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Chloride cells were identified by Na+/K+-ATPase immunocytochemistry at the light and electron microscope levels in gills of freshwater tilapia Oreochromis mossambicus. Turnover of chloride cells was enhanced by exposing the fish to waterborne copper (3.2 µmol l⁻¹) for 14 days, as indicated by a 38% increase in cells expressing proliferating cell nuclear antigen (PCNA) relative to controls. The expression of PCNA was most marked in the central area of the filamental epithelium, from where the chloride cells are thought to originate and migrate. In control fish, chloride cells were associated exclusively with the filamental epithelium. In both controls and copper-exposed fish, two chloride cell populations were seen after Na+/K+-ATPase immunostaining. These probably represent subpopulations of newly emerged chloride cells: (1) strongly stained cells (mature chloride cells) in the filamental and lamellar epithelium and (2) weakly stained cells, identified by electron microscopy as apoptotic and necrotic chloride cells, mainly in the filamental epithelium. Absolute numbers of mature chloride cells fell, while necrotic and apoptotic chloride cell numbers increased, in copper-exposed fish. A strong correlation could be established for gill Na+/K+-ATPase specific activity and the number of strongly stained chloride cells in controls and copper-exposed fish and for Na+/K+-ATPase specific activity and total numbers of immunoreactive cells in copper-exposed fish owing to an increased incidence of weakly staining cells.

Key words: teleost gill, chloride cell, Na+/K+-ATPase, copper, apoptosis, necrosis, proliferating cell nuclear antigen (PCNA).

Introduction

Chloride cells, the ion-transporting cells in gills of teleostean fish, play an important role in the maintenance of ionic balance in these animals (Perry, 1997; Wendelaar Bonga, 1997). The chloride cells are characterised by numerous mitochondria and an extensive tubular membrane system containing a high density of Na+/K+-ATPase activity. This enzyme activity plays a crucial role in branchial epithelial ion transport (McCormick, 1995; Flik et al., 1996).

The gill of freshwater fish is the prime target for the toxic actions of waterborne copper (Laurén and McDonald, 1985, 1987). In tilapia (Oreochromis mossambicus), copper accumulates in the chloride cells (Dang et al., 1999) and inhibits Na+/K+-ATPase activity (Li et al., 1996), which results in hyponatremia in freshwater fish (Li et al., 1998). To counteract the toxic actions of copper, the fish could respond with compensatory mechanisms including stimulation of Na+/K+-ATPase synthesis and enhanced chloride cell turnover (McDonald and Wood, 1993; Wendelaar Bonga, 1997), and these possibilities have been tested in this study. In seawater flounder (Platichthys flesus L.) exposed to 3.2 µmol l⁻¹ copper in the water, an increase in branchial Na+/K+-ATPase activity was demonstrated using a combination of a ouabain-binding technique and a biochemical assay of Na+/K+-ATPase activity (Stagg and Shuttleworth, 1982). In freshwater tilapia exposed for 4 weeks to acid water or to waterborne copper, branchial chloride cell density increased but Na+/K+-ATPase specific activity decreased (Wendelaar Bonga et al., 1990; Li et al., 1998). This apparent discrepancy between increased chloride cell number and decreased Na+/K+-ATPase activity was assumed to result from increased percentages of immature, necrotic and apoptotic chloride cells with a lower Na+/K+-ATPase content than mature chloride cells (Wendelaar Bonga and Lock, 1992; Pratap and Wendelaar Bonga, 1993). Copper directly induces necrosis and indirectly, via cortisol, induces apoptosis of chloride cells in tilapia gills (Bury et al., 1998; Li et al., 1998).

