

## Na<sup>+</sup>/K<sup>+</sup>-ATPase IMMUNOREACTIVITY IN BRANCHIAL CHLORIDE CELLS OF *OREOCHROMIS MOSSAMBICUS* EXPOSED TO COPPER

ZHICHAO DANG, ROBERT A. C. LOCK\*, GERT FLIK AND SJOERD E. WENDELAAR BONGA

*Department of Animal Physiology, University of Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands*

\*e-mail: roblock@sci.kun.nl

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### Summary

Chloride cells were identified by Na<sup>+</sup>/K<sup>+</sup>-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<sup>-1</sup>) 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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase specific activity and the number of strongly stained chloride cells in controls and copper-exposed fish and for Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>-1</sup> copper in the water, an increase in branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was demonstrated using a combination of a ouabain-binding technique and a biochemical assay of Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase specific activity decreased (Wendelaar Bonga et al., 1990; Li et al., 1998). This apparent discrepancy between increased chloride cell number and decreased Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was assumed to result from increased percentages of immature, necrotic and apoptotic chloride cells with a lower Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase activity is increased by increasing chloride cell density in the gills or whether the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in differentiated chloride cells is enhanced. We therefore studied the expression of Na<sup>+</sup>/K<sup>+</sup>-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  $\text{Na}^+/\text{K}^+$ -ATPase immunocytochemistry, chloride cells in the gills of tilapia were identified and quantified by light and electron microscopy, and  $\text{Na}^+/\text{K}^+$ -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; Berntssen 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  $19 \pm 6$  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  $26^\circ\text{C}$ , which was continuously aerated, filtered and recirculated by Eheim pumps at a rate of  $600 \text{ l h}^{-1}$ . The concentrations of the main ions in the water (in  $\text{mmol l}^{-1}$ ) were:  $\text{Na}^+$ , 0.5;  $\text{Ca}^{2+}$ , 0.7;  $\text{Mg}^{2+}$ , 0.2;  $\text{Cl}^-$ , 0.7;  $\text{SO}_4^{2-}$ , 0.5 (pH 7.6). Fish were fed daily with Trouvit pellets (2% of the total fish wet mass per day). Aquaria were illuminated for 12 h per day.

### Exposure to copper and sampling

The experiment was started by infusion of a  $4 \text{ mmol l}^{-1}$   $\text{Cu}(\text{NO}_3)_2$  stock solution at a rate of  $1 \text{ ml min}^{-1}$  into the tank over 4 h, followed by infusion of a  $4 \mu\text{mol l}^{-1}$  copper solution at  $10 \text{ ml min}^{-1}$ . This resulted in a constant copper level of  $3.2 \pm 0.2 \mu\text{mol l}^{-1}$ , indicating that approximately 30% of the infused copper was adsorbed to the system. Control fish were in water containing a constant  $90 \pm 6 \text{ nmol l}^{-1}$  copper (mean  $\pm$  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 phenoxyethanol (Fluka Chemicals, diluted 1:3000 in water) and sampled.

### $\text{Na}^+/\text{K}^+$ -ATPase specific activity

The specific activity of  $\text{Na}^+/\text{K}^+$ -ATPase in gill homogenate ( $H_0$ ) was determined as described by Flik et al. (1983). Saponin ( $0.2 \text{ mg mg}^{-1}$  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  $37^\circ\text{C}$  in a medium containing  $100 \text{ mmol l}^{-1}$  NaCl,  $30 \text{ mmol l}^{-1}$  imidazole-histidine, pH 7.4,  $0.1 \text{ mmol l}^{-1}$  EDTA,  $5 \text{ mmol l}^{-1}$   $\text{MgCl}_2$  and either  $15 \text{ mmol l}^{-1}$

KCl (medium A) or  $1 \text{ mmol l}^{-1}$  ouabain (medium E).  $\text{Na}_2\text{ATP}$  was added to a final concentration of  $3 \text{ mmol l}^{-1}$ . The reaction was stopped by adding 1.5 ml of an ice-cold 8.4% TCA solution. Liberated inorganic phosphate,  $\text{P}_i$ , was quantified. The specific activity of  $\text{Na}^+/\text{K}^+$ -ATPase was defined as the difference between the release of  $\text{P}_i$  in medium A and in medium E, and was expressed in  $\mu\text{mol l}^{-1} \text{ P}_i \text{ h}^{-1} \text{ mg}^{-1}$  protein.

