BRANCHIAL CHLORIDE CELLS IN LARVAE AND JUVENILES OF FRESHWATER TILAPIA Oreochromis mossambicus

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Summary
Branchial chloride cells in the developing larvae and juveniles of freshwater tilapia, Oreochromis mossambicus, were identified and the membrane Na+/K+-ATPase was localized in situ through binding of the fluorescent dye anthroylouabain. After co-labelling of the cells with the fluorescent probes DASPMI and Con-A-FITC, the mitochondria and apical crypt in the same chloride cells were visualized using confocal laser scanning microscopy. The high density of apical crypts indicated that many chloride cells were functional. The density of branchial chloride cells in larvae 10 days after hatching was approximately 6000 mm⁻². An extremely high Na+/K+-ATPase specific activity of approximately 1500 μmol P, h⁻¹ mg⁻¹ was measured in the gills 10 days after hatching. With the development of secondary lamellae and hence an increase in the amount of branchial epithelial protein, a concomitant decrease in the specific activity of the enzyme in the gill tissues was observed. Total Na+/K+-ATPase activity increased markedly in the early life stages. Our data indicate that in larval stages of fish the gills form a functional ionoregulatory organ before they start functioning as a gas-exchange organ.

Key words: chloride cells, larvae, juveniles, freshwater tilapia, Oreochromis mossambicus, fish gills, Na+/K+-ATPase, mitochondria, Concanavalin-A, confocal laser scanning microscopy.

Introduction
In the gills of adult fish, two morphologically distinct epithelia are found: the multilayered filament epithelium, which is largely involved in ion exchange, and the bilayered lamellar epithelium involved in gas exchange between the blood and the ambient water (Laurent, 1984). For a variety of species, including the tilapia studied in this paper, it has been demonstrated that the chloride cells (or ionocytes) of adult fish are mainly located in the filament epithelium (Wendelaar Bonga et al. 1990). These cells are the site of the active Ca⁺⁺ transport that underlies transepithelial Ca⁺⁺ uptake, in both freshwater and seawater fish (Marshall et al. 1992; McCormick et al. 1992; Verbost et al. 1994). Although their role in NaCl excretion in seawater species is clearly established (Foskett and Scheffey, 1982), their role in NaCl handling in freshwater fish is currently under debate: some conclude that these cells are involved in the uptake of both Na⁺ and Cl⁻ (e.g. Laurent et al. 1985; Laurent and Perry, 1990), others believe that the chloride cells are the site of Cl⁻ uptake alone and that uptake of Na⁺ occurs through respiratory cells (Goss et al. 1992a,b; Laurent et al. 1994; Morgan et al. 1994).

In general, a functional chloride cell is structurally characterized by the presence of many mitochondria, an abundant tubular system, where ion-transporting enzymes such as Na+/K⁺-ATPase, Ca²⁺-ATPase and a Ca²⁺/Na⁺ exchanger are located, and an apical crypt facing the water (Hootman and Philpott, 1979; Flik et al. 1985; Wendelaar Bonga et al. 1990; Flik and Verbost, 1993).

The embryos and larvae of teleosts are able to maintain a constant hydromineral level in their body fluids, even though their gills and kidneys appear poorly developed (Alderdice, 1988). During the early developmental stages of teleosts, extrabranchial chloride cells located in the skin have been proposed as the site of ionoregulation. Indeed, Shelbourne (1957) suggested that these integumental chloride cells play an ion-transporting role in larvae of the plaice Pleuronectes platessa. Later, several studies focused on extrabranchial chloride cells in a number of embryonic and larval stages of teleosts (Alderdice, 1988; Hwang, 1989, 1990; Ayson et al. 1994). Hwang et al. (1994) reported that the first chloride cells to appear in the skin of freshwater tilapia embryos were found 48 h after fertilization. Some chloride cells with apical crypts facing the water were observed in the skin of newly hatched larve (96h post-fertilization). They suggested that, up to 10
days after fertilization, these integumental chloride cells are the main site of active ion transport in freshwater tilapia. However, little is known about the development of branchial chloride cells in fish larvae and when they become operational.

We have applied morphological, histochemical and biochemical methods to identify functional chloride cells in the gills of larval and juvenile freshwater tilapia. Three criteria were advanced to identify mature chloride cells (i.e. cells in contact with the water). First, through their abundance of mitochondria, chloride cells were visualized with a mitochondrion-specific, fluorescent vital stain. Second, through their high Na+/K+-ATPase content, the same cells were visualized with the fluorescent dye anthroylouabain (which binds to an extracellular binding site on the enzyme). Third, using fluorescein-conjugated Concanavalin-A, which appears to bind specifically to glycoproteins in the apical crypt of chloride cells, the presence of apical crypts was demonstrated. We used live tissue for our morphological studies. The specific and total Na+/K+-ATPase activities of gills were measured biochemically.

