Differential expression of two ATPases revealed by lipid raft isolation from gills of euryhaline teleosts with different salinity preferences

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28

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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

130   distribution on LR has been reported in previous studies on mammalian tissues

131   (Welker et al., 2007). Using the Brij 98 (as a non-ionic detergent) to extract the

132   granulocytes in the brain of rat also revealed the presence of NKA α-subunits in LR

133   (Dalskov et al., 2005). However, there are few studies focused on fish NKA in LR. In

134   gills of rainbow trout (*Oncorhynchus mykiss*), NKA are expressed in LR when

135   transferred to SW but not detected in LRs in FW individuals, indicating different

136   strategies in ionoregulation between FW- and SW-acclimated rainbow trout

137   (Lingwood et al., 2005). Most reports on NKA α-subunit, however, focused on their

138   localization on the basolateral plasma membrane and their expression when fish

139   encountered different environmental salinities (Lee et al., 2003; Lin et al., 2003). The

140   NKA α-subunit protein abundance in gills of FW-preference Mozambique tilapia

141   (*Oreochromis mossambicus*) was significantly higher in SW than in FW (Lee et al.

142   2003), while in gills of SW-preference milkfish (*chanos chanos*) it was significantly

143   higher in FW than in SW (Lin et al., 2003).

144   The VHA is a multi-subunit complex organized into two domains: the 650 kDa

145   cytosolic V_1 domain and the 260 kDa membrane-embedded V_0 domain (Nishi and

146   Forgac, 2002; Forgac, 2007; Toei et al., 2010). In previous mammalian studies,

147   deprivation of cholesterol from LR resulted in the decrease of electrogenic H^+ efflux

148   by VHA and in synaptic signaling deficiency (Yoshinaka et al., 2004). On frog and

149   insect epithelial cells, VHA contributed to acid-base regulation and osmoregulation

150   (Harvey et al., 1998). In the studies on rainbow trout gills, VHA activity and

151   immunoreactivity decreased when FW trout were acclimated to SW (Lin and Randall,

152   1993; Lin et al., 1994). In the Atlantic stingray (*Dasyatis sabina*) and killifish

153   (*Fundulus heteroclitus*), VHA expressed on the cell membrane was significantly

154   higher in FW than in SW (Piermarini and Evans, 2001; Katoh et al., 2003). As a result,

155   this consistently higher expression of VHA in FW environments is linked to its role in

156 generating an electrical gradient favoring Na$^+$ uptake in FW-type ionocytes,

157 additional to its role in acid secretion. In tilapia, bafilomycin, an inhibitor of VHA,

158 has been shown to affect *in vivo* Na$^+$ influx (Fenwick et al., 1999), indicating a key

159 role of this pump in freshwater osmoregulation in tilapia and suggesting an apical

160 location in ionocytes.

161   To date, most studies on NKA and VHA in fish focus on their relationship with

162 cell membrane, while the relationship between NKA and LR in fish was only reported

163 in the rainbow trout (Lingwood et al., 2005). On the other hands, there is no reference

164 reporting the associations of VHA and LR in fish. According to previous references,

165 this study hypothesized that NKA and VHA, the major ion pumps in fish gills, may be

166 mainly distributed in the LR to be involved in ionoregulation of euryhaline teleosts

167 when acclimated to environments of different salinities. As a result, the present study

168 aims to investigate the presence of LR and their exhibition of NKA and VHA in gills

169 of tilapia (the FW euryhaline teleost) and milkfish (the marine euryhaline teleost), by

170 (i) extracting membrane LR from gills of tilapia and milkfish, with fresh water (FW)

171 and seawater (SW) preferences, respectively, and (ii) assessing differential NKA and

172 VHA expression in branchial LR between FW- and SW-acclimated milkfish and

173 tilapia. This study provides the evidence for expression of critical ion transporters in

174 LR of euryhaline teleosts with different salinity preferences. Differential expression of

175 flotillin-2 (the LR marker) and two ATPases, the ATP-consuming NKA and VHA, in

176 gills of euryhaline fish will further clarify the ionoregulation roles of branchial LR in

177 environments of different salinities.

