IN VITRO EFFECTS OF ENVIRONMENTAL SALINITY AND CORTISOL ON CHLORIDE CELL DIFFERENTIATION IN EMBRYOS OF MOZAMBIQUE TILAPIA, *OREOCHROMIS MOSSAMBICUS*, MEASURED USING A NEWLY DEVELOPED 'YOLK-BALL' INCUBATION SYSTEM

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Summary

To examine the functional differentiation of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos, we developed a 'yolk-ball' incubation system in which the yolk sac was separated from the embryonic body and subjected to incubation *in vitro*. The yolk-ball preparation consists of the yolk and the covering yolk-sac membrane, which contains a rich population of chloride cells. After appropriate cutting, the incision on the yolk ball healed during incubation in balanced salt solution for 3 h, so that the yolk-sac membrane completely enclosed the yolk. Yolk balls prepared from freshwater-acclimated embryos were transferred either to fresh water or to sea water and incubated for 48 and 96 h to elucidate the morphological changes in the chloride cells in response to environmental salinity. The chloride cells in the yolk-sac membrane were larger in sea water than in fresh water. In yolk balls transferred to sea water, chloride cells often formed multicellular complexes characteristic of seawater-type chloride cells. In those transferred to fresh water, however, the cells were small and rarely formed such complexes.

These responses of chloride cells were identical to those observed in intact embryos. Thus, chloride cells in the yolk-sac membrane could differentiate into the seawater type independent of the embryonic body. To examine the possible effects of exogenous cortisol on chloride cell differentiation, the yolk balls were incubated for 48 h in fresh water or sea water containing different doses of cortisol (0.1–10 μg ml^{-1}). Although chloride cells were consistently larger in sea water than in fresh water in all experimental groups, cortisol administration had no effect on chloride cell surface area in either medium. These findings indicate that the chloride cells in the yolk-sac membrane are equipped with an autonomous mechanism of functional differentiation that is independent of the embryonic endocrine and nervous systems. The yolk-ball incubation system established here is an excellent experimental model for further studies on chloride cell differentiation and function.

Key words: chloride cell, osmoregulation, differentiation, yolk sac, cortisol, tilapia, *Oreochromis mossambicus*, yolk ball.

Introduction

Teleost fish maintain ionic and osmotic gradients between their body fluids and the external environment. Ion regulation and osmoregulation in teleosts is achieved by integrated functions of osmoregulatory organs, such as the gills, kidney and intestine (Evans, 1993). In particular, chloride cells in the branchial epithelium function as an important site for ion secretion in sea water and for ion uptake in fresh water. Although the mechanism of chloride cell differentiation is still poorly understood, it has been suggested that osmoregulatory hormones, including cortisol, prolactin and growth hormone, may induce chloride cell differentiation during adaptation to fresh water or sea water (Foskett et al., 1983; Zadunaisky, 1984; McCormick, 1995).

The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline species able to adapt to both fresh water and sea water. Recently, Uchida et al. (Uchida et al., 2000) demonstrated morphological activation of gill chloride cells of adult tilapia in response to increasing environmental salinity. Tilapia embryos also show strong euryhalinity. The embryos are able to survive direct transfer from fresh water to sea water, even though the developing gills are not yet fully functional (Ayson et al., 1994). Previous studies have demonstrated that chloride cells located in the yolk-sac membrane are the extrabranchial site of ion exchange during the late embryonic stages of tilapia (Ayson et al., 1994; Ayson et al., 1995;

Shiraishi et al., 1997). When embryos were transferred from fresh water to sea water, chloride cells in the yolk-sac membrane became larger and frequently formed multicellular complexes with adjacent accessory cells (Shiraishi et al., 1997). Such cellular complexes are considered to be characteristic of seawater-type chloride cells with an ion-secreting function. Nevertheless, the endocrine systems involved in osmoregulation in adult fish are not fully developed in the early life stages, although some hormones of maternal origin are present in the yolk (Tagawa, 1996).

