Immune localization of prolactin receptor in the mitochondria-rich cells of the euryhaline teleost (*Oreochromis mossambicus*) gill

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Abstract The present work demonstrates, by Western blotting and immunofluorescent staining, the presence and localization of prolactin (PRL) receptor in tilapia (ti) *Oreochromis mossambicus* gills. Gill epithelial cells that reacted with PRL receptor antibody were found to be labelled concomitantly with Con A, a marker of the apical crypts in mitochondria-rich (MR) cells. No positive staining was observed in pavement cells or mucus cells with PRL receptor antibody. This indicates that PRL receptors are located specifically in the gill MR cells. Further, the tiPRL receptors were found only in the MR cells of seawater-adapted tilapia gills. The effects of salinity and ions on the expression of tiPRL receptors are discussed.

Key words: Prolactin receptor; Mitochondria-rich cells; Tilapia gill; *Oreochromis mossambicus*; Teleost

1. Introduction

Prolactin (PRL), synthesized and secreted from the pituitary, is involved in a variety of important functions including ion transport, osmoregulation, stimulation of milk protein synthesis as well as the regulation of numerous reproductive functions. PRL causes hypernatremia when injected into fresh water (FW)- and seawater (SW)-adapted teleosts [1,2]. In addition, several studies have shown that PRL has a hypercalcemic effect in a variety of teleosts [3–7]. Plasma levels of PRL are higher in fish kept in FW than in ones adapted to a hyperosmotic environment [8–11]. In tilapia pituitary, two different PRL molecules have been found with a sequence identity of 69%, one variant (tiPRL I, 188 amino acids) being more similar to other fish PRLs than to the other variant (tiPRL II, 177 amino acids) [12,13]. Ayson et al. [14] have reported a low but significant plasma level of tiPRL I in SW-adapted tilapia (*Oreochromis niloticus*). The plasma level of tiPRL II is always higher than that of tiPRL I [15].

The mitochondria-rich (MR) cells, so called chloride cells, are characterized as being rich in mitochondria and as possessing a well-developed tubular system in the epithelium of the gill and operculum. The MR cells are thought to play a major role in the hydromineral regulation of teleostean fishes. PRL has been shown to decrease Cl^- excretion by the opercular membrane of MR cells [16] and to reduce the size of MR cells in SW-adapted tilapia [17]. Accordingly, Pisam et al. [18] demonstrated that tiPRL I significantly alters the ultrastructural features of gill MR cells by shifting from a 'SW' type to a 'FW' type. The size of the MR cells was larger in the SW-adapted gills than in the FW-adapted gills.

The tiPRL receptors have been characterized in cell homogenate of gills by radiolabelled ligand binding [19–21]. Only one class of high affinity PRL receptor was found in gill homogenate when fish were kept in FW [21] or during adaptation to brackish water (BW) [22]. Sandra et al. [23] have recently identified one single gene for this tiPRL receptor. However, the cell-type specific location of the tiPRL receptor in the gills has not been identified. In the present study we applied a cell-specific marker and monoclonal antibody to the PRL receptor as well as fluorescence microscopy to identify the localization of the PRL receptor in tilapia gills.

2. Materials and methods

2.1. Animals

Euryhaline tilapia *Oreochromis mossambicus*, weighing 15–20 g, were obtained from laboratory stock. The fish were fed daily with a commercial pellet diet. The fish were reared separately in aerated local FW or artificial SW at 27–29°C with a daily 12 h photoperiod. Artificial SW was made of a mixture of synthetic sea salt Instant Ocean* (Aquarium Systems, Sarrebourg, France) and aerated FW. The ionic compositions of FW (Ca^{2+}, 0.18 mM; Mg^{2+}, 0.09 mM; Na^+, 0.37 mM; K^+, 0.07 mM; Cl^-, 0.18 mM) and SW (Ca^{2+}, 8.76 mM; Mg^{2+}, 66.65 mM; Na^+, 267.51 mM; K^-, 40.90 mM) were analyzed before use. In one experiment, the fish were transferred from FW to the low Ca^{2+} FW (Ca^{2+}, 0.02 mM) for 2 weeks as described previously [24]. The water was continuously circulated through a fabric-floss filter and partially refreshed every week.

2.2. Preparation of epithelial membrane fraction

Fish acclimatized to FW, SW and low Ca^{2+} FW were treated identically throughout. Each fish was killed by spinal transection. The gills were dissected immediately. Gill epithelium was scraped off from the underlying cartilage with a scalpel. The scrapings were homogenized in a TS buffer containing (25 mM Tris-HCl, 0.25 M sucrose, pH 7.4). Epithelial homogenate was centrifuged at $1000\times g$ for 20 min. The supernatant were further centrifuged at $6000\times g$ for 20 min. The resulting supernatant was centrifuged again at $100\,000\times g$ for 1 h. The membrane enriched pellet was resuspended in TS buffer at a protein concentration of 1 mg/ml. Aliquots of the suspension were used for protein determination and Western blotting.