Direct experimental evidence to support the statement that immature or degenerating chloride cells contain less Na+/K+-ATPase (activity) than mature and functional chloride cells is, however, lacking and the main objective of this paper is to examine this possibility. At present it is unclear how tilapia compensate for the toxic effects of copper, i.e. whether branchial Na+/K+-ATPase activity is increased by increasing chloride cell density in the gills or whether the Na+/K+-ATPase activity in differentiated chloride cells is enhanced. We therefore studied the expression of Na+/K+-ATPase in branchial chloride cells, as...
well as the epithelial density and distribution of these cells in the
gills of tilapia after exposure to waterborne copper. Using Na+/K+-ATPase immunocytochemistry, chloride cells in the
gills of tilapia were identified and quantified by light and electron microscopy, and Na+/K+-ATPase immunoreactivity
was quantified in mature chloride cells, apoptotic chloride cells
and necrotic chloride cells. Previous studies have shown
that proliferating cell nuclear antigen (PCNA) immunocytochemistry
with a mouse monoclonal antibody can successfully be applied to monitor proliferation of cells in fish
tissues (Negishi et al., 1990; Ortego et al., 1994; Alfei et al., 1994;
Berntsson et al., 1999) and we used this antibody to
quantify PCNA expression in the gill epithelium of tilapia.

Materials and methods

Animals
Male and female tilapia, Oreochromis mossambicus, with a
mean mass of 19±6 g (range 12–28; N=20) were obtained from
laboratory stocks. Groups of 10 fish each were housed in 200 l
aquaria provided with Nijmegen tap water at 26 °C, which was
continuously aerated, filtered and recirculated by Eheim pumps
at a rate of 600 l h⁻¹. The concentrations of the main ions in the
water (in mmol l⁻¹) were: Na⁺, 0.5; Ca²⁺, 0.7; Mg²⁺, 0.2; Cl⁻,
0.7; SO₄²⁻, 0.5 (pH 7.6). Fish were fed daily with Trouvit
pellets (2% of the total fish wet mass per day). Aquaria were
watered (in mmoll⁻¹) were: Na+, 0.5; Ca2+, 0.7; Mg2+, 0.2; Cl–,
4h, followed by infusion of a 4|imoll⁻¹ copper solution at

Exposure to copper and sampling
The experiment was started by infusion of a 4 mmol l⁻¹
Cu(NO₃)₂ stock solution at a rate of 1 ml min⁻¹ into the tank over
4h, followed by infusion of a 4 μmol l⁻¹ copper solution at
10 ml min⁻¹. This resulted in a constant copper level of
3.2±0.2 μmol l⁻¹, indicating that approximately 30% of the
infused copper was adsorbed to the system. Control fish were in
water containing a constant 90±6 μmol l⁻¹ copper (mean ± S.E.M.,
N=20). The levels of copper were checked twice daily using
atomic absorption spectrometry (AAS, Philips PU 9200,
connected to an electron thermal atomiser, Philips PU 9390X).
No mortality was observed during the experiments. After 14 days
of exposure to copper, i.e. when copper accumulation in the
branchial epithelium plateaued and ion balances were starting to
be restored (Dang et al., 1999), fish were removed from control
and experimental tanks, quickly anaesthetised in phenoxy-
ethanol (Fluka Chemicals, diluted 1:3000 in water) and sampled.

Na⁺/K⁺-ATPase specific activity
The specific activity of Na⁺/K⁺-ATPase in gill homogenate
(Hg) was determined as described by Flik et al. (1983). Sepionin
(0.2 mg mg⁻¹ protein) was routinely added to optimise
substrate accessibility. Membrane protein content was
determined with a reagent kit (Biorad), using bovine serum
albumin (BSA) as reference. Homogenate samples were
incubated for 20 min at 37°C in a medium containing
100 mmol l⁻¹ NaCl, 30 mmol l⁻¹ imidazole-histidine, pH 7.4,
0.1 mmol l⁻¹ EDTA, 5 mmol l⁻¹ MgCl₂ and either 15 mmol l⁻¹
KCl (medium A) or 1 mmol l⁻¹ ouabain (medium E). Na₂ATP
was added to a final concentration of 3 mmol l⁻¹. The reaction
was stopped by adding 1.5 ml of an ice-cold 8.4% TCA
solution. Liberated inorganic phosphate, Pᵢ, was quantified.
The specific activity of Na⁺/K⁺-ATPase was defined as the
difference between the release of Pᵢ in medium A and in
medium E, and was expressed in μmol l⁻¹ Pᵢ h⁻¹ mg⁻¹ protein.