The purpose of the present study was to examine whether the functional differentiation of chloride cells in the yolk-sac membrane is under embryonic control and, if not, what triggers differentiation. For this purpose, we established a 'yolk-ball' incubation system in which the yolk sac was separated from the embryonic body and subjected to incubation *in vitro*. We examined the effects of environmental salinity and cortisol administration on the morphology of chloride cells in the yolk-sac membrane using these yolk balls.

Materials and methods

Fish

Mozambique tilapia, *Oreochromis mossambicus* Peters, were collected from a pond in northern Okinawa Island, Japan, and maintained in tanks with recirculating fresh water at 25 °C at the Ocean Research Institute, University of Tokyo. Fish were fed on artificial tilapia pellets (Tilapia 41M, Shikoku Kumiai Shiryo, Tokushima, Japan) once a day. Mature fish for breeding were kept in 200 l tanks, and fertilized eggs were collected from the mouth of brooding females. At 25 °C, the eggs typically hatched 5 days after fertilization.

Preparation of the yolk ball

Tilapia embryos 2 days before hatching, which had been spawned and reared in fresh water, were used for the preparation of the yolk balls. After the chorion had been removed, the embryo was placed in tilapia balanced salt solution (BSS: NaCl, 140 mmol l^{-1}; KCl, 3 mmol l^{-1}; MgSO$_4$, 1.25 mmol l^{-1}; NaH$_2$PO$_4$, 0.4 mmol l^{-1}; NaHCO$_3$, 2 mmol l^{-1}; CaCl$_2$, 1.5 mmol l^{-1}, Hepes, 10 mmol l^{-1}; penicillin, 100 i.u. ml^{-1}; streptomycin, 0.1 mg ml^{-1}; pH 7.4). The yolk sac was then cut from the embryonic body using sterilized fine scissors. When cut appropriately, the incision closed so that no yolk material leaked through the incision. If leakage of yolk material was observed, the sample was discarded. After the surgical operation, the yolk ball was incubated at 25 °C in BSS for 3 h, to allow the wound to heal, and then used for experiments.

To examine healing of the incision during incubation in BSS, yolk balls immediately after the operation and those incubated in BSS for 3 h were stained with 0.25 % Trypan Blue solution in BSS for 3 min and observed under a dissecting microscope.

To confirm recovery from the operation, the incision was observed by scanning electron microscopy before and after incubation in BSS. The yolk balls were fixed in 2 %
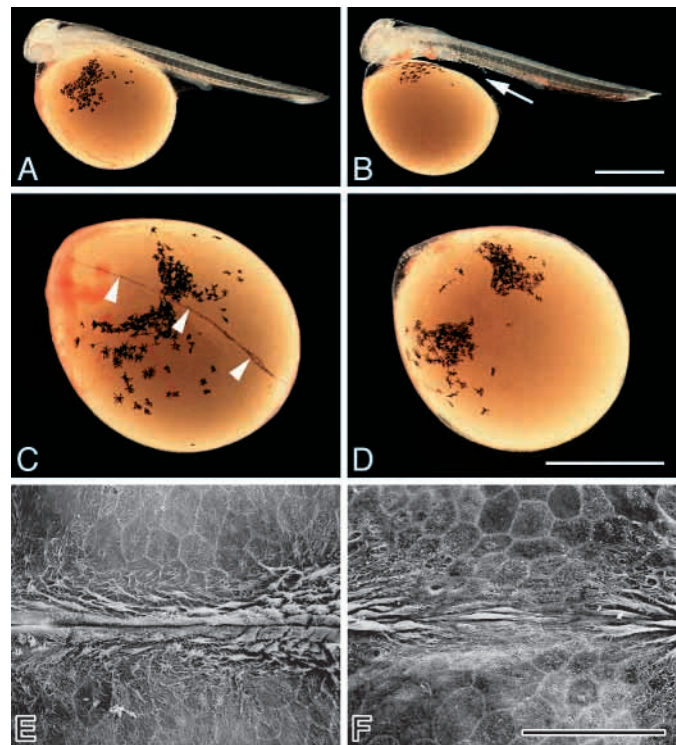

