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Abstract

In euryhaline teleosts, Na^+, K^+-ATPase (NKA) and V-type H_+-ATPase A (VHA A) are important ion-transporters located in cell membrane. Lipid rafts (LR) are plasma membrane microdomains enriched in cholesterol, sphingolipids, and proteins (e.g., flotillin). Flotillin is a LR-associated protein, commonly used as the LR marker. Previous mammalian studies showed that LR may play a crucial role in ion exchanges. Meanwhile, studies on mammals and rainbow trout showed that NKA were found to be present mainly in LR. However, little is known about LR in fish. Therefore, the present study aimed to investigate the involvement of branchial LR in osmoregulation of tilapia and milkfish, two euryhaline teleosts with different salinity preferences, by (i) extracting LR from the gills of euryhaline teleosts; (ii) detecting the abundance of LR marker protein (flotillin-2) and ion-transporters (NKA and VHA A) in branchial LR and non-LR of fresh water- and seawater-acclimated milkfish and tilapia. The results indicated that the protein abundance of LR marker, flotillin-2, changed with environmental salinities in branchial LR of tilapia. In addition, flotillin-2 and NKA were only found in LR in both tilapia and milkfish gills, while VHA A were mainly present in non-LR. Relative protein abundance of NKA was found to be significantly higher in gills of freshwater milkfish and seawater tilapia, while VHA A was significantly higher in gills of freshwater tilapia and milkfish. This study illustrated differential distribution and salinity-dependent expression of NKA and VHA A in cell membrane of gill tissues of euryhaline teleosts with different salinity preferences.

Introduction

Lipid raft (LR) are membrane microdomains rich in cholesterol and glycoshpingolipids involved in the organization and aggregation of lipid bilayer constituents including transmembrane proteins (Simons and Ikonen, 1997). LR have been reported to be involved in virus entry (Nguyen and Hildreth, 2000; Ono and Freed, 2001), protein trafficking (Brown and London, 1998), ion transport processes (Tillman and Cascio, 2003) and cell signaling (Varma and Mayor, 1998). Because of their high lipid content, i.e., high lipid-to-protein ratio (Brown and Rose, 1992) and relatively low density, the LR fraction can be extracted following non-ionic detergent treatment using density gradients. LR are highly dynamic, usually nanoscale structures that can form bigger raft by means of fusion of small entities (Lingwood and Simons, 2010). Levental et al. (2010) showed that S-acylation, referred to as 'palmitoylation' plays an important role in regulating raft affinity. The binding of palmitate with cysteine residues of proteins promotes their insertion into the LR fraction and suggests that palmitoylation plays a critical role in membrane targeting mechanisms involving transmembrane proteins (Resh, 2006). According to Levental et al. (2010) and Contreras et al. (2011), among plasma membrane proteins, about 65% were in the non-raft phase, whereas 12% required palmitoylation for raft phase inclusion, 11% were glycosylphosphatidylinositol (GPI)-anchored in the raft, and the other 11% could be bound to LR lipids such as cholesterol or sphingolipids (SLs).

Lipid compositions of the LR and non-LR fractions are considered to be different as shown in common carp (*Cyprinus carpio*), i.e., the LR fraction contained a higher percentage of cholesterol and non-polar lipids (Brogden et al., 2014). Non-ionic detergents are thus used to extract LR (also called detergent-resistant membranes (DRM)) from other cell membranes (Eckert et al., 2003; Dalskov et al., 2005; Lingwood et al., 2005). Commonly used detergents for LR extraction in mammalian

78 studies include CHAPS, Tween 20, Triton X-100, Lubrol WX, Brij96, and Brij98, and
79 different detergents that may cause different result of lipid raft isolation due to the
80 dissolvability (Drevot et al., 2002; Madore et al., 1999; Röper et al., 2000; Schuck et
81 al., 2003). Among them, Triton X-100 is the most commonly used detergent for LR
82 extraction (Foster and Chan, 2007; Pike, 2006; Schroeder et al., 1998). Triton X-100
83 has also been applied in LR research with various protocols in skate (*Raja erinacea*;
84 Musch at al., 2004), rainbow trout (Lingwood et al., 2005), Atlantic cod (*Gadus*
85 *morhua*; Gylfason et al., 2010), goldfish (*Carassius auratus*; Garcia-Garcia et al.,
86 2012), and common carp (Brogden et al., 2014). Previous studies on fish have not
87 compared different detergents and have not quantified flotillin-2 following LR
88 isolation. Brogden et al. (2014) found that lipid composition of plasma membrane in
89 common carp was different from that in human, and the lipid components were
90 organ-dependent, whether in LR or non-LR regions. In their results, even 0.1% of
91 Triton X-100 cannot perfectly isolate flotillin-2 in LR from non-LR region in all
92 tissue. As a result, it is important to test different concentration of detergents.
93 Flotillins are LR-associated proteins commonly used as markers for LR. Plasma
94 membrane targeting and clustering of flotillins on LR mainly relies on acylation
95 (myristoylation or palmitoylation) (Banning et al., 2011). Furthermore, they have also
96 been shown to be involved in some basic functions such as trafficking and transport of
97 membrane materials and proteins (Stuermer, 2010). Flotillins are divided into two
98 isoforms. Flotillin-1 (previously named reggie-2) seems to associate with raft by
99 means of the first hydrophobic domain (Liu et al., 2005) and has also been shown to
100 be palmitoylated in Cys34, which is essential for flotillin-1 to locate on the
101 cytoplasmic side of the plasma membrane (Morrow et al., 2002). Flotillin-2
102 (previously named reggie-1) interacts with the plasma membrane through several sites
103 of palmitoylations and myristoylations, and plays a significant role in the maintenance

