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Fluctuations in surface pH of maturing rat incisor enamel are a result of cycles of H^+-secretion by ameloblasts and variations in enamel buffer characteristics

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ABSTRACT

It is disputed if ameloblasts in the maturation zone of the enamel organ mainly buffer protons released by hydroxyapatite (HA) crystal growth or if they periodically secrete protons to create alternating acidic and alkaline conditions. The latter hypothesis predicts alternating pH regimes in maturing enamel, which would be affected by pharmacological interference with ameloblast H^+-secretion. This study tests these predictions.

Colorimetric pH-indicators and ratiometric fluorometry were used to measure surface pH in maturation zone enamel of rat incisors. Alternating acidic (down to pH 6.24 ± 0.06) and alkaline zones (up to pH 7.34 ± 0.08) were found along the tooth coinciding with ameloblast morphological cycles. Underlying the cyclic pattern, a gradual decrease in pH towards the incisal edge was seen. Vinblastine or FR167356 (H^+-ATPase-inhibitor) disturbed ameloblast acid-secretion, especially in the early parts of acidic zones.

Enamel surface pH reflects the titration state of surface PO_4^{3-}-ions. At the pH-values observed, PO_4^{3-} would be protonated (pKa > 12) and HA dissolved. However, by molecular dynamics simulations we estimate the pKa of HPO_4^{2-} at an ideal HA surface to be 4.3. The acidic pH measured at the enamel surface may thus only dissolve non-perfect domains of HA crystals in which PO_4^{3-} is less electrostatically shielded. During repeated alkaline/acidic cycles, near-perfect HA-domains may therefore gradually replace less perfect HA-domains resulting in near-perfect HA-crystals.

In conclusion, cyclic changes in ameloblast H^+-secretion and the degree of enamel maturation determine enamel surface pH. This is in accordance with a hypothesis implicating H^+-ATPase mediated acid-secretion by ameloblasts.

Introduction

Acid–base homeostasis of the intracellular compartment is crucial for the maintenance of most cellular functions in the body. Intracellular homeostasis is obtained by extensive transport of acid/base equivalents between the intracellular and the extracellular compartment. The acid/base status of the extracellular compartment is in turn maintained constant by appropriate function of the kidney and the lungs. However, the acid/base status of specialized parts of the extracellular space may also be controlled by nearby cells, as is the case for the lacunae formed by osteoclasts during bone resorption. In addition, acid/base conditions play an important role for the mineralization process of biological apatite, exemplified by developing enamel [1]. Tooth enamel mineralization depends on a delicate cellular control of the ionic composition and pH of the fluid surrounding the growing crystals. Control of these parameters is most likely mediated by the enamel organ cells and not least the ameloblasts (see review [2]). As the enamel organ is effectively inaccessible to direct physiological examinations, information on pH of the enamel in different stages of enamel formation is scarce. Before it became appreciated that the ameloblasts during maturation appear as either ruffle-ended (RA) or smooth-ended (SA) ameloblasts [3], the extracellular pH during the secretory stage was claimed to be 7.3–7.4 whereas the pH in the more mature enamel ranged from 8.0 to 8.5 [4].

However, studies by Takano et al. introduced a calcium-chelator dye, glyoxal bis(2-hydroxyanil) (GBHA), which demonstrated distinct red banding along the enamel at regular intervals corresponding to the location of SA bands in rat, bovine, porcine and monkey teeth [5]. Using colorimetric indicators it was shown, that this staining reflects areas of alkaline conditions [6].

Abbreviations: RA, ruffle-ended ameloblasts; SA, smooth-ended ameloblasts; HA, hydroxyapatite; GBHA, glyoxal bis(2-hydroxyanil).

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The two different morphological states of ameloblasts (RA and SA) reflect a cyclic cellular modulation process common to several species [7]. In rats, this process is characterized by the ameloblasts going through a cycle lasting about 8 h, during which the cells are in the state of RA for about 4 h before abruptly changing to become a SA type of cell for about 2 h and then gradually rebuilding the highly infolded (ruffled border) cell membrane towards the enamel. The modulation process proceeds in a wavelike pattern from the start of the maturation zone towards the incisal edge resulting in transverse bands of RA and SA conformation moving along the enamel [2]. Much effort has been invested in understanding the cellular mechanisms behind this modulation process and the functional implications. Sasaki, Takagi & Suzuki used 3 different pH staining solutions on bovine incisors and showed a banding of alternating acidic (pH ~ 6) and neutral (pH ~ 7) enamel zones and associated them with RA and SA cell bands [6]. They suggested that the acidic extracellular environment might be a result of protons released by the growth of hydroxyapatite crystals during maturation. According to this hypothesis, which was further advanced in several reviews [2,8], an important role of the ameloblasts is to transport bicarbonate to the maturing enamel to buffer the excess protons. A number of studies have aimed to support this hypothesis [9–14].

However, we have recently presented data on ion transporters in secretion and maturation stage ameloblasts, which have resulted in a new functional hypothesis of the enamel organ during maturation [15]. According to our hypothesis, H^+ is pumped by H^+-ATPases in the ruffled border towards the maturing enamel surface, which therefore becomes acidic under the RA. This hypothesis does not preclude that protons are stoichiometrically released by the appositional growth of the enamel crystals during the long lasting process of enamel maturation [2]. This passive proton release may induce a low pH in bulk enamel in the early nucleation and precipitation phase probably facilitating the gradual matrix proteolyses [2]. However, this passive proton release cannot explain the steep fluoride gradient observed in the outermost 100–150 μm of the human enamel when F^- is given during tooth development [1,3,16], and the gradual reduction in content of carbonate, magnesium etc. in surface enamel crystals during the maturation phase [1]. According to our hypothesis, the composition of surface enamel crystals is the result of an energy-demanding active process based on intricate cellular collaboration and cyclic changes of ameloblast morphology and function resulting in alternating partial dissolution and precipitation of enamel crystal subdomains by cyclic changes in enamel pH [15].