Fig. 1. (A,B) An intact tilapia embryo 2 days before hatching (A), and an embryo whose yolk sac has been almost severed from the embryonic body (B). An arrow indicates the incision. (C–F) Incised wounds on the yolk-ball preparations at 0 h (C,E) and 3 h (D,F) after surgery stained with Trypan Blue (C,D) and observed by scanning electron microscopy (E,F). The wound (arrowheads) had almost healed after incubation for 3 h in BSS. Note the symmetrical distribution of melanophores on the dorsal side of the yolk-sac membrane. Scale bars: A–D, 1 mm; E,F, 100 μm.

paraformaldehyde/2 % glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) overnight. Subsequently, the tissues were dehydrated in ethanol, transferred to 2-methyl-2-propanol and dried using a freeze-drying device (JEOL JFD-300, Tokyo). Dried samples were mounted on specimen stubs, coated with gold in an ion sputter-coater (JEOL JFC-1100) and examined with a Hitachi S-2150 scanning electron microscope.

Incubation of the yolk balls in fresh water and sea water

The yolk-ball preparations were transferred to sterilized fresh water or sea water and incubated for 48 and 96 h. The incubation was conducted in tissue culture dishes (60 mm in diameter) containing 5 ml of medium in an atmosphere of 100 % air at 25 °C. For the purpose of comparison, intact embryos from the same brood were also transferred to and maintained in fresh water or sea water at 25 °C for 48 and 96 h after removal of the chorion.

To investigate the effects of cortisol on chloride cell differentiation, the yolk balls were incubated in fresh water or sea water supplemented with cortisol (0.1, 1 or 10 μg ml^{-1}) for 48 h. Cortisol was dissolved in ethanol to a concentration of 1 mg ml^{-1} and then added to a medium.

Whole-mount immunocytochemistry

To detect chloride cells in the yolk-sac membrane of the yolk balls and intact embryos, the membrane preparations were immunocytochemically stained with an antiserum specific for Na^+/K^+-ATPase. The polyclonal antibody used here was raised in a rabbit against a synthetic peptide based on sequences of high homology and the areas of hydrophilicity of the Na^+/K^+-ATPase α-subunit (as described by Ura et al., 1996). The antigen designed was Cys-Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys-Ser. The antigen, conjugated with keyhole limpet haemocyanin, was emulsified with Freund's complete adjuvant, and immunization was performed in a New Zealand white rabbit. The antibody was affinity-purified and labelled with fluorescein isothiocyanate (FITC) as a fluorescent marker. The specificity of the antiserum has been confirmed in tilapia (Uchida et al., 2000; Katoh et al., 2000).

The yolk balls and intact embryos were fixed in 4 % paraformaldehyde in $0.1\,mol\,l^{-1}$ phosphate buffer (pH 7.4) for 1 h, and the yolk-sac membrane was then carefully peeled off using sharp-pointed forceps. The membrane preparations were fixed in the same buffer overnight and preserved in 70 % ethanol at 4 °C.

After a rinse in $0.01\,mol\,l^{-1}$ phosphate-buffered saline (PBS, pH 7.4) for 30 min, the membranes were incubated overnight with FITC-conjugated anti-Na^+/K^+-ATPase antibody diluted 1:500 with PBS containing 0.05 % Triton X-100, 10 % normal goat serum, 0.02 % keyhole limpet haemocyanin, 0.1 % bovine serum albumin and 0.01 % sodium azide at 4 °C. For double fluorescence staining for Na^+/K^+-ATPase and the nucleus, $50\,\mu g\,ml^{-1}$ propidium iodide (Molecular Probes, Eugene, OR, USA) was added to the staining solution, and the samples were processed in the same way. The yolk-sac membranes were then washed with PBS for at least 60 min, mounted on glass slides and covered with Slow Fade Light (Molecular Probes) under coverslips.