104 of membrane raft (Neumann-Giesen et al., 2004, Langhorst et al., 2006). Evidence of
105 flotillin function in fish is scarce. In zebrafish (*Danio rerio*), von Philipsborn et al.
106 (2005) suggested that flotillin might be involved in development.
107 The LR plays important roles in ionoregulation and osmoregulation, as shown by
108 proteins responsible for ion transport being localized to LR, including active transport
109 pumps/enzymes (i.e., ATPase) (Tillman and Cascio, 2003; Murtazina et al., 2006). For
110 example, the Na^+, K^+-ATPase (NKA) in fish branchial and renal cells (Lingwood et
111 al., 2005; Welker et al. 2007) and vacuolar-type H^+-ATPase (VHA) in mammalian
112 cells (Lafourcade et al., 2008).
113 Membrane structure and transmembrane enzyme function are linked because the
114 lipid environment of the enzyme can constrain protein motions required for catalysis,
115 affecting the enzyme catalytic rate (Harris, 1985; Cossins et al., 1986). Among the
116 enzymes/transporters, the NKA catalyzes the transport of Na^+ and Cl^- across epithelia
117 in both absorptive (fresh water; FW) and secretory (seawater; SW) modes in gills of
118 euryhaline teleosts (Marshall, 2002; Perry et al., 2003; Hirose et al., 2003). Changes
119 in branchial NKA activity in euryhaline fish are necessary for acclimation to
120 environmental salinity (Marshall and Bryson, 1998; Kelly et al., 1999; Marshall, 2002;
121 Mancera et al., 2002; Hirose et al., 2003; Lin et al., 2003; Scott et al., 2004).
122 Furthermore, NKA consists of α- and β-subunits (Scheiner-Bobis, 2002). The
123 α-subunit has a molecular weight of about 100 kDa and is considered the catalytic
124 center of the NKA, with binding sites for cations, ATP, and ouabain (NKA inhibitor).
125 The β-subunit, with a molecular weight of 40-60 kDa, can stabilize the structure and
126 regulate the cations affinity of the α-subunit on the plasma membrane (Skou and
127 Esmann, 1992; Abriel et al., 1999). Moreover, reciprocal interactions between NKA
128 and cholesterol or phospholipids have been proposed (Chen et al., 2011; Cornelius,
129 2008; Cornelius et al., 2015; Haviv et al., 2013; Kravtsova et al., 2015) and NKA

distribution on LR has been reported in previous studies on mammalian tissues (Welker et al., 2007). Using the Brij 98 (as a non-ionic detergent) to extract the granulocytes in the brain of rat also revealed the presence of NKA α-subunits in LR (Dalskov et al., 2005). However, there are few studies focused on fish NKA in LR. In gills of rainbow trout (*Oncorhynchus mykiss*), NKA are expressed in LR when transferred to SW but not detected in LRs in FW individuals, indicating different strategies in ionoregulation between FW- and SW-acclimated rainbow trout (Lingwood et al., 2005). Most reports on NKA α-subunit, however, focused on their localization on the basolateral plasma membrane and their expression when fish encountered different environmental salinities (Lee et al., 2003; Lin et al., 2003). The NKA α-subunit protein abundance in gills of FW-preference Mozambique tilapia (*Oreochromis mossambicus*) was significantly higher in SW than in FW (Lee et al. 2003), while in gills of SW-preference milkfish (*chanos chanos*) it was significantly higher in FW than in SW (Lin et al., 2003).

The VHA is a multi-subunit complex organized into two domains: the 650 kDa cytosolic V_1 domain and the 260 kDa membrane-embedded V_0 domain (Nishi and Forgac, 2002; Forgac, 2007; Toei et al., 2010). In previous mammalian studies, deprivation of cholesterol from LR resulted in the decrease of electrogenic H^+ efflux by VHA and in synaptic signaling deficiency (Yoshinaka et al., 2004). On frog and insect epithelial cells, VHA contributed to acid-base regulation and osmoregulation (Harvey et al., 1998). In the studies on rainbow trout gills, VHA activity and immunoreactivity decreased when FW trout were acclimated to SW (Lin and Randall, 1993; Lin et al., 1994). In the Atlantic stingray (*Dasyatis sabina*) and killifish (*Fundulus heteroclitus*), VHA expressed on the cell membrane was significantly higher in FW than in SW (Piermarini and Evans, 2001; Katoh et al., 2003). As a result, this consistently higher expression of VHA in FW environments is linked to its role in

generating an electrical gradient favoring Na^+ uptake in FW-type ionocytes, additional to its role in acid secretion. In tilapia, bafilomycin, an inhibitor of VHA, has been shown to affect *in vivo* Na^+ influx (Fenwick et al., 1999), indicating a key role of this pump in freshwater osmoregulation in tilapia and suggesting an apical location in ionocytes.

To date, most studies on NKA and VHA in fish focus on their relationship with cell membrane, while the relationship between NKA and LR in fish was only reported in the rainbow trout (Lingwood et al., 2005). On the other hands, there is no reference reporting the associations of VHA and LR in fish. According to previous references, this study hypothesized that NKA and VHA, the major ion pumps in fish gills, may be mainly distributed in the LR to be involved in ionoregulation of euryhaline teleosts when acclimated to environments of different salinities. As a result, the present study aims to investigate the presence of LR and their exhibition of NKA and VHA in gills of tilapia (the FW euryhaline teleost) and milkfish (the marine euryhaline teleost), by (i) extracting membrane LR from gills of tilapia and milkfish, with fresh water (FW) and seawater (SW) preferences, respectively, and (ii) assessing differential NKA and VHA expression in branchial LR between FW- and SW-acclimated milkfish and tilapia. This study provides the evidence for expression of critical ion transporters in LR of euryhaline teleosts with different salinity preferences. Differential expression of flotillin-2 (the LR marker) and two ATPases, the ATP-consuming NKA and VHA, in gills of euryhaline fish will further clarify the ionoregulation roles of branchial LR in environments of different salinities.