Immunocytochemistry
Gills were immersed in Bouin's fixative or 4 %
paraformaldehyde in phosphate-buffered saline (PBS, pH 7.1)
for 24h. After dehydration and embedding in paraffin, 7 μm
thick tissue sections were cut, mounted on poly-L-lysine-coated
slides (Sigma, St Louis, Mo., USA), and processed according
to the avidin–biotin–peroxidase complex (ABC) technique
(Hsu et al., 1981) to visualise chloride cells. In brief, after
dewaxing and blocking the endogenous peroxidase with 2 %
H₂O₂ for 20 min, slides were washed in TBSTX (0.05 mol l⁻¹
Tris-buffered saline with 150 mmol l⁻¹ NaCl and 0.03 % Triton
X-100, pH 7.6) solution. Non-specific sites were blocked with
20 % normal goat serum for 30 min at room temperature. Slides
were incubated overnight in a humid chamber at the room
temperature (20±2 °C) with a mouse monoclonal antibody to
Na⁺/K⁺-ATPase (IgGα5; Developmental Studies Hybridoma
Bank, Department of Biological Sciences, The University of
Iowa, USA) or to PCNA (Clone PC10; Calbiochem, catalogue
no. NA03) at working dilutions of 1:100 and 1:4000, respectively.
Biotinylated goat-anti-mouse IgG was used as the
second antiserum at a dilution of 1:200 for 1 h at room
temperature, followed by incubation with peroxidase-
conjugated streptavidin (ABC kit, Vector Laboratories, dilution
1:200, prepared at least 30 min before use) for 1 h. Between
each step, the sections were washed twice in TBSTX solution.
Subsequently, 3,3'-diaminobenzidine (DAB) in TB buffer (0.05
mol l⁻¹ Tris-buffered saline, pH 7.6) containing H₂O₂ (0.03 %)
was applied at room temperature. Finally, sections were
dehydrated and mounted. As a control for specificity the
procedure was carried out as above, with the omission of the
first antiserum.

For immunogold labelling, a piece of a second gill arch was
dissected and fixed in 1 % glutaraldehyde and 3.5 %
paraformaldehyde in 0.4 mol l⁻¹ phosphate buffer, pH 7.4 (PB),
and kept for 2 h at room temperature. The fixed specimens
were washed in distilled water, dehydrated in graded ethanols,
immersed overnight with LR White, and polymerised for 48 h
by ultraviolet light (366 nm) at 4 °C. Ultrathin sections were
cut and collected on Butvar-coated nickel grids. Grids with
sections were preincubated successively in (1) PBS containing
1 % glycine, (2) PBS containing 1 % gelatine (PBS-gelatine),
and (3) PBS containing 1 % BSA, each step lasting 15 min at
room temperature. The grids were then incubated overnight
with the Na⁺/K⁺-ATPase IgGα5 antibody at a dilution of 1:50
at 4 °C in a moist chamber. Control sections were incubated
with 1 % BSA. After thorough washing in PBS-gelatine, grids
were incubated in rabbit anti-mouse IgG, at a dilution of 1:500,
coupled to 10 nm colloidal gold for 1 h at room temperature.
I'-1 : 20 filamental chloride cells showing a nucleus and 20 lamellar chloride cells were scored for optical density measurement. Sections of gill arches were cut and every eighth section was analysed per group. The density of number of cells per millimetre length of filament. For each fish, 7 μm thick parasagittal sections of gill arches were cut and every eighth section was mounted. In every section, five different filaments containing chloride cells (Fs) and weakly stained filamental chloride cells (Fw), and filamental chloride cells, strongly stained filamental chloride cells (Ls), and weakly stained lamellar chloride cells (Lw). For each fish, at least ten electron photographs were taken (Fig. 1B). Scale bar, 30 μm.