The membranes were examined with a confocal laser scanning microscope (LSM310, Zeiss). The 488 nm line of the argon ion laser was used as the excitation wavelength, and emission was recorded at 515–565 nm for FITC and at >590 nm for propidium iodide. Confocal images

corresponding to $0.82\,mm^2$ were obtained from each sample with a 20× objective lens (numerical aperture 0.50), the width of optical sections being $12.1\,\mu m$. Since the thickness of the yolk-sac membrane was less than $10\,\mu m$, the outline of the largest cells and of cell complexes could be observed. The cross-sectional area of immunoreactive chloride cells or chloride cell complexes was measured on an Apple Macintosh computer using a public-domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/). For each sample, the mean area was calculated from at least 100 cells. The data are expressed as means ± S.E.M. (*N*=4–8 samples). Significant differences in the cross-sectional area of Na^+/K^+-ATPase-immunoreactive chloride cells between freshwater and seawater groups at 48 and 96 h were tested using the Mann–Whitney test. Significant differences in the cross-sectional area of chloride cells among cortisol-treated and control groups were examined by two-way analysis of
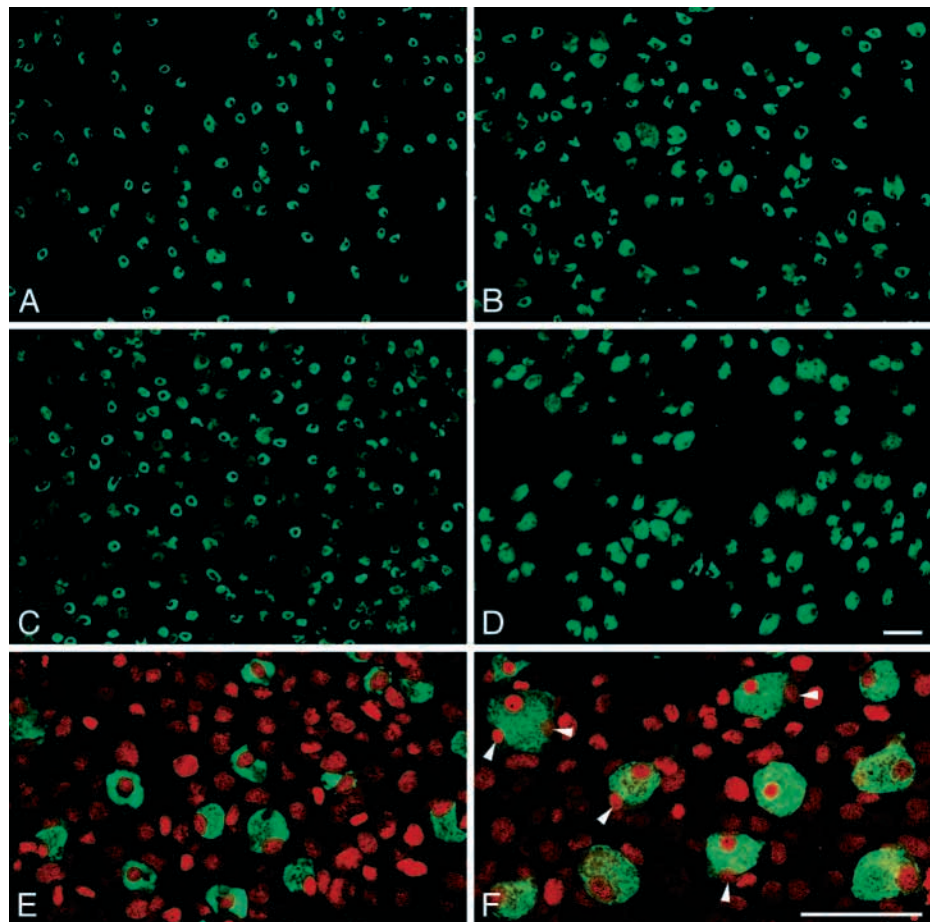

