Tohoku Journal of Agricultural Research
Vol. 47 No. 3-4, March 1997
Printed in Japan

Identification and Quantification of Chloride Cells in the Gill of Guppy *Poecilia reticulata*

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(Received, January 10, 1997)

Summary

For identification and quantification of chloride cells in the gill of the guppy, cell suspension was prepared by chopping the gill. The separated cells were distinguished into chloride cells from other cells using an acid fuchsin staining. The number and size of chloride cells were measured in freshwater-adapted S3-SW(FW) and seawater-adapted S3-SW(SW) strains. The cell number was 12.9 cells in freshwater-adapted strain and was 107.3 cells in seawater-adapted strain per 5 μl of cell suspension in an average. The cell size was 36.24 μm^2 in freshwater-adapted strain and 101.97 μm^2 in seawater-adapted strain as an average. It suggests proliferation and hypertrophy of chloride cells for seawater-adaptation of the guppy.

Adaptation of euryhaline teleosts to seawater is attended with changes in branchial, renal and intestinal functions to maintain the constancy of their internal ionic environment (1). Branchial epithelia are heterogeneous with respect to cell type and function. Chloride cells in branchial and opercular epithelia of seawater-adapted teleosts have been identified as an external site of chloride and sodium ion extrusion in seawater (2). Several electron microscope studies demonstrated that chloride cell contains numerous mitochondria and tubular systems with an expansion of their basolateral plasma membrane (3-6). Autoradiographic (7), ultracytochemical (8) and immunohistochemical (9) studies demonstrated that Na^+, K^+-ATPase locate on chloride cells and McCormick (10) reported that the enzyme plays a crucial role in ion transport of the cell.

Many studies have been done about the gill on tissue sections and have reported that branchial chloride cells increase in size and/or density in response to increased salinity in euryhaline teleosts (11, 12). However, complex structure of the gill cause an uncertainty for identification and quantification of chloride cells in tissue sections.

The guppy is a euryhaline fish and many strains are established in the laboratory (13). It has been demonstrated that seawater tolerance and adaptabil-

ity differ among the strains (14, 15). Shikano and Fujio (16) established a seawater-tolerant strain of the guppy which grows and propagates in seawater. For identification and quantification of chloride cells in the gill, branchial cells were separated in state of single cells from the gill of the guppy and stained with an acid fuchsin for demonstrating mitochondria of chloride cells. This paper describes the method for identification and quantification of chloride cells in state of single cells and shows the difference of the cell size and number between seawater- and freshwater-adapted strains of the guppy.

Materials and Methods

Animals

Seawater- and freshwater-adapted guppy strains were used in this study. S3-SW(SW) strain, which was seawater-adapted strain, was made from S3 strain by the same way with seawater-tolerant F22-SW(SW) strain reported previously (16). The origin of S3-SW(SW) strain was 29 individuals which were selected for survival in 35ppt seawater at birth out of 79 individuals born of 3 gravid females in S3 strain and the strain had been maintained in 35ppt seawater for three years. The origin of S3-SW(FW) strain, which was freshwater-adapted strain, was 28 individuals which were selected for survival in 35ppt seawater at birth out of 91 individuals born of 8 gravid females in S3 strain and the strain had been maintained in fresh water for three years. Each strain was kept as a closed colony in a 60 l aquarium at a temperature of 23 ± 2°C and was fed twice daily with ground carp pellets and dried Daphinia as a supplementary diet.

Histological observation

Gill arches were removed and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.2 (4% PFA) for 10-20 hours at 4°C. Tissue samples were dehydrated through a graded ethanol series and embedded in paraffin. Sections of 5 μm were mounted on glass slides. Sections were deparaffinized in xylene, hydrated in a graded ethanol series and stained with hematoxylin-eosin.

Preparation of cell suspension

For preparation of cell suspension, eight gill arches of a fish were removed in 100 μl of Hanks saline on a watch glass and chopped with a sharp scalpel for 5 minutes. After addition of 1,000 μl of Hanks saline, 1,000 μl of the dissociated cell suspension was pipetted to a microtube. The cell suspension was centrifuged at 1,000 rpm for 3 minutes and 600 μl of the supernatant was removed. For fixation of the cells, 600 μl of Altman's solution was added and mixed. The cell suspension was centrifuged, 800 μl of the supernatant was removed and the same

volume of Altman's solution was added. The procedure was repeated twice. The cell suspension was fixed at 0°C for an hour in a dark box. For washing the cells, the cell suspension was recentrifuged, 800 μl of the supernatant was removed and the same volume of distilled water (D.W.) was added and mixed. The procedure was repeated twice. After centrifugation, 800 μl of the suspension was removed. Then the cells were suspended finally in 200 μl.