178

179

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 ± 1℃ 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

232   The primary antibodies used in this study included (i) a mouse monoclonal

233   antibody (α5, DHSB, Iowa City, IA, USA) raised against the avian NKA α-subunit

234   (Lin et al., 2003; Yang et al., 2015), (ii) a mouse monoclonal antibody (flotillin-2,

235   Santa Cruz, CA, USA) raised against the amino acids 150-240 of human flotillin-2

236   (XP_016879883.1) with approximately 83% similarity with milkfish flotillin-2

237   (XP_030648347.1) and 80% similarity with tilapia flotillin-2 (XP_003456181.1), and

238   (iii) a rabbit polyclonal antibody (MDBio, Taipei, Taiwan) raised against the specific

239   epitope (AEMPADSGYPAYLGARLA) of the pufferfish (*Tetraodon nigroviridis*)

240   VHA A (ABX80240.1) with 100 % similarity with milkfish VHA A isoform1 and

241   isoform 2, as well as tilapia VHA A (BAF94024.1). The sequences of two isoforms of

242   VHA A found in the milkfish transcriptome database (Hu et al., 2015) were showed in

243   Fig. S1. The specificities of the VHA A antibody to gills of tilapia and milkfish were

244   tested and showed in Fig. S2. According to the primary antibodies, the secondary

245   antibodies for immunoblots included the horseradish peroxidase-conjugated (i) rabbit

246   anti-mouse IgG, (ii) goat anti-rabbit IgG, and (iii) rabbit anti-goat IgG (GeneTex,

247   Irvine, CA, USA). The similarity between flotillin-2 with milkfish.

248

249   **Immunoblotting**

250   The sample mixture of 6x sample loading dye (0.06% bromophenol blue, 30%

251   glycerol, 12% SDS, 0.6 M dithiothreitol, and 62.5 mM Tris with pH 6.8) and gill

252   proteins (1:5, v/v) was heated at 65℃ for 15 min to denature the proteins. Non-LR

253   and LR samples were separated by electrophoresis on a 7% SDS-polyacrylamide gel

254   (5 μg of protein per lane) using a Mini-protein II electrophoresis cell (Bio-Rad,

255   Hercules, CA, USA). Then, the separated proteins were transferred to polyvinylidene

256   difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After incubation in

257   the non-specific blocking solution using phosphate buffer saline with Tween 20

258   (PBST; 2.68 mM potassium chloride, 10.1 mM disodium phosphate, 137 mM sodium

259   chloride, 1.76 mM potassium dihydrogen phosphate, and 0.2 % (v/v) Tween; pH 7.4)

260   containing 5% (w/v) nonfat dried milk for 2 h at room temperature (RT), the blots

261   were incubated overnight with the primary antibody (1:5000) at 4ºC, followed by

262   washing the membranes with PBST. Then the membranes were incubated with the

263   secondary antibody (1:25000) for 1 h at RT. Images were developed with T-Pro

264   LumiLong Chemiluminescent Substrate Kit (Ji-Feng Biotechnology, New Taipei,

265   Taiwan) under a cooling-CCD (charge-couple device) camera (ChemiDoc XRS+,

266   Bio-Rad) with associated software (Quantity One version 4.6.8, Bio-Rad). The blot

267   bands were converted to numerical values by ImageLab 3.0 software (Bio-Rad) to

268   quantify and compare relative protein abundance of the immunoreactive bands.

269

270   **Statistics**

271   In this study, comparisons in flotillin-2, NKA-α or VHA A abundance between (i) LR

272   and non-LR fractions of gills in either SW or FW fish, or (ii) SW or FW groups in

273   either LR or non-LR fractions, respectively, were analyzed by the Student's *t-test*,

274   with $p < 0.05$ was set as the significant level. Values are expressed as means ± S.E.M.

275   (standard error of the mean).

276

277

278   **Results**

279   **Separation and purification of LR from tilapia and milkfish gills**

280   In tilapia (Fig. 1), the crude membrane fraction was first reacted in the non-ionic

281   detergent Triton X-100. After 30, 60, and 120 minutes of reaction with this detergent

282   at 0.1% as well as 60 minutes of reaction at 0.05%, strong immunoreactive bands

283   were found in LR for NKA α-subunit and flotillin-2. However, faint immunoreactive

284   bands of both proteins were found in non-LR, indicating that dissolvability of 0.1 and

285   0.05% Triton X-100 may not be optimal for tilapia gill tissues (Fig. 1). Meanwhile,

286   the supernatant separated by reacting with 0.05% Tween-20 for 60 minutes did not

287   show immunoreactive bands of flotillin-2 in non-LR, indicating the better dissolution

288   of this protocol for extracting LR from tilapia gills (Fig. 1). Moreover, no band for

289   NKA α-subunit was observed in the non-LR fraction.

290   In milkfish (Fig. 2), similar results were observed at 30-minutes treatments with

291   the non-ionic detergent 0.1% Triton X-100 as tilapia reacted with Triton X-100 (Fig.