Fig. 2. (A–D) Chloride cells in the yolk-sac membrane of intact tilapia embryos (A,B) and yolk-ball preparations (C,D) incubated in fresh water (A,C) and sea water (B,D) for 48 h. Chloride cells were detected by whole-mount immunocytochemistry with FITC-labelled anti-Na^+/K^+-ATPase. (E,F) Chloride cells in the yolk-sac membrane of yolk-ball preparations incubated in fresh water (E) and sea water (F) for 48 h, double-stained with FITC-labelled anti-Na^+/K^+-ATPase and propidium iodide. Chloride cells in sea water form multicellular complexes with accessory cells (arrowheads), as demonstrated by the presence of more than one propidium-iodide-labelled nucleus. Scale bars: A–D, $50\,\mu m$; E,F, $50\,\mu m$.
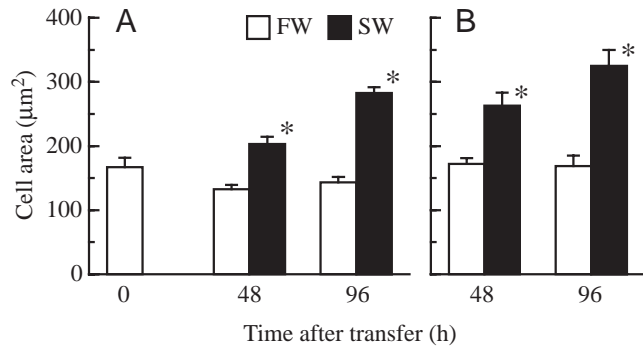
Fig. 3. Changes in the surface area of chloride cells in the yolk-sac membrane of intact tilapia embryos (A) and yolk-ball preparations (B) incubated in fresh water (FW) and sea water (SW). Values are expressed means ± S.E.M. (N=4 samples). Asterisks indicate significant differences between freshwater and seawater groups ($P<0.05$).
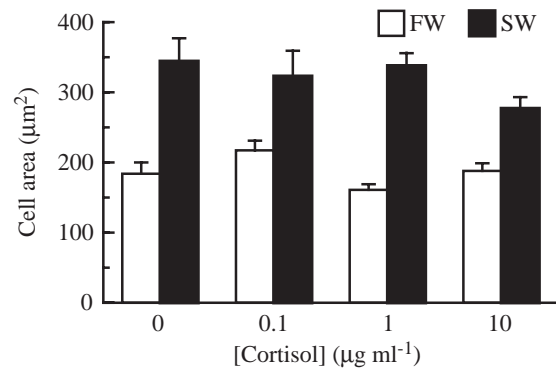

Fig. 4. Chloride cell surface area in yolk-ball preparations incubated for 48 h in fresh water (FW) and sea water (SW) supplemented with cortisol. Values are expressed as means ± S.E.M. (N=8 samples). Two-way ANOVA showed a significant effect of salinity (between FW and SW, $P<0.0001$), but no significant effect of the dose of cortisol ($P=0.2982$).

variance (ANOVA) with repetition (salinity *versus* cortisol dose) because the homogeneity of variance had been established.

Results

Preparation of the yolk ball

Fig. 1A shows a dechorionated tilapia embryo. To prepare the yolk ball, an incision was made between the embryonic body and the yolk sac from the posterior (Fig. 1B). When the yolk sac was cut off in the correct manner, the incision on the yolk sac closed but not tightly. If a newly cut yolk sac was strongly agitated, the wound often opened and yolk material leaked through the incision. After incubation in BSS for 3 h, however, the incision on the yolk sac appeared tight, since agitation no longer damaged the wound.

To examine the recovery of the incision during incubation in BSS, necrotic tissues on the yolk-sac membrane, caused by the operation, were stained with Trypan Blue. Although the incision stained intensively with Trypan Blue immediately after the operation (Fig. 1C), staining along the incision was faint or scarcely detectable after incubation in BSS for 3 h (Fig. 1D).

Scanning electron microscope observations also revealed recovery of the incised wound during incubation in BSS. Immediately after the surgical operation, the wound appeared closed, but swelling of the tissue was evident along both sides of the wound (Fig. 1E). Following incubation, the tissue swelling along the incision became less evident, although the scar was still recognizable (Fig. 1F).

Effects of transfer to fresh water and sea water on chloride cell morphology

In the yolk-sac membrane of intact embryos at 2 days before hatching, immunoreactive chloride cells were small and possessed one immunonegative nucleus. In embryos maintained in fresh water for 48 and 96 h, no apparent difference in the shape and surface area of chloride cells was observed (Fig. 2A, Fig. 3A). When transferred to sea water, however, the cell surface area increased and there were significant differences between the freshwater and seawater groups at 48 and 96 h (Fig. 2B, Fig. 3A). Chloride cells in sea water often formed multicellular complexes, as demonstrated by the presence of more than one immunonegative nucleus, whereas chloride cells in fresh water rarely formed complexes (Fig. 2A,B).