### Immunocytochemistry

Gills were immersed in Bouin's fixative or 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.1) for 24 h. After dehydration and embedding in paraffin,  $7 \mu\text{m}$  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%  $\text{H}_2\text{O}_2$  for 20 min, slides were washed in TBSTX ( $0.05 \text{ mol l}^{-1}$  Tris-buffered saline with  $150 \text{ mmol l}^{-1}$  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 ( $20 \pm 2^\circ\text{C}$ ) with a mouse monoclonal antibody to  $\text{Na}^+/\text{K}^+$ -ATPase (IgG $\alpha$ 5; 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 ( $0.05 \text{ mol l}^{-1}$  Tris-buffered saline, pH 7.6) containing  $\text{H}_2\text{O}_2$  (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  $0.4 \text{ mol l}^{-1}$  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  $4^\circ\text{C}$ . 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  $\text{Na}^+/\text{K}^+$ -ATPase IgG $\alpha$ 5 antibody at a dilution of 1:50 at  $4^\circ\text{C}$  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.

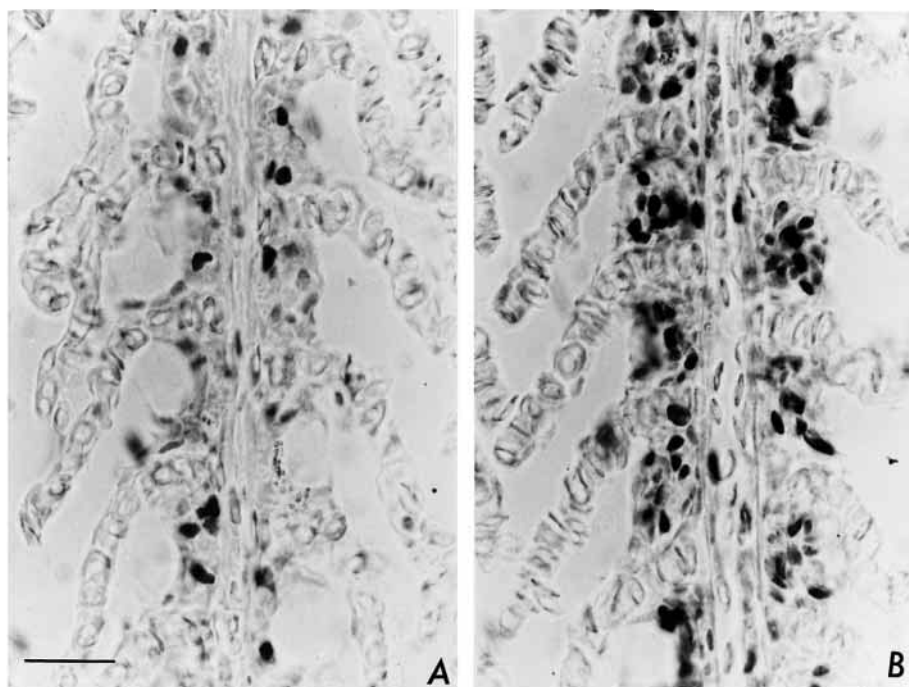


Fig. 1. PCNA immunostaining in the gills of tilapia and of tilapia exposed for 14 days to  $3.2\mu\text{mol l}^{-1}$  copper. In control fish, the PCNA-positive cells are mainly located in the central layer of the filamental epithelium (A). In copper-exposed fish, more PCNA-positive cells are found in this region and they are also frequently present in the more basal and apical layers of this epithelium (B). Scale bar,  $30\mu\text{m}$ .

