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Sites of calcium uptake of fish otoliths correspond with macular regions rich of carbonic anhydrase

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Abstract

Based on pharmacological data, it has been suggested that the enzyme carbonic anhydrase (CAH) plays a prominent role in the mineralization of fish otoliths. To directly test this proposal, the topographical distribution of CAH was histochemically analyzed in the utricular and saccular maculae of larval cichlid fish *Oreochromis mossambicus*. Further investigations were focussed on the sites of otolithic calcium uptake using the fluorescent calcium tracer alizarin-complexone (AC). Both in the utricle and the saccule, CAH-reactivity was prominent in regions on both sides of the sensory macula (centrifugal (cf) and centripetal (cp) areas), which reportedly contain ionocytes, specialized cells regulating the ionic composition of the endolymph. (The terms centrifugal and centripetal were chosen instead of lateral and medial, because the saccule is positioned perpendicular to the utricle; "lateral" and "medial" thus do not allow an unambiguous allocation of the respective regions.) In the saccule, the size of cf and cp did not differ from each other, whereas, in the utricle, cp was considerably larger as compared to cf (CAH-reactivity per μm^2 was nearly identical in both areas of both endorgans). AC-incubation resulted in a fluorescent band on the proximal surface of the otoliths (this surface lies next to the sensory epithelium). In saccular otoliths (sagittae), the area of the band did not differ between centrifugal and centripetal otolith regions, whereas in the utricular otoliths (lapilli), the area of the centripetal AC-band was larger in size as compared to the centrifugal one (AC-fluorescence per μm^2 did not differ between the areas analyzed in both types of otoliths). These results strongly suggest that calcium/carbonate uptake of otoliths takes place especially in those regions of their proximal face which are located adjacent to CAH-rich areas of the macular epithelium. It is thus concluded that CAH is directly involved in otolith calcification. The differences in CAH-localization/AC-uptake in the two endorgans analyzed are discussed on the basis of their different functional properties (saccule, hearing; utricle, graviperception).

Keywords: Utricle; Saccule; Inner ear; Otolith growth; Mineralization

1. Introduction

Teleost otolith endorgans comprise three macular compartments, i.e., the saccule, the utricle, and the lagena, which contain crystalline inclusions. These otoliths are mainly composed of $CaCO_3$ (Borelli et al., 2001; Degens et al., 1969).

It is a widely accepted concept that precursors of the otoliths are secreted by the supporting cells of the sensory epithelium and that particular glycoconjugates within these precursors play their role in $CaCO_3$ deposition. Acidic amino acids, present on glycoproteins, are known to attract both calcium and carbonate ions, facilitating crystal nucleation and potentially providing a template for crystallization (Crenshaw, 1982; Murayama et al., 2002; Steyger and Wiederhold, 1995).

The calcium entry into the otocyst is performed by the macular sensory epithelium which transfers calcium directly into the proximal endolymphatic compartment being located between the sensory epithelium and the otolith (Ibsch et al., 2004; Mugiya, 1974; Payan et al., 2002). Besides its role for Ca supply, this proximal zone of the endolymph is also of importance for the formation of

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organic compounds (Borelli et al., 2001; Gauldie and Nelson, 1988; Payan et al., 1999), indicating its importance for $CaCO_3$ deposition.

Calcium carbonate deposition itself has been proposed to be regulated by the carbon status in the endolymph (Gauldie and Nelson, 1988; Tohse and Mugiya, 2001). Therefore, it has been suggested that carbonic anhydrase (CAH) as a catalyst for the conversion of CO_2 to HCO_3^- may play a role in carbonate production for otolith calcification (Fermin et al., 1998; Tohse and Mugiya, 2001) in supplementing bicarbonate ions in the epithelial cells (i.e., mostly ionocytes). Findings, according to which the degree of otolith growth is correlated with vestibular CAH-reactivity (Beier et al., 2002, 2004a), support this concept.

The present study was undertaken to reinforce the proposed role of CAH in otolith mineralization, especially in order to possibly demonstrate a direct, topographical relationship between CAH-reactivity and otolithic calcium uptake.

2. Material and methods

Larval cichlid fish siblings (*Oreochromis mossambicus* PETERS, Perciformes) of stage 14 (2 days after hatch, ah, and 6 days post fertilization, pf, at 28 °C; prominent yolk-sac; Anken et al., 1993) and of stage 23 (16 days ah and 20 days pf; primordia in pelvic fin, gross-morphology basically completed, body length ca. 11 mm; Anken et al., 1993) were used.