In the yolk balls incubated in fresh water, the surface area of the chloride cell was equivalent to that in intact embryos in fresh water (Fig. 2C, Fig. 3B). Chloride cells in yolk balls incubated in sea water were significantly larger than those in fresh water and comparable in size with those in intact embryos in sea water at both 48 and 96 h (Fig. 2D, Fig. 3B). As in intact embryos, the formation of multicellular complexes was evident in the yolk ball in sea water; two or three immunonegative, propidium-iodide-labelled nuclei were observed in the cellular complex (Fig. 2F). In contrast, chloride cells rarely formed complexes in freshwater yolk balls (Fig. 2E).

Effects of cortisol on chloride cell differentiation

To examine the possible effects of cortisol on chloride cell differentiation, yolk-ball preparations were incubated for 48 h in fresh water or sea water supplemented with cortisol. Although chloride cells were larger in sea water than in fresh water in all experimental groups, cortisol treatment at doses of 0.1, 1 and 10 μg ml^{-1} failed to affect the size of the chloride cells (Fig. 4). Two-way analysis of variance (ANOVA) showed a significant effect of salinity (between fresh water and sea water, $P<0.0001$), but no significant effect of the dose of cortisol ($P=0.2982$). Irrespective of cortisol treatment, multicellular complexes of chloride cells were consistently observed in sea water, whereas the cells were small and no complexes were formed in fresh water.

Discussion

We have developed the yolk-ball incubation system, a unique *in vitro* experimental model in which to examine the functional differentiation of chloride cells. The yolk ball consists of the yolk and the covering yolk-sac membrane, which contains a rich population of chloride cells. To prepare the yolk ball, the yolk sac was cut from the embryonic body. During incubation in BSS, the wound healed within 3 h, as confirmed by Trypan Blue staining and scanning electron microscope observations. Consequently, the yolk-sac membrane completely enclosed the yolk, and the serosal side of the membrane was separated from the external medium.

The yolk-ball preparation is a useful model in which to examine whether environmental salinity induces the differentiation of chloride cells in the absence of the influence of the developing embryo. When yolk balls were incubated in fresh water or sea water for up to 96 h, the chloride cells became significantly larger in sea water than in fresh water. In yolk balls transferred to sea water, the chloride cells often formed multicellular complexes, characteristic of seawater-type chloride cells (Shiraishi et al., 1997). In those transferred to fresh water, chloride cells occurred individually without forming complexes. These morphological changes in response to external salinity coincide well with those observed in intact tilapia embryos in our present and previous studies (Ayson et al., 1994; Shiraishi et al., 1997). Recently, Hiroi et al. (Hiroi et al., 1999) found that, when freshwater embryos were transferred to sea water, single chloride cells enlarged and were indented by newly differentiated accessory cells to form multicellular complexes. Thus, the formation of multicellular complexes may result both from enlargement of pre-existing chloride cells and from concomitant development of adjacent accessory cells. Taken together, the present findings suggest that chloride cells in the yolk-sac membrane possess an autonomous mechanism of functional differentiation in response to environmental salinity that is independent of the embryonic endocrine and nervous systems.

Although the precise mechanism of chloride cell differentiation is not clear, environmental salinity is probably detected in a direct or indirect manner, and this triggers cellular differentiation. One possible way is to detect a fluctuation in the environmental salinity *via* the apical membrane of chloride cells facing the external medium. It is also possible, however, that increased environmental salinity results in a slight increase in internal osmolality, which could be detected by the basolateral membrane of chloride cells. In fact, changes in osmolality on the basolateral side have been shown to affect the rate of Cl^- secretion by opercular epithelia of *Fundulus heteroclitus* (Zadunaisky et al., 1995; Marshall et al., 2000). In mammalian renal cells, incubation in a hyperosmotic medium decreases cell volume, which leads to alterations in intracellular ion concentrations and an increase in Na^+/K^+-ATPase activity (Bowen, 1992; Yordy and Bowen, 1993). It would be interesting to examine changes in chloride cell volume and Na^+/K^+-ATPase activity when external and internal osmolalities are individually manipulated in the yolk balls.