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<sup>+</sup>/K<sup>+</sup>-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,  $7\mu\text{m}$  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 Wendelaar Bonga et al. (1990). The density of immunogold particles (number per  $\mu\text{m}^2$ ) 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  $\pm$  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

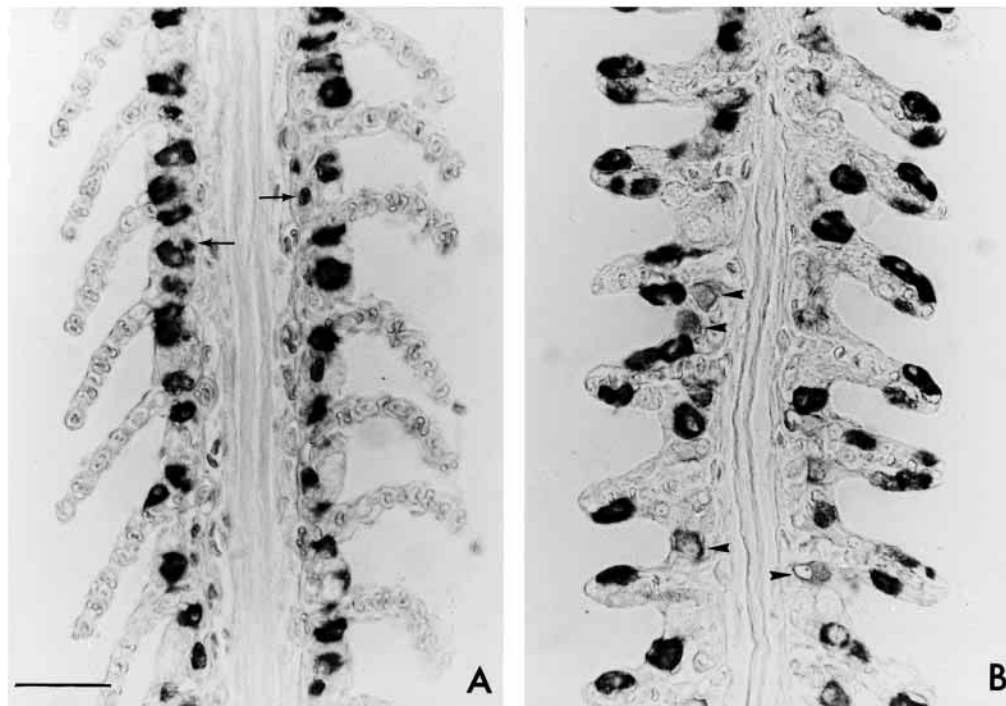
#### PCNA immunoreactive cells

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  $77.8\pm 7.8\text{ cells mm}^{-1}$  (mean  $\pm$  S.E.M., range 40–130 cells  $\text{mm}^{-1}$ ,  $N=6$ ) in control fish to  $107.7\pm 10.9\text{ cells mm}^{-1}$  (mean  $\pm$  S.E.M., range 50–163 cells  $\text{mm}^{-1}$ ,  $N=6$ ) 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<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactive staining intensity

In control fish, Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactive cells (chloride cells) were exclusively found in the filamental epithelium, mostly at its surface, with occasional small,

Fig. 2.  $\text{Na}^+/\text{K}^+$ -ATPase antibody staining of gill chloride cells in control tilapia (A) and in tilapia after exposure to  $3.2\ \mu\text{mol l}^{-1}$  copper for 14 days (B) showing strong  $\text{Na}^+/\text{K}^+$ -ATPase immunoreactivity in the filaments of control fish. Some small  $\text{Na}^+/\text{K}^+$ -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,  $50\ \mu\text{m}$ .