Acid fuchsin staining and observation

Five microliter of the cell suspension was dropped on glass slide. After drying for over night, the dissociated cells on the glass slide were exposed to 0.1% potassium permanganate solution for 2 minutes and 0.1% oxalic acid solution for 2 minutes and then washed with flowing water for 10 minutes. After washing procedure, the glass slide was exposed to acid fuchsin-aniline solution (acid fuchsin 1.0 g, aniline 0.5 ml and D.W. 10.0 ml) and incubated for 30 minutes at 60° C. After cooling down at room temperature, the glass slide was exposed to two mixtures (picric acid saturated in absolute ethanol : 20% ethanol = 1 : 4, 1 : 7) in turn for 3 minutes, respectively, and then rinsed with 95% ethanol. The preparations were used for observation and measurement of chloride cells. On the other hand, isolated cells were also stained with hematoxylin-eosin after cell isolation as in acid fuchsin stain and fixation with 4% PFA. The cells were observed with a light microscope. The number of chloride cells was expressed by counting the cells contained in 5 μl of the cell suspension. The size of the cell was expressed as the product of major axis by minor axis of the cell. One hundred cells were measured per each individual.

Results

Identification of chloride cells

The gill section in freshwater-adapted S3-SW(FW) strain was stained with hematoxylin-eosin as shown in Fig. 1-A. Large spherical eosinophilic cells were observed on primary lamelae as shown by arrows, suggesting chloride cells. Separated cells from the gill were stained with hematoxylin-eosin and some of them were characterized as sphere and eosinophil as shown by arrows (Fig. 1-B). Some of the separated cells were darkly stained with acid fuchsin in both S3-SW(FW) and S3-SW(SW) strains (Fig. 2). Darkly staining with acid fuchsin indicates the cells are mitochondria rich and could be identified as chloride cells. The darkly stained cells were larger in S3-SW(SW) strain than in S3-SW(FW) strain.

Quantification of chloride cells

Table 1 shows the number and size of chloride cells in S3-SW(FW) strain.

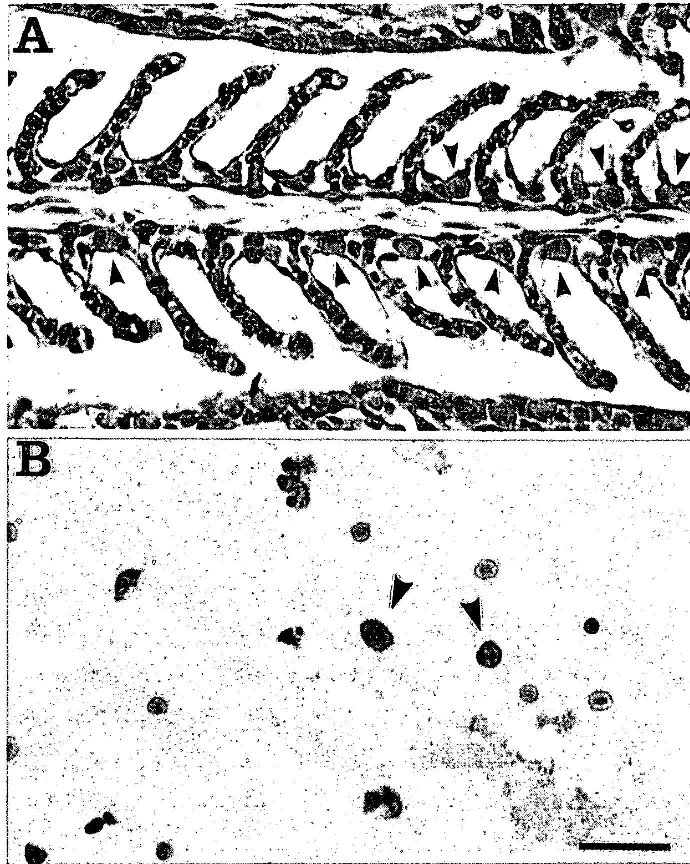

FIG. 1. Hematoxylin-eosin staining of gill section (A) and isolated branchial cells (B) in S3-SW(FW) strain. Arrows; large spherical eosinophilic cells. Bar; 20 μm.

In regardless of sex, number and size of the cells showed larger values in large body sizes than in small body sizes. There were no differences ($p>0.05$) at the same body length (18.0 ± 1.0 mm) between males and females. The result indicates that the cell number and size could be compared between experimental groups at the same body length, regardless of sex.

Table 2 shows the number and size of chloride cells in S3-SW(FW) and in S3-SW(SW) strains at the same body length (18.0 ± 1.0 mm). The cell number was 12.9 cells in S3-SW(FW) strain and was 107.3 cells in S3-SW(SW) strain per 5 μl of cell suspension in an average. The cell size was 36.24 μm^2 in S3-SW(FW) strain and 101.97 μm^2 in S3-SW(SW) strain in an average. The chloride cells in S3-SW(SW) strain were about eight times in number and about three times in size of S3-SW(FW) strain. It indicates proliferation and hypertrophy of chloride cells for seawater adaptation of the guppy.