Materials and methods

Animals

Batches of eggs close to hatching were collected from adult female tilapia *Oreochromis mossambicus* (Peters). At 27 °C, hatching occurs approximately 4 days after fertilization (Lanzing, 1976; Hwang et al. 1994). Groups of at least six larvae and juveniles 3, 10 and 24 days after hatching, ranging in mass from 4 to 150 mg, were used. Masses refer to the animals after their yolk sac had been removed. A comparison was made with 180-day-old fish (adult). Animals were kept in Nijmegen tap water containing (in mmol l⁻¹): Na⁺, 5.0; K⁺, 0.06; Ca²⁺, 0.8; Mg²⁺, 0.2; Cl⁻, 4.2; SO₄²⁻, 0.5; pH 7.6; the water temperature was kept constant at 27 °C. Lights were on for 12 h per day. Fish were killed by spinal transection. For biochemical studies, gill arches were excised under a binocular microscope and kept in a Ringer’s solution containing (in mmol l⁻¹) NaCl, 120; KCl, 5.0; CaCl₂, 1.25; MgCl₂, 1.0; Heps, 10.0; glucose, 10.0; pH 7.4 (adjusted with Tris) at 4 °C until further use.

Vital staining and density measurement of chloride cells

When 'baby' fish were studied, they were allowed to swim in vital stain dissolved in the water. The fish were first placed in Concanavalin-A–fluorescein conjugate (Con-A-FITC; 1 mg ml⁻¹) for 45 min. This probe binds to the carbohydrate groups of glycoproteins (Blithe et al. 1982; Angelides et al. 1988) that are present in the apical crypts of the chloride cells (Pisam, 1981). The procedure was completed by co-staining with the specific mitochondrial probe 2-(4-dimethylamino-styryl)-1-methylpyridinium iodide (5 μmol l⁻¹; DASPMI, Molecular Probes, Eugene, USA) for 5 min. The fish were then killed by spinal transection and the gill arches excised, rinsed three times in Ringer’s solution and placed in 20 μl of Ringer’s solution in a rounded silicone chamber on a glass microscope coverslip. Using this method, we prevented the Con-A-FITC from entering the tissue through the cut edges (DASPMI, in contrast, is very lipophilic and will penetrate the tissue easily from the water). The successful staining of the apical crypts of chloride cells by this *in vivo* labelling technique suggests that the gills of the very young fish are being ventilated. When adult specimens were examined, individual filaments of the first and second gill arches were cut and stained using Ringer’s solution to which Con-A-FITC and DASPMI had been added (at the same concentrations as above).

A confocal laser scanning microscope (CSLM, MRC-600, Bio-Rad) was employed to identify the chloride cells. Imaging conditions were as follows: we used the 488 nm line of the argon ion laser as the excitation wavelength and a filter block that allowed the concurrent collection of the emission wavelengths of Con-A-FITC (530 nm) and DASPMI (609 nm). All images were recorded and processed with an IBM-compatible computer. The objective lenses used were a 10× and a 40× oil-immersion type. The thickness of optical sections was 0.5 μm.

In gills of juvenile fish, chloride cells were equally distributed throughout the (single layer) branchial epithelium. Optical sections were made in a plane parallel to the surface of the filament. For chloride cell counting, at least four optical sections of different filaments from each fish were randomly photographed. In gills of adult fish (where secondary lamellae have developed, the chloride cells occur mainly on the trailing edge of the filament and where the epithelium has become multilayered), optical sections were made through the superficial cell layer of the epithelium.

All DASPMI-stained cells in the surface layer of the filaments were counted. From each recorded image (0.5 mm²), three areas of 2500 μm² were analysed and the density of stained cells was expressed per mm² of section area. In total, three filaments from each fish and at least four different fish per group were analysed in this way. For comparison of chloride cell densities during development, we refer to the total filament surface in juvenile stages and to the trailing edge area of the filament in adult fish. Sites were chosen at a point one-third of the way along the filament.