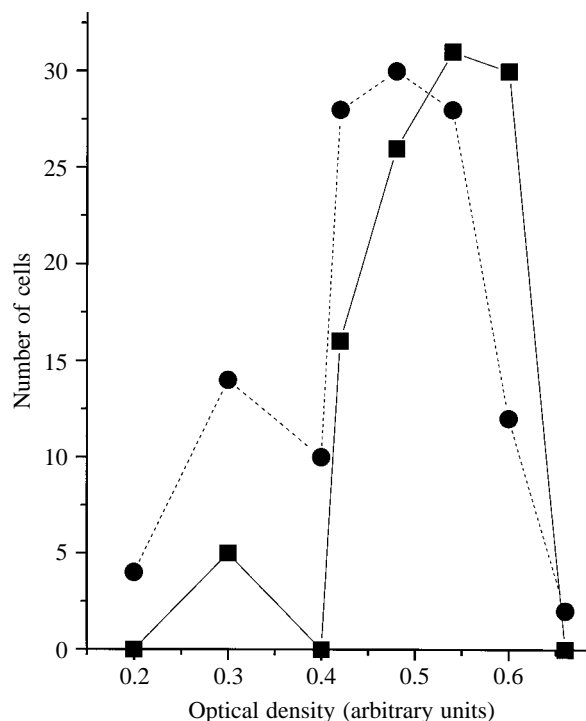
$\text{Na}^+/\text{K}^+$ -ATPase immunoreactive cells in the inner part (Fig. 2A). After exposure to copper, chloride cells were found not only in the filamental epithelium but also in the lamellar epithelium (Fig. 2B). Two populations of  $\text{Na}^+/\text{K}^+$ -ATPase immunoreactive cells, strongly 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  $\text{Na}^+/\text{K}^+$ -ATPase-stained chloride cells in filamental epithelium of controls was  $0.545 \pm 0.017$  ( $N=6$ ). After copper exposure, the mean optical density of  $\text{Na}^+/\text{K}^+$ -ATPase stained chloride cells had decreased significantly by 14% to  $0.471 \pm 0.015$  ( $P < 0.01$ ). The mean optical density of strongly stained chloride cells in controls ( $0.555 \pm 0.015$ ) was not significantly different from that of the strongly stained filamental chloride cells ( $0.517 \pm 0.012$ ) and strongly stained lamellar chloride cells ( $0.526 \pm 0.011$ ) in copper-exposed fish. The mean optical density of weakly stained chloride cells ( $0.352 \pm 0.021$ ; 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  $111.4 \pm 5.2$  cells  $\text{mm}^{-1}$  in control to  $121 \pm 16.9$  cells  $\text{mm}^{-1}$  in copper-exposed fish. The appearance of chloride cells in the lamellar epithelium accounted for the increase (Figs 2B, 4).

#### *$\text{Na}^+/\text{K}^+$ -ATPase immunogold labelling of chloride cells*

Immunogold labelling of  $\text{Na}^+/\text{K}^+$ -ATPase at the electron microscope level was essentially restricted to mitochondria-

Fig. 3. Distribution of  $\text{Na}^+/\text{K}^+$ -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).



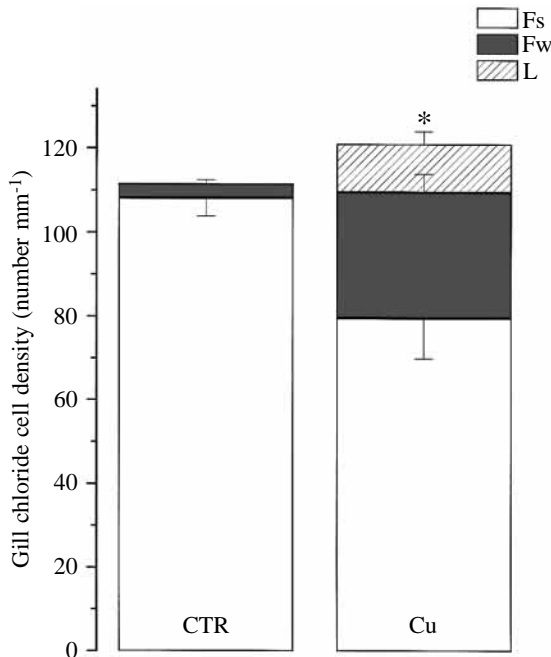


Fig. 4. Gill chloride cell numbers in control tilapia (CTR) and in fish exposed to  $3.2 \mu\text{mol l}^{-1}$  copper (Cu) for 14 days. Values are means  $\pm$  S.E.M. ( $N=6$ ). Significant difference in total chloride cell numbers compared with controls are indicated by an asterisk,  $P<0.05$ ; Fs, strongly stained filamental chloride cells; Fw, weakly stained filamental chloride cells; L, chloride cells in lamellae.