Materials and methods

Experimental fish and design

Mozambique tilapia (*Oreochromis mossambicus*), and juvenile milkfish (*Chanos chanos*) were obtained from laboratory stocks and a local fish farm, respectively, with average standard lengths of 5.5 ± 0.5, and 10.6 ± 0.4 cm, respectively. Seawater (SW; 35 ‰) and brackish water (BW; 15 ‰) used in this study were prepared from aerated tap water (FW) with artificial sea salt (Synthetic Sea Salt; Blue Treasure, Qingdao, China). The water was continuously circulated through fabric-floss filters and quarter of water was changed every two weeks. The ammonia contents were routinely examined using the commercial API® ammonia test kit. Quarter of water was changed when the ammonia level was higher than 0 ppm. Each species of fish was reared in four separated tanks at 28 ± 1ºC in BW for two weeks before transfer to either FW or SW, with a daily 12 h photoperiod and a daily diet of commercial pellets. The holding densities of tilapia and milkfish in each tank were 0.4 fish/L and 0.2 fish/L, respectively.

For establishing the protocol of lipid raft (LR) extraction, tilapia and milkfish were reared in FW and SW, respectively, for at least four weeks before sampling the gills. Differential expression of NKA and VHA in branchial LR was further assessed between FW and SW in each species after one-month acclimation. For subsequent analyses, six individuals of either FW or SW groups were used for experiments and four gill arches from the right opercular side of each fish were sampled. Before sampling, experimental fish were not fed for one day and anesthetized in 500 μL/L 2-phenoxyethanol. The protocol used for the experimental fish was reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 102-09R and 105-130 to T.H. Lee).

Preparation of crude membrane fractions, lipid raft and non-lipid raft fractions

Gill tissues from the experimental fish were steeped in a mixture of homogenization buffer (SEI buffer, 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.4) with commercial protease inhibitor cocktail (Roche, Mannheim, Germany; 1:50, v/v). Homogenization was performed with a Polytron PT1200E (Kinematica, Lucerne, Switzerland). After the first centrifugation (1,500 *g* at 4ºC for 10 min), the pellets were discarded and the supernatants were centrifuged again (13,000 *g* at 4ºC for 12 min). After the second centrifugation, the supernatants were retained and centrifuged a third time (20,800 *g* at 4ºC for 60 min). Then after the third centrifugation, the supernatants as the cytosol was discarded. Meanwhile, the pelleted fraction containing large fragments of the plasma membrane was retained and considered as the crude membrane fraction (Tang and Lee, 2010). In this study, the crude membrane fraction was further dissolved to obtain the lipid raft using SEI buffer with 0.1% or 0.05%Triton X-100 or 0.05% Tween 20 (abbreviated as SEIT) at 4ºC (Brogden et al., 2014). After dissolution in detergents at different time points (30, 60 and/or 120 min) to determine the best duration of digestion, the aliquots were centrifuged (20,800 g at 4ºC for 30 min). The supernatants of the fourth centrifugation was considered as the detergent-soluble membrane (non-LR). The pellet as the detergent-insoluble membrane (LR) was then dissolved by the protease inhibitor cocktail (Roche) contained SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.5% sodium deoxycholate, pH 7.4) because LR can be dissolved in deoxycholate (McGuinn and Mahoney, 2014). Protein concentrations were measured with reagents from the Pierce™ BCA protein assay kit (Thermo Fisher, Rockford, IL, USA) using bovine serum albumin (Thermo Fisher) as standard.

Antibodies

The primary antibodies used in this study included (i) a mouse monoclonal antibody (α5, DHSB, Iowa City, IA, USA) raised against the avian NKA α-subunit (Lin et al., 2003; Yang et al., 2015), (ii) a mouse monoclonal antibody (flotillin-2, Santa Cruz, CA, USA) raised against the amino acids 150-240 of human flotillin-2 (XP_016879883.1) with approximately 83% similarity with milkfish flotillin-2 (XP_030648347.1) and 80% similarity with tilapia flotillin-2 (XP_003456181.1), and (iii) a rabbit polyclonal antibody (MDBio, Taipei, Taiwan) raised against the specific epitope (AEMPADSGYPAYLGARLA) of the pufferfish (*Tetraodon nigroviridis*) VHA A (ABX80240.1) with 100 % similarity with milkfish VHA A isoform1 and isoform 2, as well as tilapia VHA A (BAF94024.1). The sequences of two isoforms of VHA A found in the milkfish transcriptome database (Hu et al., 2015) were showed in Fig. S1. The specificities of the VHA A antibody to gills of tilapia and milkfish were tested and showed in Fig. S2. According to the primary antibodies, the secondary antibodies for immunoblots included the horseradish peroxidase-conjugated (i) rabbit anti-mouse IgG, (ii) goat anti-rabbit IgG, and (iii) rabbit anti-goat IgG (GeneTex, Irvine, CA, USA). The similarity between flotillin-2 with milkfish.

Immunoblotting

The sample mixture of 6x sample loading dye (0.06% bromophenol blue, 30% glycerol, 12% SDS, 0.6 M dithiothreitol, and 62.5 mM Tris with pH 6.8) and gill proteins (1:5, v/v) was heated at 65℃ for 15 min to denature the proteins. Non-LR and LR samples were separated by electrophoresis on a 7% SDS-polyacrylamide gel (5 μg of protein per lane) using a Mini-protein II electrophoresis cell (Bio-Rad, Hercules, CA, USA). Then, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After incubation in the non-specific blocking solution using phosphate buffer saline with Tween 20

(PBST; 2.68 mM potassium chloride, 10.1 mM disodium phosphate, 137 mM sodium chloride, 1.76 mM potassium dihydrogen phosphate, and 0.2 % (v/v) Tween; pH 7.4) containing 5% (w/v) nonfat dried milk for 2 h at room temperature (RT), the blots were incubated overnight with the primary antibody (1:5000) at 4ºC, followed by washing the membranes with PBST. Then the membranes were incubated with the secondary antibody (1:25000) for 1 h at RT. Images were developed with T-Pro LumiLong Chemiluminescent Substrate Kit (Ji-Feng Biotechnology, New Taipei, Taiwan) under a cooling-CCD (charge-couple device) camera (ChemiDoc XRS+, Bio-Rad) with associated software (Quantity One version 4.6.8, Bio-Rad). The blot bands were converted to numerical values by ImageLab 3.0 software (Bio-Rad) to quantify and compare relative protein abundance of the immunoreactive bands.

