHA reactions**
The pretreatment of sections for 10 h with 80% ethanol (pH 11) caused no difference in GBHA reactions; however, no GBHA reaction was observed when 80% ethanol was saturated either with EDTA or EGTA (pH 10) (Fig. 4).

Sections treated for 15 min with 80% ethanol saturated with Na₂CO₃ and KCN showed no change in GBHA reactions (data not shown).

**DISCUSSION**
KASHIWA and SIGMAN (1966) were the first to introduce the GBHA staining method for localizing calcium in odontogenic cells, demonstrating calcium in the enamel organ of mandibular molar tooth germs of suckling rats. In their experiment, however, it was difficult to correlate GBHA-reactive structures with histology at cellular levels due to the drastic tissue processing.

In the present study, a remarkable improvement in the demonstration of GBHA reactions and the preservation of morphological tissue features were made possible by introducing rapid-freezing and freeze-substitution of tissues and their subsequent embedding in epoxy resin. Current control tests clearly indicated the specificity of GBHA reactions for calcium.

It is well known that, in the ruffle-ended ameloblast in the rat incisor enamel organ, mitochondria assemble at both basal and apical ends of the cytoplasm, whereas they are fairly randomly distributed in the smooth-ended ameloblast (JOSEPHSEN and FEJERSKOV, 1977). Most of the tubulo-vesicular, GBHA-reactive structures observed in both types of maturation ameloblasts in the present study apparently correspond to the mitochondria, judging from their characteristic localizations.

KALLENBACH (1970) observed an increase in the size of the basal mitochondria of rat incisor ameloblasts during the enamel maturation phase and suggested a connection between basal mitochondria and calcium transport. GBHA-reactive long, tubular structures in the infranuclear cytoplasm of ruffle-ended ameloblasts as shown in Figure 2 may correspond to the basal giant mitochondria as described by KALLENBACH (1970).

The high calcium content in mitochondria of maturation stage ameloblasts, both ruffle-ended and smooth-ended, as revealed by the GBHA staining apparently contradicts previous autoradiographic (MUNHOZ and LEBLOND, 1974), electron probe analytical (BOYDE and REITH, 1978), as well as cytochemical studies (REITH and BOYDE, 1985) of the rat incisor enamel organ, in which the authors of each study stressed the absence of substantial amounts of intracellular calcium. In our observations, calcium thus demonstrated in the enamel organ was shown to be highly labile since intracellular GBHA reactions were localized only in extremely well-frozen portions of the specimen and, in fact, were easily lost by aqueous treatment (data not shown). The discrepancy between our results and
previous ones, regarding the presence or absence of intracellular calcium in the enamel organ, may therefore be explained by the difference in the efficacy of tissue preparation methods used in the individual studies, whereby labile calcium was expected to remain in its original location. For example, Munhoz and Leblond (1974) used conventional aqueous methods for tissue fixation and subsequent processing for their 40Ca autoradiography which, presumably, might have caused a drastic loss or dislocation of cellular calcium. In the case of the pyroantimonate method by Reith and Boyde (1985), one of the most commonly used cytochemical methods for the electron microscopic localization of cations (Komnick, 1962; Klein et al., 1972), there is a potential problem that the reagent must penetrate various cellular membrane systems before it reaches the reaction site in the intracellular compartment, such as the mitochondria.

One must also bear in mind that, in calcified tissue forming cells, a high concentration of intramitochondrial calcium might result from an artificial increase of the cytosolic calcium level due to leakage through a damaged plasma membrane (Reith and Boyde, 1978; Burger and de Bruin, 1979). It is known that non-energy supported calcium ion accumulation in skeletal tissue mitochondria is much greater than that by mitochondria from non-calcifying tissues (Shapiro and Lee, 1975). Accordingly, the possibility exists in the current study that GBHA reactions in the enamel organ as well as those in the adjacent connective tissue cells represent an artifactual accumulation of calcium in mitochondria, caused by cell injury suffered during tissue preparation.

In our specimens, however, there was no indication of any positive correlation between (macroscopic) mechanical tissue damage and the intensity of GBHA reactions at the respective sites. In fact, at the periphery of each specimen, where cells would likely have suffered severe mechanical damage, GBHA reactions were considerably less than those in microscopically intact zones showing no sign of cell injury, nor of ice crystal damage (not depicted). Hence, within the limits of this study, the discrete GBHA reactions at the well-frozen portions of our specimens may represent the sites of high calcium concentration in vivo, at the instance of rapid freezing.

The presence of the smooth-surfaced endoplasmic reticulum showing tubular conformations, and their occasional association with mitochondria have been reported in maturation ameloblasts of the rat incisor (Ozawa et al., 1983). At least some of the GBHA-reactive, tubular structures extending through the mid portion of the cytoplasm of ruffle-ended ameloblasts as shown in the current study (Fig. 4) may correspond to the smooth endoplasmic reticulum interconnecting the basal and apical compartments where numerous mitochondria are assembled.

Our recent autoradiographic study has shown that the intercellular junctions between the ruffle-ended ameloblasts of rat incisors restrict the passage of radiocalcium, and implied the presence of transcellular pathways for calcium in this type of ameloblast (Takano et al., 1987). Hence, although the limitation of methods does not allow any conclusive remarks regarding the exact location of calcium at the ultrastructural level, our findings of the exclusive localization of granular GBHA reactions in the ruffled border, and the above mentioned distribution of GBHA-reactive, tubulo-vesicular structures may prompt one to hypothesize a transcellular calcium transportation by the ruffle-ended ameloblasts in which mitochondria may play a central role in the regulation of intracellular calcium.

It should be stressed, however, that the histochemical reaction presented thus far shows no evidence of calcium movement in the ameloblast, but merely the location of calcium in cells at the time of staining. Evidently, it is not known whether the intracellular GBHA reactions represent calcium in transit or in stability. To pursue this fundamental question, 40Ca autoradiography combined with the current tissue preparation method should be performed in future investigations. The significance of the high calcium concentration in the smooth-ended ameloblasts should be likewise examined.

The papillary cells have been implicated as playing a significant role in the regulation of minerals because of their cytological features typical of ion-transporting cells (Kallenbach, 1966; Elwood and Bernstein, 1968; Garant, 1972; Sasaki and Higashi, 1983; Garant et al., 1984; Sasaki and Garant, 1986). The weakness of GBHA reactions in the mitochondria of papillary cells, relative to those of adjacent ameloblasts, does not impair the value of such a speculation, but simply indicates possible differences in calcium-related biological features of mitochondria in both types of cells.

Concerning the fact that, in each specimen, the papillary layer is located farthest from each of the frozen surfaces, the above phenomenon may be related to minor differences in the freezing conditions within the specimen. The elucidation of the significance of differences in mitochondrial GBHA reactions between two cell layers awaits further study.
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