2.3. Western blotting of tiPRL receptors

The membrane-enriched protein (50 μg) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide gels) and then electrophoretically transferred onto polyvinylidene difluoride membrane (PVDF-plus, MSI). The blot was blocked with 3% nonfat dried milk in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% Tween 20, pH 7.4), washed with PBST, and then incubated with monoclonal anti-prolactin receptor antibody (U5) [25] for 1 h at room temperature. The blot was extensively washed with PBST and incubated with the secondary antibody conjugated alkaline phosphatase for 1 more hour at room temperature. After washes with PBST, the blot was developed with 0.015% nitroblue tetrazolium and 0.07%

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bromochloroindolyl phosphate in 100 mM Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$.

2.4. Immunofluorescent staining of prolactin receptor

Fish gills were freshly excised and rinsed with PBS. The gills were incubated with 1 mg/ml Con A-FITC in a dark box for 30 min. Con A binds specifically to the carbohydrate groups of glycoprotein [26,27] in the apical crypts of the MR cells [28–30]. After two washes in PBS, the Con A-labelled gills were fixed and permeabilized with ethanol (15%, 25%, 50%, and 75%, 2 min each in order) and washed with PBS (2 min). The specimen was then incubated with monoclonal anti-prolactin receptor antibody (U5) at 37°C for 1 h in a dark wet chamber. One specimen was incubated without U5 (as a negative control) at the same procedure. After a 2 min wash in PBS, the specimen was reacted with secondary antibody conjugated fluorescein or Texas Red (37°C, 10 min) in a dark wet chamber. The specimen was finally rinsed with PBS (2 min) and subjected to a fluorescent microscopy examination. The localization of positive staining in the specimen was also examined with a confocal laser scanning microscope (BioRad MRC-600) equipped with an argon laser.

3. Results

To identify the presence of the PRL receptor in the gill of tilapia, the gill epithelial homogenate was subjected to Western blotting. To localize the PRL receptor in gill epithelial cells, Con A and antibody to tiPRL receptor were co-applied to the gill epithelial section for immunofluorescent staining.

Fig. 1 shows the Western blotting of the tiPRL receptor using mouse anti-PRL receptor antibody (U5). Epithelial homogenates of SW-, FW- or low Ca^{2+} FW-adapted tilapia gills as well as rat kidney homogenate, as positive control, all immunoreacted with the mouse anti-PRL receptor antibody (U5). At least two major bands (molecular weights of 42 and 84 kDa) were seen in the blot of rat kidney homogenate. However, one major band, with a molecular weight of 42 kDa, was observed in the blot of all tilapia gill homogenates. By comparison, the homogenate from SW-adapted and low Ca^{2+} FW-adapted tilapia gills possessed a higher amount of PRL receptors than the homogenate from FW-adapted tilapia gills.

Fig. 2 demonstrates Con A labelling and anti-PRL receptor immunoreactive distribution in tilapia gills. The apical crypts of the MR cells were specifically labelled with Con A-FITC (Fig. 2a), while no positive staining was observed in one specimen without first antibody (U5) incubation (as negative control, result not shown). The immunofluorescence of the tiPRL receptor is located exclusively in the same cells as Con A in SW-adapted tilapia gills (Fig. 2b), indicating that tiPRL receptors localize in MR cells. The colocalization of Con A and PRL receptors in the MR cells of the gills was also confirmed using a confocal laser scanning microscope (not shown). In contrast, no positive staining of anti-PRL receptors was observed in the FW-adapted tilapia gills although the MR cells were still labelled with Con A (Fig. 2c). However, the positive signal of stained tiPRL receptors increased in the MR cells when the fish were adapted in the low Ca^{2+} FW for 2 weeks (Fig. 2d).

4. Discussion

The present study is the first to present the specific localization of tiPRL receptors on MR cells, labelled with Con A, in tilapia gills with the method of immunofluorescent staining. The mouse monoclonal anti-PRL receptor has been successfully used to characterize PRL receptors in various mammalian tissues, in which two major bands, with molecular weights of 42 and 84 kDa, were seen by Western blotting [25]. In the present study, it also specifically recognizes the tiPRL receptor either by Western blotting or by immunofluorescent staining. However, only a 42 kDa immunoreactive band was present in the blots of all tilapia gill homogenates. This is consistent with the published results of radioligand binding assays [19,21,22]. Notably, the tiPRL level in the epithelial homogenate of FW-adapted tilapia gills is much less than that of Ca^{2+} FW-adapted or SW-adapted tilapia gills.