Statistics

In this study, comparisons in flotillin-2, NKA-α or VHA A abundance between (i) LR and non-LR fractions of gills in either SW or FW fish, or (ii) SW or FW groups in either LR or non-LR fractions, respectively, were analyzed by the Student's *t-test*, with $p < 0.05$ was set as the significant level. Values are expressed as means ± S.E.M. (standard error of the mean).

Results

Separation and purification of LR from tilapia and milkfish gills

In tilapia (Fig. 1), the crude membrane fraction was first reacted in the non-ionic detergent Triton X-100. After 30, 60, and 120 minutes of reaction with this detergent at 0.1% as well as 60 minutes of reaction at 0.05%, strong immunoreactive bands were found in LR for NKA α-subunit and flotillin-2. However, faint immunoreactive bands of both proteins were found in non-LR, indicating that dissolvability of 0.1 and 0.05% Triton X-100 may not be optimal for tilapia gill tissues (Fig. 1). Meanwhile, the supernatant separated by reacting with 0.05% Tween-20 for 60 minutes did not show immunoreactive bands of flotillin-2 in non-LR, indicating the better dissolution of this protocol for extracting LR from tilapia gills (Fig. 1). Moreover, no band for NKA α-subunit was observed in the non-LR fraction.

In milkfish (Fig. 2), similar results were observed at 30-minutes treatments with the non-ionic detergent 0.1% Triton X-100 as tilapia reacted with Triton X-100 (Fig. 1). However, after 60 and 120 minutes of reaction with 0.1% Triton X-100, there was no flotillin-2 and NKA α-subunit immunoreactive band found in the non-LR membrane fraction, indicating that the LR of milkfish gills could be separated after 0.1% Triton X-100 dissolution following 60 minutes of incubation. The NKA α-subunit abundance was high in the LR fraction (Fig. 2). Hence, extraction of LR by 0.1% Triton X-100 for 60 minutes is an appropriate procedure for LR purification from milkfish gills.

Comparisons of flotillin-2 and NKA in branchial LR and non-LR fractions between FW and SW tilapia and milkfish

As illustrated in Fig. 1, tilapia gills treated with 0.05% Tween-20 for 60 minutes revealed better dissolvability in LR membrane fractions. This condition has thus been

used to compare salinity effects on LR and non-LR gill fractions of tilapia. Flotillin-2 and NKA α-subunit protein levels were only detected in the LR of both FW and SW tilapia. The abundance of flotillin-2 in LR was significantly (2 times) higher in the FW group than in the SW group (Fig. 3A). On the contrary, the abundance of NKA α-subunit in LR of SW tilapia was 1.4 times higher than that of FW fish (Fig. 3B).

Similarly, flotillin-2 and NKA α-subunit protein levels were only detected in the LR of both FW and SW milkfish using 0.1% Triton X-100 as detergent for 60 minutes (Fig. 4). In milkfish LR, no significant difference was found in the abundance of flotillin-2 between FW and SW groups (Fig. 4A). Significantly higher (4.4 times) NKA α-subunit abundance was detected in LR of FW milkfish compared to the SW LR group (Fig. 4B).

Comparisons of VHA A abundance in branchial LR and non-LR fractions between FW and SW tilapia and milkfish

In tilapia, a specific immunoreactive band of 69 kDa was detected in branchial LR and non-LR fractions (Fig. 5A). Most VHA A was located in the non-LR (mem) compared to LR. In addition, significant higher VHA A abundance was detected in both non-LR (mem) and LR fraction of the FW group compared to those of the SW group. (Fig. 5B).

Two immunoreactive bands corresponding to 69 and 60 kDa were detected as the isoform 1 and 2, respectively, of the VHA A subunit of milkfish gills (Fig. 6A). Similar to tilapia VHA A, relative abundance of milkfish VHA A isoform 1 and 2 was significantly higher in the non-LR fraction rather than the LR fraction (Fig. 6B, 6C). Moreover, significantly higher amounts of both isoform 1 (about 2 times; Fig. 6B) and isoform 2 (about 1.5 times; Fig. 6C) of milkfish VHA A subunit were found in the non-LR fraction of the FW group compared to the SW group. In the LR fraction

330 of gills, however, no significant difference was found between FW and SW milkfish

331 in either isoform 1 (Fig. 6B) or isoform 2 (Fig. 6C) of VHA A subunit.

332

Discussion

This study revealed that Triton X-100 had better dissolvability for extracting LR from gills of milkfish rather than tilapia due to the weakly immunoreactive bands of flotillin and NKA found in the non-LR fractions of tilapia gills. LR and non-LR membrane regions of tilapia were isolated more efficiently using Tween 20 as detergent. LR extraction efficacy from Madin-Darby canine kidney (MDCK) cells has been previously reported for various surfactants. It was found that the membrane dissolving effects of Triton X-100 and CHAPS used to extract the LR-protein were 10 times higher than using Tween 20, Brij58, and Lubrol WX. Moreover, the Madin-Darby canine kidney (MDCK) cell and MDCK cells expressing human PLAP (MDCK-PLAP) cell revealed better results in LR extraction by using Triton X-100 as a detergent compared to Tween 20, indicating that Triton X-100 had a higher ability to dissolve lipid structures than Tween 20 (Schuck et al., 2003).The detergent efficacy results in this study also showed that the strength of the detergents required for LR extraction in various fish species differed and thus had to be adjusted for each species in order to have optimal LR isolations. It has to be noted that when different detergents are used, LR composition might be slightly different (Schuck et al., 2003). Differences in SLs and cholesterol-rich fatty acids abundance found among various species may lead to different efficacy of detergents (Schuck et al., 2003; Lingwood et al., 2005; Brogden et al., 2014). As a result, different efficacy of detergents between tilapia and milkfish found in this study might be attributed to more abundant SLs and cholesterol-rich fatty acids in LR of milkfish than tilapia, requiring a more efficient detergent to extract LR from other portions of the membranes.