rich cells, and within these cells labelling was largely restricted to the tubular system of chloride cells (Fig. 5A). This Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity was found in mature chloride cells (Fig. 5A) as well as in necrotic (Fig. 5B) and apoptotic chloride cells (Fig. 5C). Apoptotic cells were identified at the electron microscope level on the basis of nuclear chromatin condensation and nuclear shrinkage, the presence of condensed cytosol, condensation of membranes and an increase in the diameter of the lumina of the tubular system; in later stages of the apoptotic event, macrophages are found in association with the degenerating cells. Necrotic cells are recognised by disrupted nuclear and plasma membranes, transparent cytoplasm, swollen mitochondria, often with fragmented cristae, and electron-transparent nuclei with dense patches of chromatin (Wendelaar Bonga and Van der Meij, 1989; Wendelaar Bonga et al., 1990).

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  $3340 \pm 760$  immunogold particles  $\mu\text{m}^{-2}$  (mean  $\pm$  S.E.M.,  $N=6$ ; range 2680–4320  $\mu\text{m}^{-2}$ ), while apoptotic chloride cells only contained  $470 \pm 260$  (range 140–740  $\mu\text{m}^{-2}$ ) and necrotic chloride cells  $680 \pm 350$   $\mu\text{m}^{-2}$  (range 310–860  $\mu\text{m}^{-2}$ ).

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

As shown in Fig. 6, the Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activities

had decreased after 14 days of copper exposure: mean specific activities were  $13.1 \pm 0.9 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$  protein for controls and  $9.2 \pm 0.6 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$  protein for copper-exposed fish ( $N=6$ ,  $P<0.01$ ). The number of strongly stained Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactive cells present and the branchial Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase specific activity and total number of Na<sup>+</sup>/K<sup>+</sup>-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).

#### 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<sup>+</sup>/K<sup>+</sup>-ATPase antigenicity than mature chloride cells. Fourth, branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activity is strongly and positively correlated with the number of strongly stained Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactive chloride cells in control and copper-exposed fish; in copper-exposed fish, Na<sup>+</sup>/K<sup>+</sup>-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.

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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase activity, which are probably apoptotic and necrotic cells. As a result, the overall Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activity and the Na<sup>+</sup>/K<sup>+</sup>-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 *Lebistes reticulatus* (Chrétien and Pisam, 1986) and chum salmon *Oncorhynchus keta* (Uchida and Kaneko, 1996) during seawater adaptation. This means that the chloride cell