For CAH-histochemistry in the saccular and utricular maculae, the larvae were sacrificed in ice water and immediately fixed for 2 h at 4 °C in a solution containing 4% paraformaldehyde and 3% glutaraldehyde, buffered with 0.05 M Na-cacodylate (pH 7.4). Subsequently, samples were decalcified (2 days in 5% EDTA at 4 °C, buffered with 0.05 M Na-cacodylate, pH 7.4) and cryoprotected by increasing concentrations of sucrose (10, 12.5, 15, and 20% sucrose each in 0.05 M Na-cacodylate, pH 7.4, for 30 min at 4 °C), followed by an incubation in 2 parts 20% sucrose: 1 part OCT (Tissue Tec Compound Medium, Ted Pella, CA, USA) for 30 min at 4 °C. After cryoprotection, the samples were frozen at −80 °C in a medium containing 2 parts OCT: 1 part 20% sucrose in isopentane.

The histochemical demonstration of CAH on 14 μm cryostat transverse sections was modified after an earlier published method (Hoyle, 1983; Loveridge, 1978).

A solution containing 5.8×10^{-2} M sulfuric acid, 4×10^{-3} M cobalt sulfate and 4×10^{-3} M potassium dihydrogen orthophosphate was mixed with a solution of 9×10^{-3} M sodium hydrogen carbonate in bidistilled water immediately prior to use. After the pH had risen to 6.8, sections were incubated for 20 min at 26 °C. After incubation, sections were rinsed in PBS (8% NaCl, 0.2% KCl, 1.4% Na_2HPO_4, and 0.2% KH_2PO_4 in double distilled water, pH 7.4) for 30 s, immersed in an aqueous solution of 1% ammoniumsulfide for 3 min, rinsed in distilled water and mounted in Hydromatrix (Micro-Tech-Lab, Graz, Austria).

In a preliminary methodological study, CAH-reactivity was densitometrically determined (optical density) at 550 nm by use of a digital imaging system (Axio Cam MRC, Axiovision 3.06, Zeiss Oberkochen, Germany) within peripheral areas of the utricular and saccular maculae (centrifugal and centripetal areas; cf and cp, respectively; Fig. 1; we have chosen the terms centrifugal and centripetal rather than lateral and medial, because the latter terms apply to the circumstances when the maculae and the otoliths are placed on microscopical slides; in the animal, however, "lateral" and "medial" cannot be used for a correct allocation of the respective ares, because the utricle is positioned perpendicular to the saccule). These areas abound of ionocytes (cells specialized for maintaining a pH-value necessary for carbonate deposition), where CAH-activity is reportedly especially pronounced (Beier et al., 2002, 2004a; Mayer-Gostan et al., 1997; Payan et al., 1997). CAH-reactivity per μm^2 did not differ between the areas analyzed. Thus, for a comparison of saccular vs. utricular features, the size of these areas was determined planimetrically. For comparative purposes, cf and cp per animal were added and set 100%. (We wanted to analyze the correlation of cf/cp in the macular areas and in the otoliths of the same animals. Since the absolute sizes of cf and cp vary between animals, it was necessary to use such a relative measure.)

To determine otolithic calcium uptake, further animals of the same batch were maintained in a 100 mg/L solution of the calcium-tracer alizarin-complexone (AC; Sigma, Germany) for 3 h at room temperature. After AC-incubation, animals were sacrificed in ice-water and saccular and utricular otoliths (i.e., sagittae and lapilli, respectively) were dissected and mounted (distal side up; the proximal face in intact animals is located directly adjacent to the sensory epithelium) in Mowiol/Dabco (Sigma) on microscopical slides. Under an incident fluorescence microscope (Axiovert 135, Zeiss) with excitation bands from 450 to 490 nm, the AC fluorescence was visualized. During otolith growth, $CaCO_3$ and thus AC is found around the otoliths's surface. Using a computer-based image analyzing system (Axiovision 3.06, Zeiss) the surface of the bands within

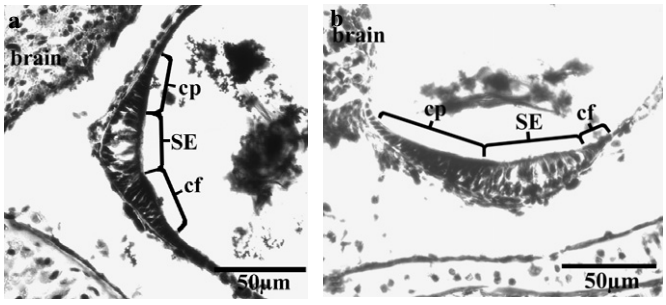

Fig. 1. Carbonic anhydrase-reactivity within the saccule (a) and the utricle (b) of a cichlid fish larva (stage 23; cryostat transverse section; left in the figures points to medial). The centripetal (cp) and the centrifugal (cf) regions of the maculae are especially reactive. SE, sensory epithelium.