LR are known to act as signaling and sorting platforms for numerous molecules (Simons and Toomre, 2000; Smart et al., 1999), and proteins forming the scaffolding of LR (Babuke et al., 2009). Flotillins have generally been used as marker proteins for

LR (Bickel et al., 1997; Neumann-Giesen et al., 2004, 2007). In addition, specific GPI-anchored proteins have been observed to co-cluster with flotillins. So, flotillins have been proposed to represent centers for GPI-anchored proteins' communication with intracellular signal transduction molecules (Stuermer and Plattner, 2004). They have also been reported to play the roles in trafficking and transport of membrane proteins and materials (Stuermer, 2010). Our results in different species provided evidence that LR are differentially expressed in different euryhaline fish regarding salinity acclimation by assessing the expression patterns of the LR marker, flotillin-2. LR abundance (i.e., flotillin-2 abundance) in gills was significantly higher in FW than in SW tilapia while there was slightly but not significantly higher expression of flotillin-2 in gills of FW compared to SW milkfish. Differential LR abundance might affect transporter function, as the membrane lipid environment is essential for the functioning of transporters and channels. Until now, evidence on the link between LR and salinity acclimation was only reported in few species. In skate, the anion exchanger 1, for example, was present in intracellular vesicles in detergent-insoluble lipid raft in isotonic conditions and shifted to detergent-soluble plasma membrane regions through exocytosis following hypotonicity treatment (Musch et al., 2004).

NKA α-subunit protein abundance (membrane fractions protein) in gills of FW-preference tilapia was significantly higher in SW than in FW (Lee et al. 2003; Lin et al., 2004), while in gills of SW-preference milkfish it was significantly higher in FW than in SW (Lin et al., 2003). This study further revealed that average LR abundance in tilapia gills was significantly higher (two folds) in the FW group than the SW group, while in milkfish gills LR abundance was similar between FW and SW individuals. In addition, the presence of NKA proteins in LR is positively correlated to NKA activities because they were also reported to be higher in gills of the FW milkfish (Lin et al., 2003) as well as SW tilapia (Uchida et al., 2000). Our results thus

385 echoed previous studies on NKA expression in membrane fractions of fish gills in
386 tilapia and milkfish (Lee et al. 2003; Lin et al., 2003; Lin et al., 2004).
387 The immunoblots of this study clearly showed high and low abundance of VHA
388 in the non-LR and LR fraction, respectively, of milkfish and tilapia. Contrary to NKA,
389 VHA is more abundant in non-lipid raft fractions and salinity modulation affects only
390 the expression of VHA in those membrane fractions. In FW non-LR fractions, VHA
391 expression was increased compared to SW non-LR fractions in both species.
392 Moreover, this study is the first to reveal the distribution of VHA in both LR and
393 non-LR regions of cell membrane in gills of euryhaline teleosts. The presence of VHA
394 mostly found in non-LR fractions may be due to differential membrane compositions
395 between apical and basolateral membranes with remains to be clarified in future
396 studies.
397
398 **Conclusion**
399 The present study set up the method of LR isolation by extracting the LR from
400 two species of euryhaline teleosts with different salinity preferences. The results
401 indicated that lipid raft isolation should be optimized in different species using
402 different detergents or detergent concentration. Accordingly, NKA was found to
403 distributed exclusively in the LR fraction of both tilapia and milkfish gills. The FW
404 euryhaline species (tilapia) has more abundant (two folds) LR in its native media and
405 about 25% lower NKA expression. The marine euryhaline species (milkfish), however,
406 does not alter LR abundance following salinity changes but increases massive
407 amounts of NKA in the LR fraction. Meanwhile, VHA was first found to be mostly
408 distributed in non-LR fraction where salinity-mediated alterations also occurred in
409 both euryhaline species. Relative VHA abundance in the non-LR fraction was
410 significantly higher in the FW group than the SW group of both euryhaline teleosts

regardless of their salinity preferences. With successful separation of LR and non-LR fractions from the crude membrane of fish gills, this study deepened our understanding in distribution of two important ion transporters, NKA and VHA, in cell membranes of euryhaline fish gills under different salinities.

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Competing interests

No competing interests declared.

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Figure legends

Fig. 1 Expression of NKA α-subunit (NKA) and flotillin-2 protein in lipid raft of fresh water (FW) tilapia gills after digestion with 0.1% Triton X-100 for 30, 60, and 120 min, as well as 0.05% Triton X-100 and 0.05% Tween-20 for 60 min. Immunoblots revealed an immunoreactive band in each condition with a molecular mass of 100 kDa (NKA α-subunit) and 48 kDa (flotillin-2). "mem", non-lipid raft; "raft", lipid raft. The tilapia were reared in brackish water (15 ‰) for two weeks and then acclimated to FW for four weeks before experiments.

Fig. 2 Expression of NKA α-subunit (NKA) and flotillin-2 protein in lipid raft of seawater (SW) milkfish gills after digestion with 0.1% Triton X-100 for 30, 60, and 120 min. The immunoblots revealed an immunoreactive band in each condition with a molecular mass of 100 kDa (NKA α-subunit) and 48 kDa (flotillin-2), respectively. "mem", non-lipid raft; "raft", lipid raft. The milkfish were reared in brackish water (15 ‰) for two weeks and then acclimated to SW for four weeks before experiments.