After incubation, the grids were washed in PBS and MilliQ water. Samples were examined in a transmission electron microscope (Jeol CX11) at 60 kV.

Quantification

Gill chloride cells and PCNA-immunoreactive cells in the filamental and lamellar epithelia were quantified using a video image data-analysing system (VIDAS; Kontron, Germany). The chloride cells were categorised as strongly stained or weakly stained according to their Na+/K+-ATPase staining intensity (a chloride cell with an optical density above 0.41 (arbitrary units) was considered to be strongly stained; see Fig. 3). The chloride cells were divided into two groups of filamental chloride cells, strongly stained filamental chloride cells (Fs) and weakly stained filamental chloride cells (Fw), and two groups of lamellar chloride cells, i.e. strongly stained lamellar chloride cells (Ls), and weakly stained lamellar chloride cells (Lw). For each fish, 7μm thick parasagittal sections of gill arches were cut and every eighth section was mounted. In every section, five different filaments containing lamellae were randomly selected for quantification. Six fish were analysed per group (N=6). The results are expressed as the number of cells per millimetre length of filament. For each fish, 20 filamental chloride cells showing a nucleus and 20 lamellar chloride cells were scored for optical density measurement.

For each fish, at least ten electron photographs were taken in the trailing edge area of the filament to score mature and degenerating (apoptotic and necrotic) chloride cells according to the description of Wendelaar Bonga and Van der Meij (1989) and Wendehaar Bonga et al. (1990). The density of immunogold particles (number per μm²) was determined using Kontron MOP integration equipment (Kontron, Munich, Germany). Density was based on ten chloride cells per cell type per fish. Six fish were counted per sampling group.

Statistics

Data are presented as means ± s.e.m. Differences between groups were assessed by Student's two-tailed t-test for unpaired observations using Instat software. Significance levels are indicated as follows: *P<0.05, **P<0.01 and ***P<0.001.

Results

PCNA immunoreactive cells

In controls, PCNA immunoreactive cells were mainly found in the centre of the filamental epithelium spanning from leading to trailing edge. Occasionally, they were present in more apical areas of the filamental epithelium or in the lamellar epithelium (Fig. 1A). In gill filaments from fish exposed to copper, the number of PCNA immunoreactive cells in the branchial epithelium had increased by 38% from 77.8±7.8 cells mm⁻¹ (mean ± s.e.m., range 40–130 cells mm⁻¹, N=6) in control fish to 107.7±10.9 cells mm⁻¹ (mean ± s.e.m., range 50–163 cells mm⁻¹, N=6) in copper-exposed fish (P<0.05). The PCNA immunoreactivity in gills of copper-exposed fish was distributed mainly in the basal and central areas of the filamental epithelium (Fig. 1B).

Branchial chloride cell density and Na⁺/K⁺-ATPase immunoreactive staining intensity

In control fish, Na⁺/K⁺-ATPase immunoreactive cells (chloride cells) were exclusively found in the filamental epithelium, mostly at its surface, with occasional small,
Fig. 2. Na⁺/K⁺-ATPase antibody staining of gill chloride cells in control tilapia (A) and in tilapia after exposure to 3.2 μmol L⁻¹ copper for 14 days (B) showing strong Na⁺/K⁺-ATPase immunoreactivity in the filaments of control fish. Some small Na⁺/K⁺-ATPase positive cells (arrows) are present in the inner part of filaments (A). In copper-exposed fish gills, strongly stained as well as weakly stained cells (see arrowheads) are found in the filament, while strongly stained chloride cells are also frequent on the lamellae. Scale bar, 50 μm.