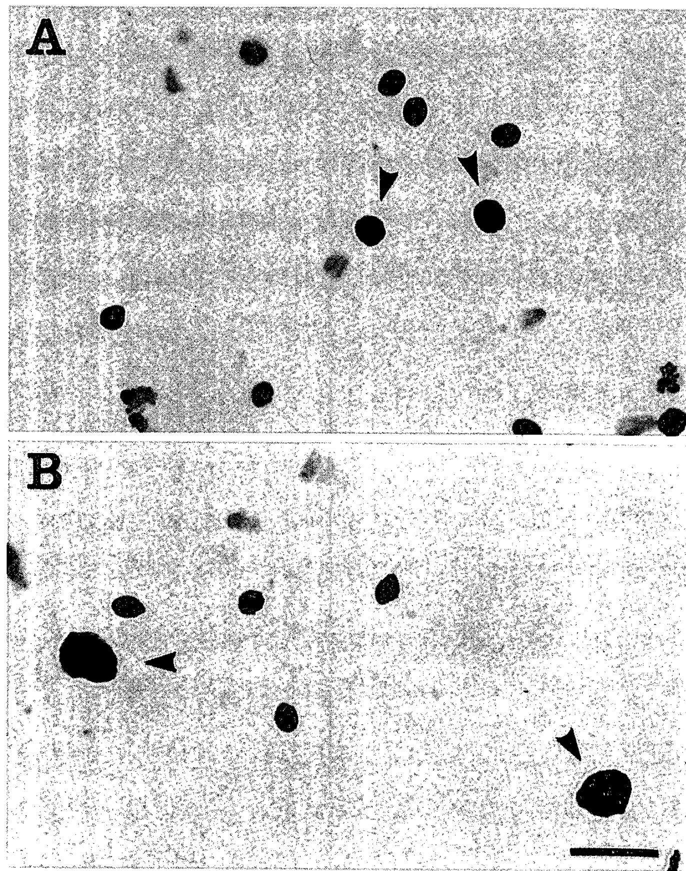

FIG. 2. Acid fuchsin staining of isolated branchial cells in S3-SW(FW) strain (A) and S3-SW(SW) strain (B). Arrows; darkly stained cells with acid fuchsin. Bar; 20 μm.

TABLE 1 *Number and size of chloride cells in the gills of small and large body sizes in S3-SW(FW) strain*

Sex	Body length (mm)	N	Number (cell/5 μl)**	Size (μm^2)**
Female	30.0±1.0	5	27.9±0.3	47.37±0.56
	18.0±1.0	5	12.5±0.6*	35.84±0.32*
Male	18.0±1.0	5	13.3±1.6	36.64±0.98
	14.0±1.0	5	6.3±0.3*	34.81±0.74

N: Number of tested fish.
* Significant difference ($p<0.05$) between large and small size in each sex (t-test).
** Values represent the mean±standard error.

Discussion

Copeland (17) has identified chloride cells in the gill by demonstrating the mitochondria of the cells with an acid fuchsin stain. By immunohistochemical methods, Ura *et al.* (9) have shown that Na^+, K^+-ATPase-positive cells correspond

TABLE 2. *Number and sjze of chloride cells in S3-SW(FW) and S3-SW(SW) strains*

Strain	N	Number (cell/5 μl)**	Size (μm^2)**
S3-SW(FW)	10	12.9±0.9	36.24±0.53
S3-SW(SW)	6	107.3±5.9*	101.97±1.70*

N: Number of tested fish.
* Significant difference ($p<0.05$) between strains (t-test).
** Values represent the mean±standard error.

to chloride cells in the gill and the cells are also characterized as spherical and eosinophilic cells with hematoxylin-eosin stain. These studies have shown precise identification and localization of chloride cell on fixed tissue section of the gill with a light microscope. In the present study, the chloride cells were observed as large spherical eosinophilic cells on primary lamellae in gill preparation and an acid fuchsin staining of the separated cells allow distinction of chloride cells from other cells as shown in the fixed section of the gill (11, 17).

Quantification of chloride cells in the gill has been mainly performed on fixed tissue section (11, 12). However, fixed tissue section is not suitable for a measurement of the cells because of influences of observational points and angles of tissue sections. Thomson and Sargent (18) have isolated branchial cells from the tissue and have measured chloride cells on the basis of their size and granular appearance. The present study showed precise identification and quantification of chloride cells in the gill using an acid fuchsin staining of separated cells.

In a comparison of the number and size of chloride cells between freshwater- and seawater-adapted strains of the guppy, seawater-adapted strain showed about eight times in cell number and about three times in cell size of freshwater-adapted strain. Daikoku (12) reported that the number of chloride cells in the gill was about four times more in seawater-adapted guppies than in freshwater-adapted guppies using gill sections. Compared with the report, the difference of chloride cell number between freshwater- and seawater-adapted guppies were twice larger in the present study. This supports an availability of the present method.

Although both seawater- and freshwater-adapted strains were originated from selected individuals for seawater resistance from S3 strain, freshwater-adapted guppies could not survive in 35ppt seawater upon direct transfer from fresh water (Unpublished observation). The environmental salinity might cause the differences in the number and size of chloride cells between two strains. The increase of the number and/or the size of branchial chloride cells has been reported in euryhaline teleosts transferred from fresh water to seawater (5, 11, 12, 18). The present study suggests the guppy need proliferation and hypertrophy of chloride cells for their marine life.

The present study demonstrates a precise and ease method for quantitative

analysis of chloride cells in the gill of teleost.

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