Fig. 3 Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA α-subunit (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and freshwater (FW) tilapia. The representative immunoblots showed an immunoreactive band in each environment with a molecular mass of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA α-subunit). The asterisk indicated the significant difference of protein abundance in branchial lipid raft (raft) fractions between the SW and the FW groups. The pound signs indicated significant differences of protein abundance between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW or FW tilapia gills. N/A,

not detected. (n = 6, mean ± S.E.M., t-test, $P < 0.05$). The tilapia were reared in brackish water (15 ‰) for two weeks and then acclimated to either FW or SW for four weeks before experiments.

Fig. 4 Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA α- subunit (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and freshwater (FW) milkfish. The representative immunoblots showed an immunoreactive band in each environment with a molecular mass of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA α-subunit). The asterisk indicated the significant difference of NKA abundance between the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions. The pound signs indicated significant differences of protein abundance between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW or FW milkfish gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, $P < 0.05$). The milkfish were reared in brackish water (15 ‰) for two weeks and then acclimated to either FW or SW for four weeks before experiments.

Fig. 5 Relative protein abundance of V-type H^+-ATPase A subunit (VHA A) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and freshwater (FW) tilapia. (A) The representative immunoblot showed an immunoreactive band in each group with a molecular mass of 68 kDa. (B) The asterisks indicated significant differences of VHA A abundance between the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions. The pound signs indicated significant differences of VHA A abundance between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW or FW tilapia gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, $P < 0.05$). The tilapia were reared in brackish water (15 ‰) for two weeks and then

703 acclimated to either FW or SW for four weeks before experiments.

704

705 **Fig. 6** Relative protein abundance of V-type H^+-ATPase A subunit (VHA A) in lipid

706 raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and

707 freshwater (FW) milkfish. The representative immunobglot of V-type

708 H^+-ATPase A subunit (VHA A) showed two bands at molecular weights of 69

709 kDa (isoform 1) and 60 kDa (isoform 2) (A). Relative protein abundance of

710 VHA A isoform 1 (B) and isoform 2 (C) revealed the significant difference

711 indicated by the asterisk between the SW and FW groups in non-lipid raft

712 (mem) fractions of gills. Meanwhile, the pound signs indicated significant

713 differences in VHA A isoform 1 (B) and isoform 2 (C) between the lipid raft

714 (raft) and non-lipid raft (mem) fractions of either SW or FW milkfish gills. (n

715 = 6, mean ± S.E.M., t-test, $P < 0.05$). The milkfish were reared in brackish

716 water (15 ‰) for two weeks and then acclimated to either FW or SW for four

717 weeks before experiments.

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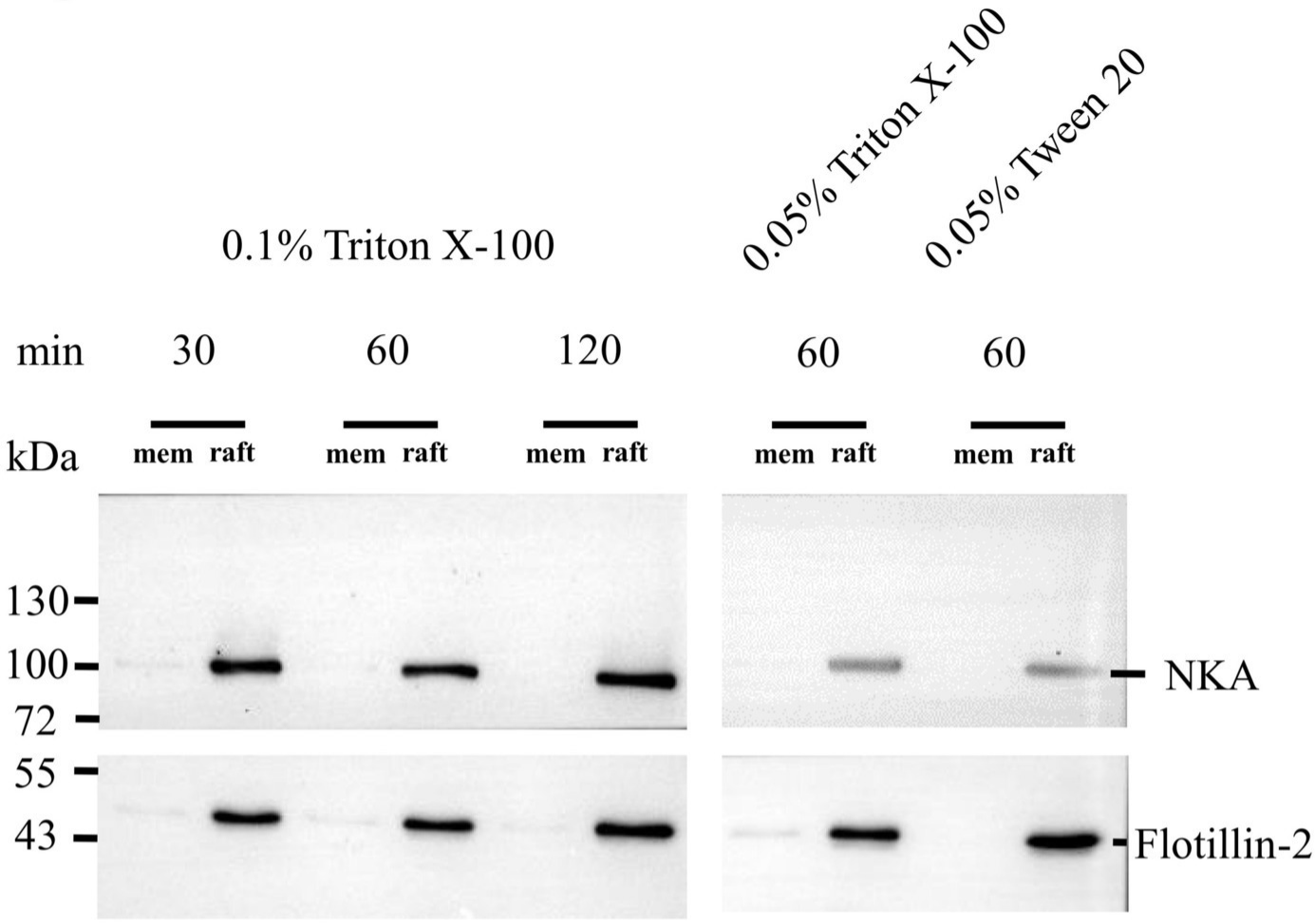
Fig. 1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NKA
Flotillin-2