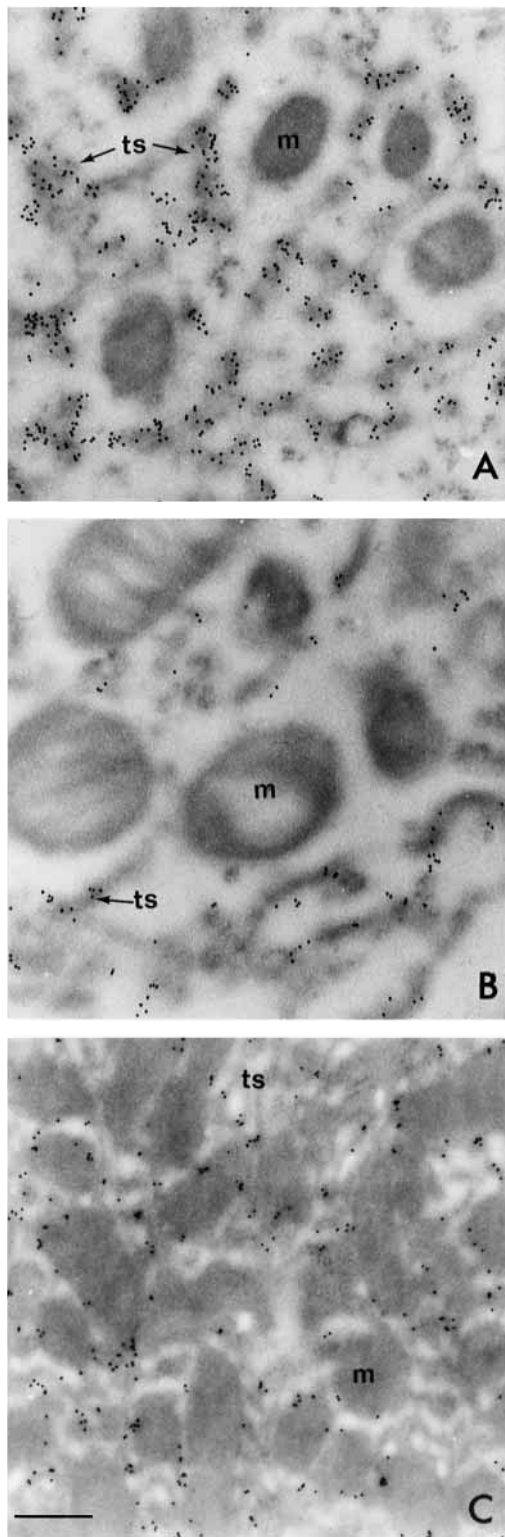


Fig. 5. The distribution of  $\text{Na}^+/\text{K}^+$ -ATPase immunogold labelling in gill mature chloride cells (A), necrotic chloride cells (B) and apoptotic chloride cells (C) of fish gills after 14 days of exposure to copper ( $3.2 \mu\text{mol l}^{-1}$ ). Labelling is associated with the tubular membrane system (ts). Most immunogold particles are present in mature chloride cells; m, mitochondria. Scale bar,  $0.1 \mu\text{m}$ .

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  $\text{Na}^+/\text{K}^+$ -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  $\text{Na}^+/\text{K}^+$ -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  $\text{Na}^+/\text{K}^+$ -ATPase similar to those found in mature filamental chloride cells. Since chloride cells were

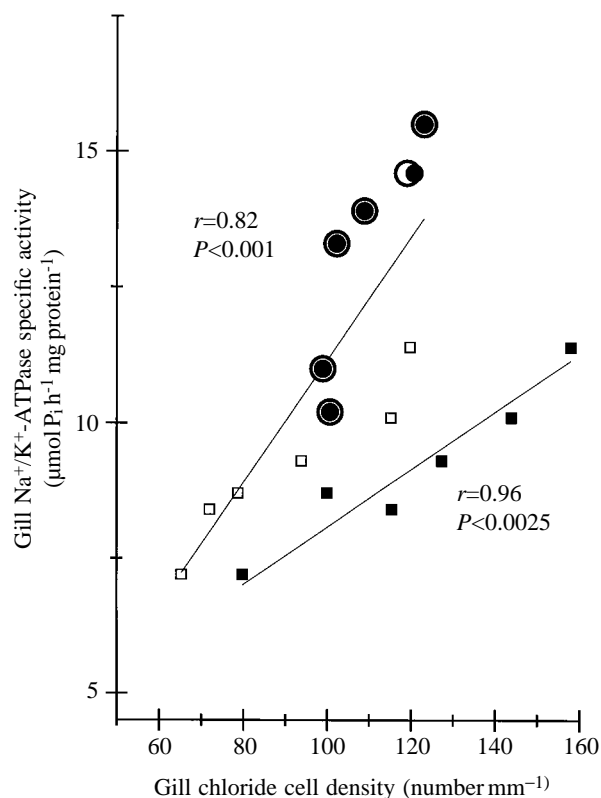


Fig. 6. Correlations between gill  $\text{Na}^+/\text{K}^+$ -ATPase specific activity and gill chloride cell numbers in controls and tilapia exposed to copper for 14 days. In controls, very few weakly stained cells were found (overlap of open circles and filled circles). In copper-exposed fish, two populations were observed (open squares and filled squares were separated). Significant positive correlations between  $\text{Na}^+/\text{K}^+$ -ATPase specific activity and cell number were found for strongly stained cells in controls and copper-exposed fish ( $y=0.11x-0.16$ ;  $r=0.82$ ,  $P<0.001$ ) and for the total number of immunoreactive cells in copper-exposed fish ( $y=0.05x+3.3$ ;  $r=0.96$ ,  $P<0.01$ ). 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.