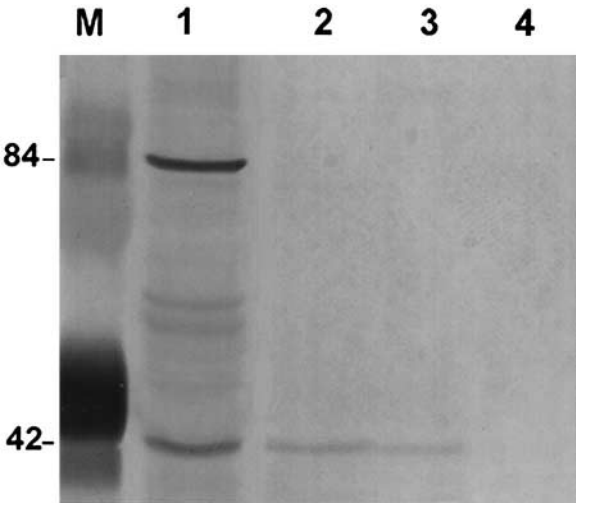

Fig. 1. Western blotting of tilapia gill and rat kidney PRL receptors using mouse anti-PRL receptor antibody (U5). Membrane-enriched proteins were run on 7.5% SDS-PAGE and electrophoretically blotted onto the transfer membrane. The membrane was incubated with U5 for 1 h at room temperature with gentle shaking. The membrane was washed and incubated with the secondary antibody conjugated alkaline phosphatase for 1 more hour at room temperature. After washes with PBST, the blot was developed by 0.015% nitro-blue tetrazolium and 0.07% bromochloroindolyl phosphate. Lane M, molecular markers; lane 1, rat kidney (positive control); lane 2, low Ca^{2+} FW-adapted tilapia gills; lane 3, SW-adapted tilapia gills; lane 4, FW-adapted tilapia gills.

Double labelling immunofluorescence revealed that the tiPRL receptors became dominant in the MR cells of the SW-adapted tilapia gills. In contrast, tiPRL receptors were not present in MR cells of the FW-adapted tilapia gills although the intensity of Con A labelling was similar. Two explanations are possible for this. First, the tiPRL receptors may be down- or up-regulated by various levels of tiPRL in waters of different salinity. Plasma prolactin levels are reportedly higher in fish kept in FW than in ones adapted to a hyperosmotic environment [8–11,14]. Following transfer of teleosts into SW, a drastic reduction in PRL secretion has been demonstrated [8,10]. This phenomenon has also been observed in the gills of rainbow trout [31] and coho salmon [32], in which the high cortisol levels down-regulate the corticosteroid receptors. In addition, the number of tiPRL receptors in tilapia (*Oreochromis niloticus*) was shown to increase after the fish were transferred from FW to BW or hypophysectomized [22]. The up-regulation of PRL receptors has been demonstrated in rats and rabbits [33–35]. Second, the intensity of fluorescence staining was below the detection limit so that it could not be identified by fluorescence microscopy. The tiPRL receptors do indeed exist, although in smaller amounts,