Fig. 2

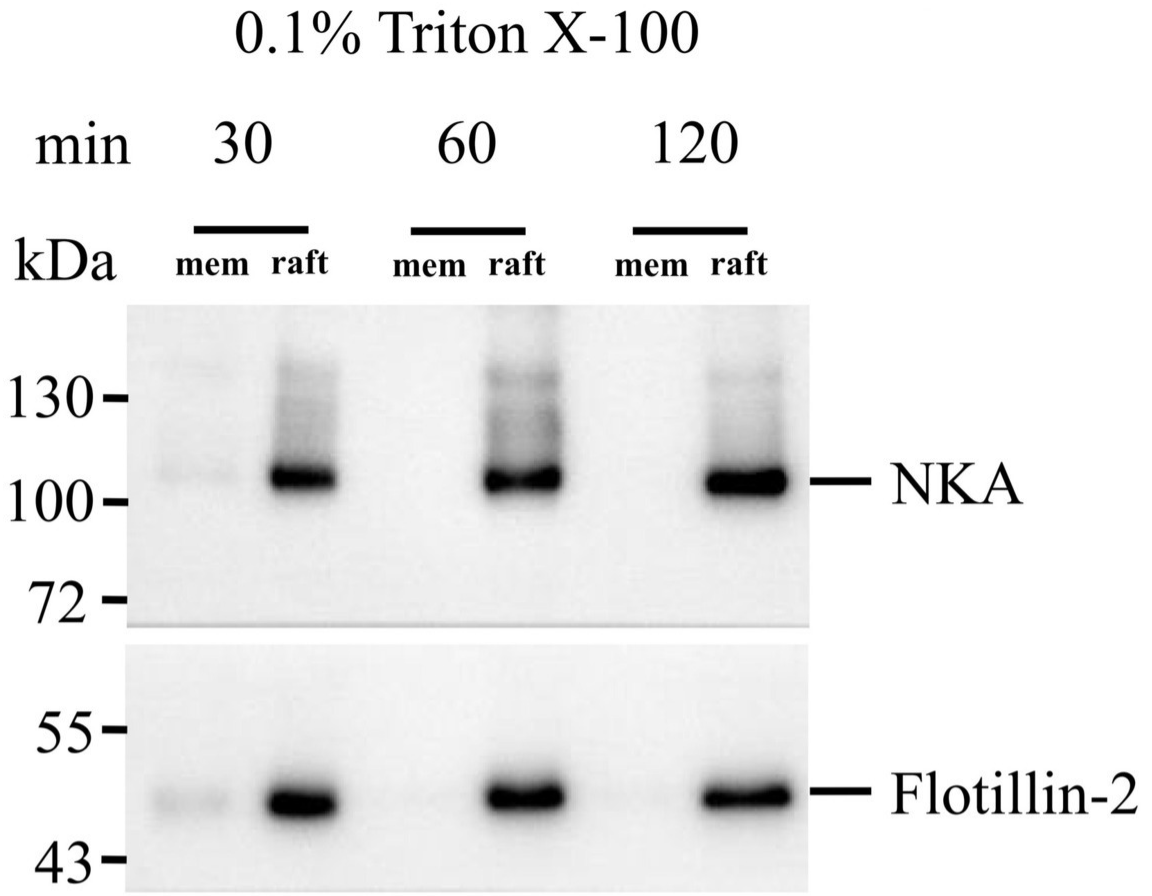
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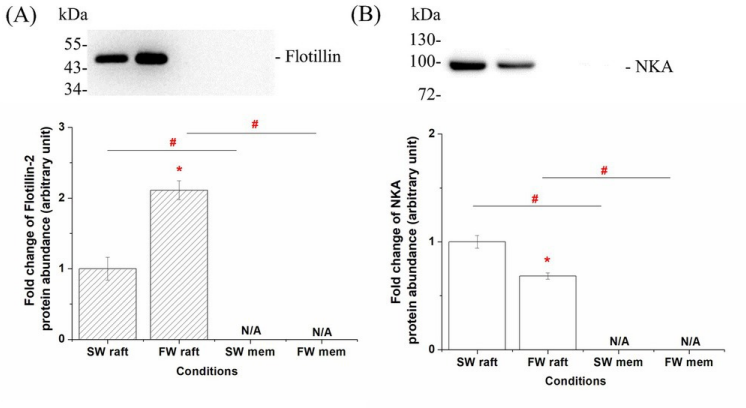
Fig. 3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FW raft
SW mem
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Conditions
(B)
kDa
130-
100-
72-
- NKA
Fold change of NKA protein abundance (arbitrary unit)
SW raft
FW raft
SW mem
FW mem
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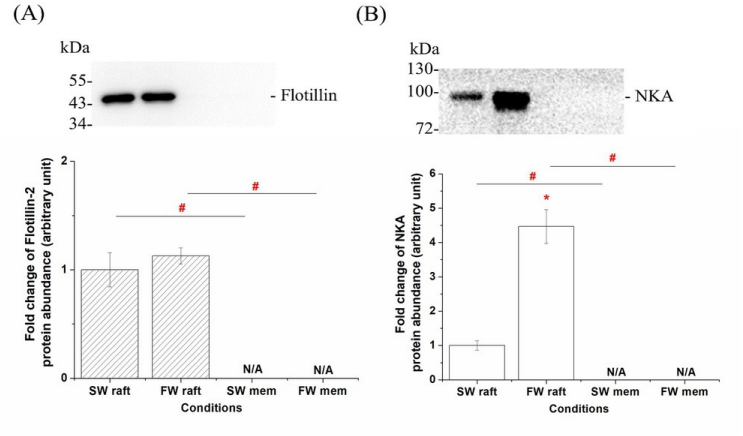
Conditions