Na⁺/K⁺-ATPase immunoreactive cells in the inner part (Fig. 2A). After exposure to copper, chloride cells were found not only in the filamental epithelium but also in the lamellar epithelium (Fig. 2B). Two populations of Na⁺/K⁺-ATPase immunoreactive cells, strongly stained chloride cells and weakly stained chloride cells, were scored according to an analysis of cell optical density in copper-exposed fish and controls (Fig. 3). The number of filamental strongly stained chloride cells decreased from 97% in controls to 72% in copper-exposed fish (Fig. 4). In copper-exposed fish, 27.5% of filamental chloride cells showed weak staining, whereas 8% of lamellar chloride cells were weakly stained. The mean optical density of Na⁺/K⁺-ATPase-stained chloride cells in filamental epithelium of controls was 0.545±0.017 (N=6). After copper exposure, the mean optical density of Na⁺/K⁺-ATPase-stained chloride cells had decreased significantly by 14% to 0.471±0.015 (P<0.01). The mean optical density of strongly stained chloride cells in controls (0.555±0.015) was not significantly different from that of the strongly stained filamental chloride cells (0.517±0.012) and strongly stained lamellar chloride cells (0.526±0.011) in copper-exposed fish. The mean optical density of weakly stained chloride cells (0.352±0.021; these cells are essentially confined to the filamental epithelium) in copper-exposed fish was significantly lower (P<0.001) than that of the strongly stained chloride cells. The total number of chloride cells in the gill filaments of fish exposed to copper had increased by 9.1% from 111.4±5.2 cells mm⁻¹ in control to 121±16.9 cells mm⁻¹ in copper-exposed fish. The appearance of chloride cells in the lamellar epithelium accounted for the increase (Figs 2B, 4).

Na⁺/K⁺-ATPase immunogold labelling of chloride cells

Immunogold labelling of Na⁺/K⁺-ATPase at the electron microscope level was essentially restricted to mitochondria-

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Fig. 3. Distribution of Na⁺/K⁺-ATPase immunoreactive cells in the filamental and lamellar epithelia of the gills of control (squares) and copper-exposed (circles) tilapia. In both controls and copper-exposed fish, two populations of chloride cells can clearly be distinguished on the basis of their optical density (arbitrary units).
In studies on the effects of heavy metals on fish ionoregulation, two compensatory mechanisms have been reported with respect to branchial ion transport activity: an increase of chloride cell numbers and an increase in Na⁺/K⁺-ATPase activity per chloride cell (Stagg and Shuttleworth, 1982; McDonald and Wood, 1993). Our results demonstrate that the branchial compensatory response to copper exposure in tilapia involves an increase in chloride cell turnover, with a decrease in the number of mature chloride cells and an increase in the number of chloride cells with low Na⁺/K⁺-ATPase activity, which are probably apoptotic and necrotic cells. As a result, the overall Na⁺/K⁺-ATPase specific activity and the Na⁺/K⁺-ATPase density per cell decreased. This conclusion is based on a decreased optical density in subpopulations of chloride cells and increased numbers of chloride cells in the copper-exposed fish. The increased chloride cell turnover is substantiated by the increased PCNA expression observed after exposure to copper and the higher number of apoptotic and necrotic cells present; the latter finding is consistent with earlier studies in our laboratory on copper-exposed tilapia (Li et al., 1998). Although the number of chloride cells is less than 5–10% of the total branchial cells (Perry and Laurent, 1993; Verbost et al, 1994), it has been shown that approximately 30% of the proliferating cells differentiate into chloride cells in guppy Poecilia reticulata (Chàtrien and Pisam, 1986) and chum salmon Oncorhynchus keta (Uchida and Kaneko, 1996) during seawater adaptation. This means that the chloride cell

### Discussion

We draw four major conclusions from this study. First, when fish are exposed to copper, increased numbers of chloride cells are observed in the gills of freshwater tilapia. Second, after copper exposure, chloride cells appear in the lamellar epithelium. Third, copper exposure increases levels of necrosis and apoptosis of chloride cells and necrotic and apoptotic cells have much lower Na⁺/K⁺-ATPase antigenicity than mature chloride cells. Fourth, branchial Na⁺/K⁺-ATPase specific activity is strongly and positively correlated with the number of strongly stained Na⁺/K⁺-ATPase immunoreactive chloride cells in control and copper-exposed fish; in copper-exposed fish, Na⁺/K⁺-ATPase specific activity is still strongly, but less positively, correlated to the total number of chloride cells as a result of an increased density of weakly stained cells.