It is generally accepted that chloride cell activity is under the control of hormones such as cortisol, growth hormone, insulin-like growth factor-1, prolactin, thyroid hormones and sex steroids (McCormick, 1995). Daily injections of cortisol, one of the seawater-adapting hormones, increased the number and/or size of chloride cells in the opercular and branchial epithelia of tilapia *Oreochromis mossambicus* (Foskett et al., 1981), coho salmon *Oncorhynchus kisutch* (Richman and Zaugg, 1987), rainbow trout *Oncorhynchus mykiss* (Laurent and Perry, 1990; Madsen, 1990a) and brown trout *Salmo trutta trutta* (Madsen, 1990b). In the present study, however, we detected no such effect of cortisol on chloride cells in the yolk balls.

Ayson et al. (Ayson et al., 1995) established an incubation system for the tilapia yolk-sac membrane and examined the effects of cortisol on chloride cell morphology *in vitro*. In sharp contrast to the present results, their findings showed that cortisol was essential for maintaining chloride cells *in vitro*: chloride cells disappeared from the cultured yolk-sac membrane, and administration of cortisol in the culture medium partially prevented this disappearance. The discrepancy might be attributable to the presence or absence of the yolk in the tissue preparations. Although endocrine systems are not yet developed or not fully functional in the early life stages of fish, the yolk contains substantial concentrations of hormones of maternal origin. Cortisol has been detected in the yolk of tilapia (Hwang et al., 1992; Hwang and Wu, 1993; Ayson et al., 1995; Hiroi et al., 1997; Tagawa, 1996) and some other teleost species (Tagawa, 1996), indicating that maternal cortisol may be readily available for chloride cells in the yolk-ball preparation. This is not the case in the yolk-sac membrane preparation, from which the yolk is absent. Most probably, the yolk contains an adequate concentration of cortisol to maintain chloride cells in the yolk ball, and this may be the reason why additional treatment with cortisol failed to modify chloride cell morphology.

Although many studies in the past have focused on the effects of environmental salinity and hormones, their direct effects on chloride cell differentiation are not yet clear. This may be partly due to the absence of suitable *in vitro* experimental models. Primary cultures of cells isolated from gill epithelia have been utilized in physiological studies; however, dispersed chloride cells do not survive *in vitro* (Battram et al., 1991; Perry and Walsh, 1989; Pärt and Bergström, 1995; Avella and Ehrenfeld, 1997; Wood and Pärt, 1997). Our preliminary experiments showed that the yolk-ball incubation system established in the present study allowed chloride cells to survive in fresh water or sea water for at least 1 week. Another problem of primary cultures is the loss of cellular polarity, since the location of ion-transporting proteins in the plasma membrane is critical for the ion-transporting mechanisms of chloride cells (Silva et al., 1977; Zadunaisky, 1984; Towel, 1990; Marshall, 1995; McCormick, 1995). Although the opercular and yolk-sac membrane preparations maintain cellular polarity, chloride cells in these preparations can neither survive for long nor differentiate *in vitro* (McCormick, 1990; Ayson et al., 1995). Recently, Fletcher et al. (Fletcher et al., 2000) have established a double-seeded

insert technique for the culture of branchial epithelial preparations from freshwater rainbow trout. This technique allows chloride cells to survive *in vitro*, although the ion-transporting properties of the double-seeded insert epithelia are not the same as those of intact gills.

Compared with *in vitro* systems for chloride cell research, the newly developed yolk-ball incubation system has the following advantages: (i) the tissue, including chloride cells, can survive for a long time so that morphological changes in chloride cells can be followed; (ii) the chloride cells maintain their capacity for cellular differentiation; (iii) the tissue preparation is free from the embryonic endocrine and nervous systems; and (iv) the cellular polarity can be maintained and the serosal side of the yolk-sac membrane is separated from the external environment. Thus, the yolk-ball incubation system will be an excellent experimental model for further studies on chloride cell differentiation and function.

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