292   1). However, after 60 and 120 minutes of reaction with 0.1% Triton X-100, there was

293   no flotillin-2 and NKA α-subunit immunoreactive band found in the non-LR

294   membrane fraction, indicating that the LR of milkfish gills could be separated after

295   0.1% Triton X-100 dissolution following 60 minutes of incubation. The NKA

296   α-subunit abundance was high in the LR fraction (Fig. 2). Hence, extraction of LR by

297   0.1% Triton X-100 for 60 minutes is an appropriate procedure for LR purification

298   from milkfish gills.

299

300   **Comparisons of flotillin-2 and NKA in branchial LR and non-LR fractions**

301   **between FW and SW tilapia and milkfish**

302   As illustrated in Fig. 1, tilapia gills treated with 0.05% Tween-20 for 60 minutes

303   revealed better dissolvability in LR membrane fractions. This condition has thus been

304 used to compare salinity effects on LR and non-LR gill fractions of tilapia. Flotillin-2

305 and NKA α-subunit protein levels were only detected in the LR of both FW and SW

306 tilapia. The abundance of flotillin-2 in LR was significantly (2 times) higher in the

307 FW group than in the SW group (Fig. 3A). On the contrary, the abundance of NKA

308 α-subunit in LR of SW tilapia was 1.4 times higher than that of FW fish (Fig. 3B).

309   Similarly, flotillin-2 and NKA α-subunit protein levels were only detected in the

310 LR of both FW and SW milkfish using 0.1% Triton X-100 as detergent for 60 minutes

311 (Fig. 4). In milkfish LR, no significant difference was found in the abundance of

312 flotillin-2 between FW and SW groups (Fig. 4A). Significantly higher (4.4 times)

313 NKA α-subunit abundance was detected in LR of FW milkfish compared to the SW

314 LR group (Fig. 4B).

315

316 **Comparisons of VHA A abundance in branchial LR and non-LR fractions**

317 **between FW and SW tilapia and milkfish**

318   In tilapia, a specific immunoreactive band of 69 kDa was detected in branchial

319 LR and non-LR fractions (Fig. 5A). Most VHA A was located in the non-LR (mem)

320 compared to LR. In addition, significant higher VHA A abundance was detected in

321 both non-LR (mem) and LR fraction of the FW group compared to those of the SW

322 group. (Fig. 5B).

323   Two immunoreactive bands corresponding to 69 and 60 kDa were detected as the

324 isoform 1 and 2, respectively, of the VHA A subunit of milkfish gills (Fig. 6A).

325 Similar to tilapia VHA A, relative abundance of milkfish VHA A isoform 1 and 2 was

326 significantly higher in the non-LR fraction rather than the LR fraction (Fig. 6B, 6C).

327 Moreover, significantly higher amounts of both isoform 1 (about 2 times; Fig. 6B)

328 and isoform 2 (about 1.5 times; Fig. 6C) of milkfish VHA A subunit were found in

329 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

359   LR (Bickel et al., 1997; Neumann-Giesen et al., 2004, 2007). In addition, specific

360   GPI-anchored proteins have been observed to co-cluster with flotillins. So, flotillins

361   have been proposed to represent centers for GPI-anchored proteins' communication

362   with intracellular signal transduction molecules (Stuermer and Plattner, 2004). They

363   have also been reported to play the roles in trafficking and transport of membrane

364   proteins and materials (Stuermer, 2010). Our results in different species provided

365   evidence that LR are differentially expressed in different euryhaline fish regarding

366   salinity acclimation by assessing the expression patterns of the LR marker, flotillin-2.