absent 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  $\alpha$  and  $\beta$  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 sea water according to the Na<sup>+</sup>/K<sup>+</sup>-ATPase staining intensity in chloride cells (Uchida et al., 1996; Shikano and Fujio, 1998). However, this conclusion is not corroborated by observations on Na<sup>+</sup>/K<sup>+</sup>-ATPase staining for chloride cells after cortisol administration in brown trout *Salmo trutta* because the number of lamellar chloride cells did not change (Seidelin et al., 1999). Our results demonstrate that filamental and lamellar chloride cells have similar Na<sup>+</sup>/K<sup>+</sup>-ATPase activities, and thus may have a similar ion uptake function in the copper-exposed tilapia. Compared with weakly stained filamental chloride cells, the low proportion of apoptotic and necrotic chloride cells in the lamellar epithelium suggested either that the lamellar chloride cells are more resistant to copper or that the lamellar chloride cells degenerate and disappear rapidly, by sloughing or migration to the filament.

The expression of PCNA in branchial epithelium occurs mainly in central layers of tilapia gill filaments, as shown here and in our earlier study on tilapia (Dang et al., 1999) and in accordance with similar observations on rainbow trout (Laurent et al., 1994), chum salmon *Oncorhynchus keta* (Uchida and Kaneko, 1996) and guppy *Lebistes reticulatus* (Chrétien and Pisam, 1986). After copper exposure, increased cell numbers are observed mainly in these central layers, although increases were also seen in the basal and the apical cell layers of the filament epithelium. This indicates that the chloride cells appearing in the lamellar epithelium have migrated from the filaments, as previously concluded by Laurent (1984).

Antibodies to Na<sup>+</sup>/K<sup>+</sup>-ATPase have been widely used to identify chloride cells in fish gills (Witters et al., 1996; Uchida et al., 1996; Shikano and Fujio, 1998; Seidelin et al., 1999) and Na<sup>+</sup>/K<sup>+</sup>-ATPase immunocytochemistry at the light and electron microscope level allowed us to study the Na<sup>+</sup>/K<sup>+</sup>-ATPase expression during the chloride cell phase. In this paper, we provide direct evidence that the activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase may vary in subpopulations of branchial chloride cells, which include immature, mature and apoptotic chloride cells (Wendelaar Bonga et al., 1990). We realise that other cells (and probably most cells in any organism) will also have Na<sup>+</sup>/K<sup>+</sup>-ATPase in their plasma membranes. However, the techniques applied to visualise the enzyme do not appear to result in significant staining of cells other than chloride cells in these

experiments. In addition, our analyses included criteria other than immunoreactivity to identify chloride cells (at the electron microscope level, mitochondria and the tubular system; at the light microscope level, location in the tissue). Pavement cells sometimes contain many mitochondria but do not demonstrate Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity. In addition, pavement cells are defined as the superficial cells of the epithelium, which makes their localisation simple. The fate of the cells that appear in deeper layers of the epithelium and that show Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity were followed over time in a previous study (Dang et al., 1999) and such cells never appeared to end up as pavement cells. Our electron microscope analysis strongly supports the supposition that weakly stained cells are degenerating chloride cells. As discussed above, chloride cells stem from undifferentiated cells located in the middle areas of filaments (Laurent, 1984), which is where we observed the greatest PCNA immunoreactivity. The small Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase specific activities 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<sup>+</sup>/K<sup>+</sup>-ATPase. A less positive correlation was observed between the branchial Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup> pump activity is dependent on the total number of available pumps (Ewart and Klip, 1995). It may well be that not all Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity reflects Na<sup>+</sup> pump activity in vivo. The presence of such silent Na<sup>+</sup>/K<sup>+</sup>-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),

supporting the body of evidence for decreased Na<sup>+</sup>/K<sup>+</sup>-ATPase activities and hyponatremia in copper-exposed fish (Laurén and McDonald, 1985, 1987; Li et al., 1998). We conclude that copper-induced hyponatremia in a freshwater fish is associated with decreased branchial Na<sup>+</sup>/K<sup>+</sup>-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<sup>+</sup>/K<sup>+</sup>-ATPase activity than mature chloride cells, and this explains the discrepancy between the increase in chloride cell total numbers and the reduction in Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activity in copper-exposed fish.

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