Localization of Na⁺/K⁺-ATPase in chloride cells

Freshly excised first and second gill arches were incubated in 5 μmol l⁻¹ (final concentration) anthroylouabain (Sigma) in low-K⁺ tilapia Ringer’s solution (LKTR) for 60 min at room temperature in the dark. The LKTR solution contained (in mmol l⁻¹): NaCl, 140; NaHCO₃, 10; MgSO₄, 1; CaCl₂, 2; Na₂HPO₄, 1; KCl, 0.1; Heps, 10; glucose, 5, adjusted to pH 7.4 with Tris. As a control, in some samples, a high concentration (1 mmol l⁻¹) of non-fluorescent ouabain (Sigma) was added to the incubation medium to confirm the specific binding of anthroylouabain to membrane Na⁺/K⁺-ATPase (McCormick, 1990). After labelling with the fluorescent dye, the tissues were rinsed for 2 min in LKTR and placed on a
microscope coverslip as described above. Anthroyloaubain has an excitation wavelength of 365–368 nm and an emission wavelength of 480 nm (Fortes, 1977). For these observations, a Zeiss epifluorescence microscope was used at a magnification of 250×.

Determination of Na⁺/K⁺-ATPase activity

The Na⁺/K⁺-ATPase activity in whole gill tissue homogenate was determined as described by Flik et al. (1983). Freshly dissected gills of each larval, juvenile or adult fish were placed in 100 μl of membrane isolation medium containing (in Branchial chloride cells in baby tilapia

![Fig. 1. Confocal laser scanning micrographs of the gill filaments of a 3-day-old freshwater tilapia after in vivo co-labelling with Con-A-FITC (red) and DASPMI (green).](image-url)

(A) Second gill arch, transmission light microscopy; the gill arch can be seen in the gill arch; filament tips point to the left of the picture; magnification 100×. (B) Confocal cross section (section thickness 0.2 μm); magnification 100×. Note that the DASPMI-positive cells form an essentially confluent sheet. The apical crypts (red spots) are found only at the interface with the water. The central part of the filament is almost free of dye. Filament tips point towards the bottom of the picture. (C) Confocal cross section (section thickness 0.2 μm) near the gill arch; apical crypts, indicated by Con-A-FITC staining, face the water; filament tips point to the bottom left of the picture; magnification 400×. (D) Superficial optical section (thickness 0.2 μm) through the filament tip; rounded DASPMI-positive cells with a nuclear shadow are seen; some of the cells in this section show a red spot (red spots); magnification 480× (including a zoom factor of 1.2×). The filament tip points towards the bottom of the picture. (E) A single DASPMI-positive cell co-labelled with Con-A-FITC. This picture is composed of 31 superimposed and subsequent optical sections (0.5 μm each) collected in the z-direction. Note the absence of DASPMI staining adjacent to the Con-A-FITC-labelled space, in accordance with the absence of mitochondria in this region shown by electron microscopy (see Fig. 2). Magnification 900× (including a zoom factor of 1.5×). (F) Reconstructed cross section (90° tilted) of the cell shown in E; the cross section was made perpendicular to the plane of view shown in E, from left to right. This side view shows the depth of the apical crypt. Magnification 900× (including a zoom factor of 1.5×).
centrally located nucleus, which resembled chloride cells, could be observed in the epithelium of filaments (Wendelaar Bonga et al. 1990; McCormick, 1990). Lamellae had just started to develop on day 3. In 10-day-old larvae, the yolk sac was essentially absorbed and lamellae had begun to develop on both sides of all filaments. In 24-day-old larvae, the yolk sac had completely disappeared and adult-like lamellae were observed.

Chloride cells

The numerous large, ovoid cells with prominent nuclei seen on the newly developed gill filaments in 3-day-old tilapia using phase-contrast microscopy were identified as chloride cells by DASPMI staining using the CLSM; apical crypts were demonstrated by co-labelling the cells with Con-A-FITC (Fig. 1). The filamental epithelium consisted of a single layer of DASPMI-positive cells. Essentially all the cells had apical crypts as indicated by Con-A-FITC staining. The presence of apical crypts was confirmed by electron microscopical observations (Fig. 2). Fig. 3 presents the density of chloride cells (mm\(^{-2}\)):

- 3-day-old fish: 4630±489
- 10-day-old fish: 6280±271
- 24-day-old fish: 6600±219
- Adult fish: 6519±710

Electron microscopy

Gill filaments from juvenile tilapia were dissected and fixed for electron microscopy as described by Wendelaar Bonga and Van der Meij (1989).

Results

General observations

Three days after hatching, larvae had not yet absorbed their yolk sac. Without the yolk sac, they weighed about 4 mg and had a body length of approximately 7 mm. On each side of the head, four newly developed gill arches could be distinguished microscopically. The first and most exterior arch was slightly bigger than the second one; the third and fourth arches were smaller still. Gill filaments were already present, in particular on the first two arches. Under a phase-contrast microscope, numerous large, spherical or ovoid granular cells with a centrally located nucleus, which resembled chloride cells, could be observed in the epithelium of filaments (Wendelaar Bonga et al. 1990; McCormick, 1990). Lamellae had just started to develop on day 3. In 10-day-old larvae, the yolk sac was essentially absorbed and lamellae had begun to develop on both sides of all filaments. In 24-day-old larvae, the yolk sac had completely disappeared and adult-like lamellae were observed.