The density of gold particles was considerably lower in the degenerating cells than in the mature chloride cells. In both controls and copper-exposed fish, the mature chloride cells contained 3340±760 immunogold particles μm⁻² (mean ± s.e.m., N=6; range 2680–4320 μm⁻²), while apoptotic chloride cells only contained 470±260 (range 140–740 μm⁻²) and necrotic chloride cells 680±350 μm⁻² (range 310–860 μm⁻²).

### Relationship between branchial chloride cell number and Na⁺/K⁺-ATPase activity

As shown in Fig. 6, the Na⁺/K⁺-ATPase specific activities had decreased after 14 days of copper exposure: mean specific activities were 13.1±0.9 μmolP₂h⁻¹mg⁻¹ for controls and 9.2±0.6 μmolP₂h⁻¹mg⁻¹ protein for copper-exposed fish (N=6, P<0.01). The number of strongly stained Na⁺/K⁺-ATPase immunoreactive cells present and the branchial Na⁺/K⁺-ATPase specific activities in controls and copper-exposed fish were significantly correlated (r=0.82, P<0.001). However, in copper-exposed fish, the correlation between Na⁺/K⁺-ATPase specific activity and total number of Na⁺/K⁺-ATPase immunoreactive cells was even stronger (r=0.96, P<0.01), but with a lower slope because of the greater numbers of weakly stained cells (Fig. 6).

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**Fig. 4. Gill chloride cell numbers in control tilapia (CTR) and in fish exposed to 3.2 μmolL⁻¹ copper (Cu) for 14 days. Values are means ± S.E.M. (N=6). Significant difference in total chloride cell numbers compared with controls are indicated by an asterisk, P<0.05; Fs, strongly stained filamental chloride cells; Fw, weakly stained filamental chloride cells; L, chloride cells in lamellae.**

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population in particular must have a higher turnover than the other cell types that make up the branchial epithelium. In a previous study, we demonstrated that metallothionein was exclusively induced in the immature stages and not in the mature branchial cells after an exposure to copper similar to that used in this study (Dang et al., 1999). Metallothionein can protect Na⁺/K⁺-ATPase against the toxic actions of heavy metals, including copper (Hussain et al., 1995). Copper promotes the expression of metallothionein in the gills, where it is first activated in the chloride cells (Dang et al., 1999). Thus, increased chloride cell turnover may contribute to the protection of branchial Na⁺/K⁺-ATPase activity in new chloride cells that express metallothionein.

In copper-exposed tilapia, we observed strongly stained chloride cells in the lamellae. These lamellar chloride cells contained amounts of Na⁺/K⁺-ATPase similar to those found in mature filamental chloride cells. Since chloride cells were

![Fig. 5. The distribution of Na⁺/K⁺-ATPase immunogold labelling in gill mature chloride cells (A), necrotic chloride cells (B) and apoptotic chloride cells (C) of fish gills after 14 days of exposure to copper (3.2 μmol l⁻¹). Labeling is associated with the tubular membrane system (ts). Most immunogold particles are present in mature chloride cells; m, mitochondria. Scale bar, 0.1 μm.](image)