As pointed out above, the knowledge of enamel surface pH is scarce and based on traditional colorimetric methods or analysis of sectioned teeth. Based on the current knowledge, which has limited spatial resolution, it appears that pH is higher under the SA, than under the RA. This is in accordance with our hypothesis, but as the colorimetric measurements may be influenced by other factors including the concentration of the indicator, more precise knowledge of enamel surface pH is needed.

In the present study the hypothesis of ameloblast proton secretion described above is tested in two ways:

1) The spatial variability and range of pH on the surface of maturing enamel of rat incisors are measured by a ratiometric fluorescent pH indicator (BCPCF) and conventional colorimetric pH indicators. The hypothesis predicts lower pH under RA than SA.
2) The spatial variability in pH and GBHA staining is assessed in vinblastine and H^+-ATPase-inhibitor-treated (FR167356) rats. The hypothesis predicts that both treatments should result in higher pH under RA since the acid-secretion of RA is predicted to be reduced.

Materials and methods

Enamel staining with GBHA and colorimetric pH-indicators

Eight male Wistar rats (bw 274 ± 24 g) were anesthetized with 20% chloral hydrate (0.2 ml/100 g bw) and decapitated. The mandibles were removed and cleaned of soft tissues. The lower incisors with adhering enamel organs were then carefully dissected from the surrounding alveolar bone [17]. In each animal one tooth was used for GBHA staining and one for pH-indicators. For GBHA staining the periodontal connective tissue adhering to the enamel organ were pulled off with a pair of fine tweezers. Each incisor was stained at room temperature for 7–8 min by immersion in 2 ml solution containing 3.75% GBHA (Sigma Chemical Co., St. Louis) and 1.275% sodium hydroxide dissolved in 75% ethanol. Subsequently the incisors were briefly rinsed in 75% ethanol, the enamel organ peeled off the enamel surface, further rinsed in 96% ethanol, and air dried. For staining with pH-indicators the enamel organ was gently wiped from the enamel surface with lightly moistened ear cotton sticks. Immediately after this, the incisors were soaked into 2 ml solutions of either methyl red (0.1% in 96% ethanol) or bromocresol purple (0.1% in 20% ethanol) for 2 min. Excess solution was removed and the teeth air dried. Color standards at different pH values (ranging from 4 to 7.5) were made from adding 200 μl pH-indicator to 4 ml of PBS buffer stem solutions adjusted with hydrochloric acid or sodium hydroxide. All incisors were photographed shortly after staining and air drying with a Nikon D1 digital camera attached to a Wild M8 Macroscope.

Enamel surface pH measurements using ratiometric fluorometry

A series of pilot experiments were performed to optimize the labeling and ratiometric pH measurement procedure. For the final series of experiments, incisors from 3 untreated rats (bw 197 ± 8 g), 5 rats (bw 160 ± 5 g) treated with vinblastine (0.5 mg/rat) and 3 rats (bw 208 ± 7 g) treated with H^+-ATPase blocker FR167356 [18] (50 mg/rat) were investigated. Vinblastine and FR167356 were given by IP injection 4 h before the animals were anesthetized with isoflurane and decapitated. Animal experiments were conducted according to animal experiment license # 2012-15-2934-00240 issued by the Danish Animal Experiment Authorities.

One lower incisor was removed and stained with GBHA as described above. The contralateral incisor was removed and cleaned as described above for colorimetric pH indicators. The enamel was air dried and quickly formed an opaque region with a sharply defined boundary. This opaque white boundary was marked with a scalpel and served as a reference point. The tooth was loaded for 60 s in 50 μM 2′,7′-bis(3-carboxypropyl)-5(6)-carboxyfluorescein (BCPCF, Molecular Probes) dissolved in saline. Excess dye was gently removed by using a filter paper. Each tooth was separately placed on a glass coverslip and mounted on an inverted microscope. The dye was excited using 495 nm and 440 nm light from a monochromator (Till Photonics) and the light emitted at 510 to 535 nm was recorded by a 12-bit cooled monochrome CCD-camera system (IMAGO, Till photonics). For overview images, exposure time was for 50 and 100 ms, for 495 and 440 nm images respectively, using a 1× objective. For detailed measurements of RA and SA zones, a 10× objective was used and exposure time was reduced to 1 ms for both wavelengths. QED InVivo imaging software (Media Cybernetics) was used to control wavelength, light exposure time, and binning.

The distances from the start of the maturation zone and the center of each SA-band were measured on overview images. The SA-bands were numbered from the start of the maturation zone and high magnification images were obtained from each. For each SA-band, regions of interest were selected at 5 positions: A: in the newly formed RA close to the SA, B: in the terminal part of the SA band, C: in the middle of the band, D: in the newly formed SA and E: in the RA just preceding the SA band (Fig. 1E).

The images were analyzed using ImageJ Software. The fluorescence intensities following excitation with both 495 and 440 nm within each region were measured. For translation of fluorescence to pH, background was subtracted from both images and the ratio of emission following excitation at the two wavelengths (495/440 ratio) was calculated. The ratios were calibrated using HEPES buffered saline (145 mM