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Density of branchial chloride cells

![Graph showing density of branchial chloride cells over time](image)

**Discussion**

In this paper, we present evidence that numerous functional chloride cells are present in the gills of freshwater tilapia (yolk-sac) larvae, as early as 3 days after hatching. We have based this conclusion on four observations. First, in addition to the numerous mitochondria indicated by DASPMI-staining, most chloride cells possess an apical crypt in contact with the water, as shown by electron microscopy and by *in vivo* Con-A-FITC staining. Second, the chloride cells possess a high density of ouabain-binding sites, as shown by their ability to bind fluorescent anthroylouabain. Third, an extremely high Na⁺/K⁺-ATPase specific activity was found in the gill tissue of these larvae. Fourth, electron micrographs of the chloride cells confirm the presence of numerous mitochondria and an extensive tubular membrane system where transport ATPases and carriers have been shown to reside (Flik *et al.* 1985; Flik and Verbost, 1993).

Indeed, functional chloride cells in teleosts are structurally characterized by many small mitochondria and anastomosing tubules, where ion-transporting enzymes such as Na⁺/K⁺-ATPase, transport Ca²⁺-ATPase and a Na⁺/Ca²⁺ exchanger are located (Hootman and Philpott, 1979; Flik *et al.* 1985; Wendelaar Bonga *et al.* 1990; Kültz and Jürss, 1993; Verbost *et al.* 1994). The tubules have a lumen that is continuous with the extracellular space through orifices in the basolateral membrane (Sardet *et al.* 1979; Laurent and Dunel, 1980; Wendelaar Bonga *et al.* 1990), and the cell apical plasma membrane surface forms a crypt (or pit) facing the water. The latter contains glycoproteins and from its structure is considered to be an ion gradient region, a typical feature of functional chloride cells (Alderdice, 1988). To confirm the presence of intact functional chloride cells, a combination of three specific vital fluorescent probes (DASPMI, labelling mitochondria, Con-A-FITC labelling apical crypts, and anthroylouabain for indicating Na⁺/K⁺-ATPase) was applied successfully in this study. Since chloride cells are the *only* cells in gill epithelia containing an abundance of mitochondria,
Time after hatching (days)

<table>
<thead>
<tr>
<th>Time after hatching (days)</th>
<th>( V_{\text{total}} ) (( N = 6 ))</th>
<th>( V_{\text{spec}} ) (( N = 6 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>57±17</td>
<td>1558±366</td>
</tr>
<tr>
<td>24</td>
<td>233±42</td>
<td>914±192</td>
</tr>
<tr>
<td>180 (adult)</td>
<td>371±58</td>
<td>4±0.6</td>
</tr>
</tbody>
</table>

\( V_{\text{total}} \) is the total \( \text{Na}^+\text{K}^-\text{ATPase} \) activity in \( \mu \text{mol P}_1\text{ h}^{-1} \) (\( V_{\text{spec}} \times \text{mg protein} \)).

\( V_{\text{spec}} \) is the specific \( \text{Na}^+\text{K}^-\text{ATPase} \) activity in \( \mu \text{mol P}_1\text{ h}^{-1} \text{mg}^{-1} \).

Data are means ± s.d. for six fish.

Table 1. Total and specific \( \text{Na}^+\text{K}^-\text{ATPase} \) activity in gills of larval, juvenile and adult freshwater tilapia

These cells may provide the energy required for active transepithelial ion transport (\( \text{Na}^+ \), \( \text{Cl}^- \), \( \text{Ca}^{2+} \)) against the sizeable electrochemical gradients known to exist between the blood and the external medium in freshwater fish (for a review, see Evans, 1982). Therefore, the specific vital dyes for blood and the external medium in freshwater fish (for a review, these cells may play a major role in active ion transport during the early developmental stages.

This conclusion is further supported by the presence of numerous ouabain-binding sites and significant \( \text{Na}^+\text{K}^-\text{ATPase} \) activity in vitro. The \( \text{Na}^+\text{K}^-\text{ATPase} \) specific activity
in gills of 10-day-old tilapia is almost 400 times higher than that of an adult tilapia. In 24-day-old tilapia, more lamellae had developed, while the density of branchial chloride cells in the filaments did not change. This resulted in the observed decrease of Na+/K+-ATPase specific activity an increase of total Na+/K+-ATPase activity of the gills.

In summary, our results demonstrate the presence of mature branchial chloride cells in very young freshwater tilapia and that these cells are equipped to participate in ion transport, even before the gills have adult morphological characteristics.

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References