![Fig. 6. Correlations between gill Na⁺/K⁺-ATPase specific activity and gill chloride cell numbers in controls and tilapia exposed to copper for 14 days. In controls, very few weakly stained cells were found (overlap of open circles and filled circles). In copper-exposed fish, two populations were observed (open squares and filled squares were separated). Significant positive correlations between Na⁺/K⁺-ATPase specific activity and cell number were found for strongly stained cells in controls and copper-exposed fish (r=0.11, P=0.16; r=0.82, P<0.001) and for the total number of immunoreactive cells in copper-exposed fish (r=0.05, x=3.3; r=0.96, P<0.01). open circles, strongly stained chloride cells in control; closed circles, total number of chloride cells in control; open squares, strongly stained chloride cells in copper-exposed fish; closed squares, total number of chloride cells in copper-exposed fish.](image)
abundant in the lamellae of the control fish, the lamellar chloride cells must have migrated from the germinal centres (the PCNA immunoreactive area) of the filamental epithelium (Dang et al., 1999). The lamellar chloride cells, which are in close contact with lamellar blood spaces, may play an important role in active branchial ion absorption, as has been suggested for freshwater rainbow trout Oncorhynchus mykiss (Perry and Wood, 1985; Avella et al., 1987; Perry and Laurent, 1989). On the basis of their structure and position in the gills during freshwater to seawater transfer of fish, chloride cells have been classified into two different subtypes, the α and β cells (Pisam et al., 1995). Several researchers concluded from the freshwater and seawater transfer experiment that chloride cells in the lamellae are the site of ion uptake in fresh water and that chloride cells in the filament the site of salt secretion in seawater according to the Na+/K+-ATPase specific activity and the total number of Na+/K+-ATPase immunoreactive cells located in these areas (Laurent, 1984), which is where we observed the greatest PCNA immunoreactivity. The small Na+/K^+ ATPase immunoreactive cells located in these areas (Fig. 1A) are probably immature chloride cells (Wendelaar Bonga et al., 1990). The hypothesis that immature and degenerating chloride cells (including apoptotic and necrotic chloride cells) may contain less Na+/K+-ATPase was proposed earlier by Wendelaar Bonga et al. (1990) and is widely accepted (Perry and Laurent, 1993; Witters et al., 1996), yet has never been verified experimentally. Our results at both the light and electron microscope level provide direct evidence that apoptotic and necrotic chloride cells contain less Na+/K+-ATPase activity. It is well established that stressors such as copper exposure induce apoptosis and necrosis of chloride cells in tilapia gills (Wendelaar Bonga et al., 1990; Li et al., 1998; Bury et al., 1998). Such increases in levels of apoptosis and necrosis were also found here and could be directly correlated with a reduced expression of Na+/K^-ATPase. At the light microscope level, we found more weakly stained chloride cells after copper exposure. At the electron microscope level, weakly stained (low density of immunogold particles) cells proved to be chloride cells showing signs of either apoptosis or necrosis.

An important new finding is the positive correlation between branchial Na+/K^-ATPase specific activity and the numbers of the strongly stained chloride cells. The strongly stained chloride cells both in controls and in copper-exposed fish showed all the ultrastructural characteristics of mature chloride cells and contained the highest amount of Na+/K^-ATPase. A less positive correlation was observed between the branchial Na+/K^-ATPase specific activity and the total number of chloride cells in copper-exposed fish, which resulted from the large increase in weakly staining cells. Na+ pump activity is dependent on the total number of available pumps (Ewart and Klip, 1995). It may well be that not all Na+/K^-ATPase immunoreactivity reflects Na+ pump activity in vivo. The presence of such silent Na+/K^-ATPase was recently indicated in the gills of shore crab Carcinus maenas (Lucu and Flik, 1999). Our observations support the idea that immature and degenerating cells are no longer functional in the ion-transporting activity of gills (Wendelaar Bonga et al., 1990),

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supporting the body of evidence for decreased Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activities and hyponatremia in copper-exposed fish (Lauren\u00e9 and McDonald, 1985, 1987; Li et al., 1998). We conclude that copper-induced hyponatremia in a freshwater fish is associated with decreased branchial Na\textsuperscript{+}/K\textsuperscript{+}-ATPase specific activity and decreasing numbers of mature (strongly stained) chloride cells.

In summary, increased chloride cell turnover and migration to the lamellae are important mechanisms of branchial adaptation to copper exposure in tilapia. Apoptotic and necrotic chloride cells, whose numbers increase after copper exposure, contain less Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity than mature chloride cells, and this explains the discrepancy between the increase in chloride cell total numbers and the reduction in Na\textsuperscript{+}/K\textsuperscript{+}-ATPase specific activity in copper-exposed fish.

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