Fig. 4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Fold change of Flotillin-2 protein abundance (arbitrary unit)
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N/A
SW raft
FW raft
SW mem
FW mem
Conditions
(B)
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72-
- NKA
Fold change of NKA protein abundance (arbitrary unit)
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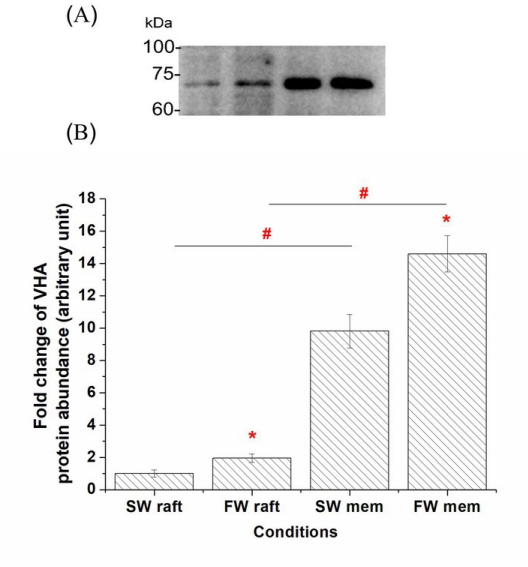
SW raft
FW raft
SW mem
FW mem
Conditions

Fig. 5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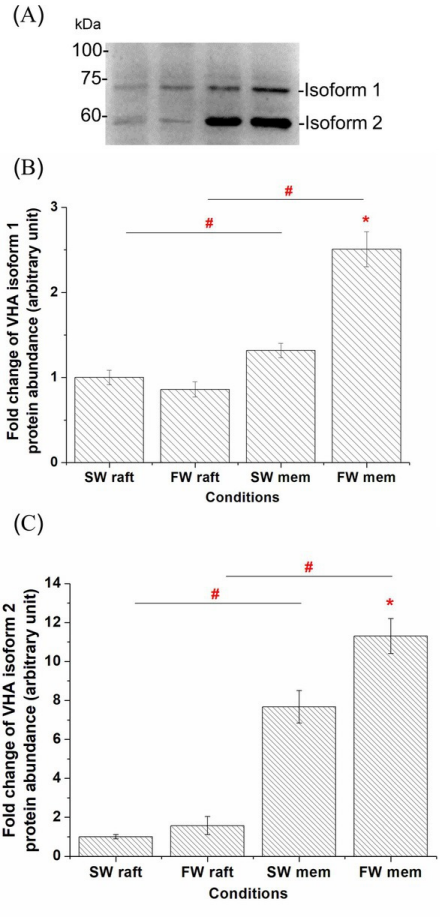
SW raft
FW raft
SW mem
FW mem
Conditions

Fig. 6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SW mem
FW mem
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(C)
Fold change of VHA isoform 2 protein abundance (arbitrary unit)
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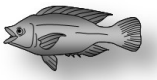
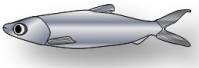


Tilapia (*Oreochromis mossambicus*)



Milkfish (*Chanos chanos*)

Fresh water

VS

Seawater (35‰)

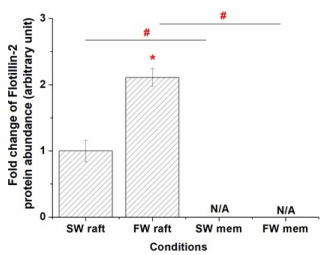
Gills

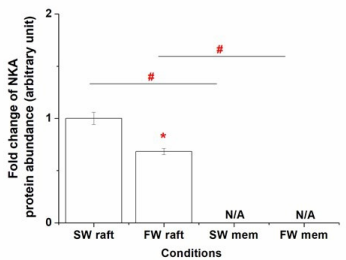
Establishing lipid raft (LR) isolation method

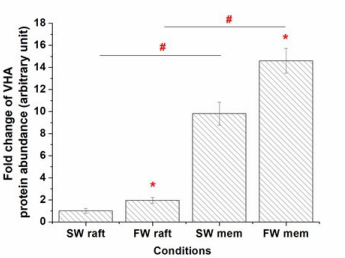
Flotillin-2 (LR marker)

Na^+, K^+-ATPase (NKA)

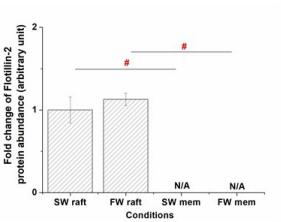
V-type H^+-ATPase (VHA)

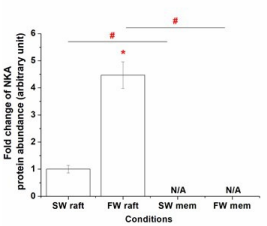


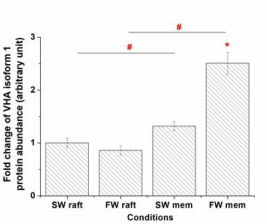


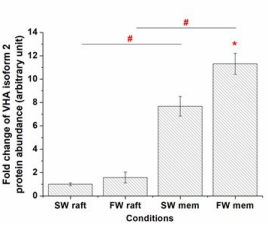


Tilapia









Milkfish