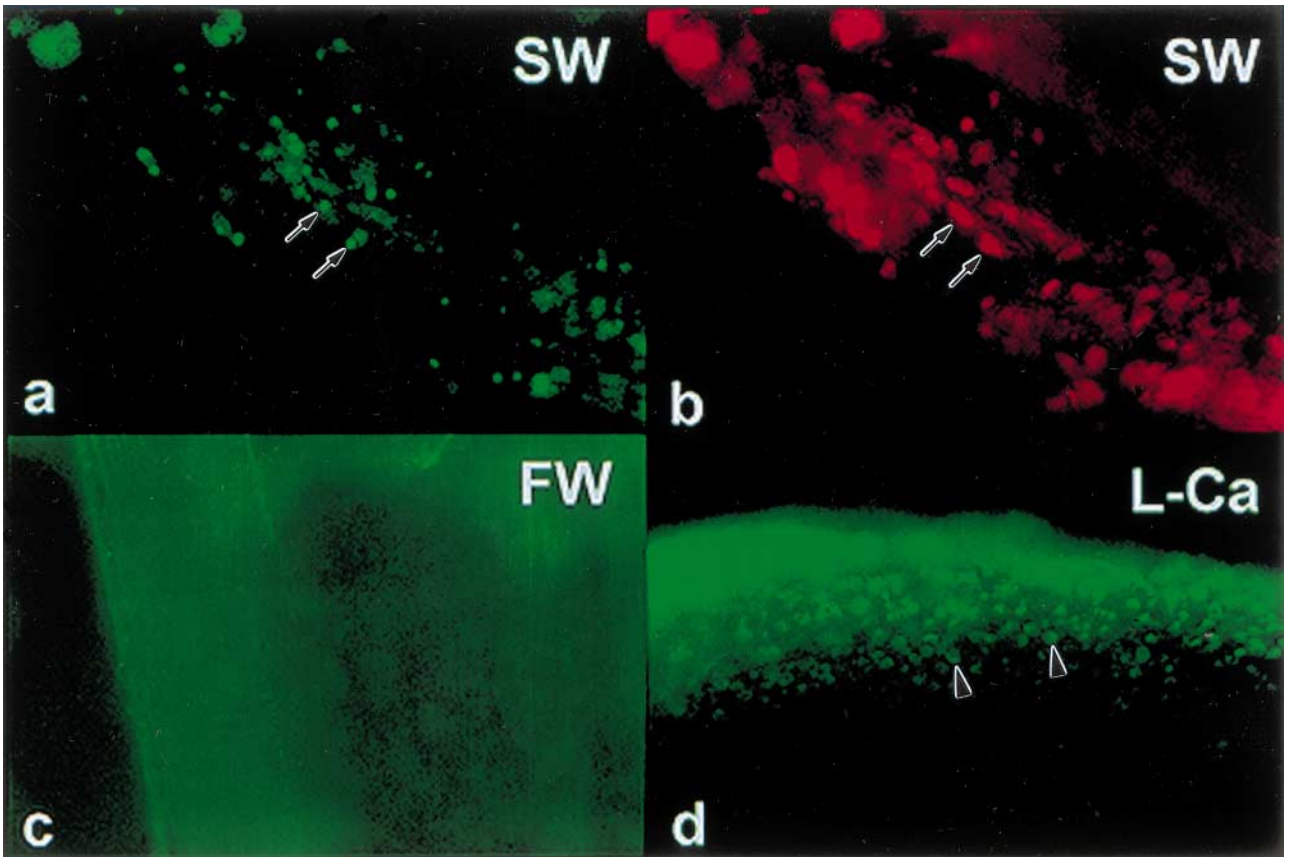

Fig. 2. Con A labelling and anti-PRL receptor immunoreactive distribution in tilapia gills (×410). a and b are double stained. a: Arrow indicates MR cells (Con A-FITC labelling, green) in SW-adapted tilapia gills. b: Arrow indicates positive staining of PRL receptors in MR cells (anti-PRL receptor with secondary antibody conjugated Texas Red, red) in SW-adapted tilapia gills. c: Non-positive staining of PRL receptors in MR cells (anti-PRL receptor with secondary antibody conjugated fluorescein, green) in FW-adapted tilapia gills. d: Arrowhead indicates positive staining of PRL receptors in MR cells (the immunofluorescence as in c, green) in low Ca^{2+} FW-adapted tilapia gills (L-Ca).

in the epithelial homogenate of FW-adapted tilapia gills as revealed by Western blotting in the present study or by traditional radioligand binding assay [19,21,22]. However, these two methods could not demonstrate what cell type of the gill epithelium contains the tiPRL receptor. On the other hand, other cells such as pavement, mucous or undifferentiated cells may contribute to the osmoregulation of gills and also contain the tiPRL receptors. However, this seems unlikely because we could not see any positive staining of tiPRL receptors in other epithelial cells of the gill.

The MR cells of the gill epithelium in goldfish [39] and in FW trout [40] have a function in the major Ca^{2+} influx. Injection of ovine PRL (oPRL) has been shown to boost the net Ca^{2+} uptake rate in FW tilapia [4] and in eel [38]. There is much evidence to show that PRL affects the osmoregulation (flux of water and ions) of gills and produces characteristic ultrastructural changes in the MR cells [18,36,37]. Interestingly, after the fish were adapted in the low Ca^{2+} FW for 2 weeks, the tiPRL receptors in MR cells increased in the present study. This indicates that the alteration of ion in the medium could regulate the expression of PRL receptors in the MR cells of the gills. The underlying mechanism of ion regulation on the PRL receptor remains to be determined.

The physiological functions of PRL and subsequent signal transduction (tyrosine kinase, JAK1 and JAK2) after binding to its receptor have been extensively studied in cell lines [41–43] and in pancreatic islets of rat [44]. However, no studies have been reported in teleosts. The present study demonstrates the existence of tiPRL receptors on the MR cells of tilapia gills. This provides an approach to understanding the physiological role of tiPRL and its own receptor which trigger signal transduction in relation to the osmoregulation of gills in vivo.

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