367   LR abundance (i.e., flotillin-2 abundance) in gills was significantly higher in FW than

368   in SW tilapia while there was slightly but not significantly higher expression of

369   flotillin-2 in gills of FW compared to SW milkfish. Differential LR abundance might

370   affect transporter function, as the membrane lipid environment is essential for the

371   functioning of transporters and channels. Until now, evidence on the link between LR

372   and salinity acclimation was only reported in few species. In skate, the anion

373   exchanger 1, for example, was present in intracellular vesicles in detergent-insoluble

374   lipid raft in isotonic conditions and shifted to detergent-soluble plasma membrane

375   regions through exocytosis following hypotonicity treatment (Musch et al., 2004).

376   NKA α-subunit protein abundance (membrane fractions protein) in gills of

377   FW-preference tilapia was significantly higher in SW than in FW (Lee et al. 2003; Lin

378   et al., 2004), while in gills of SW-preference milkfish it was significantly higher in

379   FW than in SW (Lin et al., 2003). This study further revealed that average LR

380   abundance in tilapia gills was significantly higher (two folds) in the FW group than

381   the SW group, while in milkfish gills LR abundance was similar between FW and SW

382   individuals. In addition, the presence of NKA proteins in LR is positively correlated

383   to NKA activities because they were also reported to be higher in gills of the FW

384   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

411   regardless of their salinity preferences. With successful separation of LR and non-LR

412   fractions from the crude membrane of fish gills, this study deepened our

413   understanding in distribution of two important ion transporters, NKA and VHA, in

414   cell membranes of euryhaline fish gills under different salinities.

415

416

426

427   **Competing interests**

428   No competing interests declared.

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652

653 **Fig. 1** Expression of NKA α-subunit (NKA) and flotillin-2 protein in lipid raft of fresh

654 water (FW) tilapia gills after digestion with 0.1% Triton X-100 for 30, 60,

655 and 120 min, as well as 0.05% Triton X-100 and 0.05% Tween-20 for 60 min.

656 Immunoblots revealed an immunoreactive band in each condition with a

657 molecular mass of 100 kDa (NKA α-subunit) and 48 kDa (flotillin-2). "mem",

658 non-lipid raft; "raft", lipid raft. The tilapia were reared in brackish water (15

659 ‰) for two weeks and then acclimated to FW for four weeks before

660 experiments.

661 **Fig. 2** Expression of NKA α-subunit (NKA) and flotillin-2 protein in lipid raft of

662 seawater (SW) milkfish gills after digestion with 0.1% Triton X-100 for 30,

663 60, and 120 min. The immunoblots revealed an immunoreactive band in each

664 condition with a molecular mass of 100 kDa (NKA α-subunit) and 48 kDa

665 (flotillin-2), respectively. "mem", non-lipid raft; "raft", lipid raft. The

666 milkfish were reared in brackish water (15 ‰) for two weeks and then

667 acclimated to SW for four weeks before experiments.

668 **Fig. 3** Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA α-subunit

669 (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of

670 seawater (SW) and freshwater (FW) tilapia. The representative immunoblots

671 showed an immunoreactive band in each environment with a molecular mass

672 of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA α-subunit). The asterisk

673 indicated the significant difference of protein abundance in branchial lipid

674 raft (raft) fractions between the SW and the FW groups. The pound signs

675 indicated significant differences of protein abundance between the lipid raft

676 (raft) and non-lipid raft (mem) fractions of either SW or FW tilapia gills. N/A,

677   not detected. (n = 6, mean ± S.E.M., t-test, $P < 0.05$). The tilapia were reared

678   in brackish water (15 ‰) for two weeks and then acclimated to either FW or

679   SW for four weeks before experiments.

680   **Fig. 4** Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA α- subunit

681   (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of

682   seawater (SW) and freshwater (FW) milkfish. The representative

683   immunoblots showed an immunoreactive band in each environment with a

684   molecular mass of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA α-subunit).

685   The asterisk indicated the significant difference of NKA abundance between

686   the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions.

687   The pound signs indicated significant differences of protein abundance

688   between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW

689   or FW milkfish gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, $P <$

690   0.05). The milkfish were reared in brackish water (15 ‰) for two weeks and

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

692

693   **Fig. 5** Relative protein abundance of V-type H^+-ATPase A subunit (VHA A) in lipid

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

695   freshwater (FW) tilapia. (A) The representative immunoblot showed an

696   immunoreactive band in each group with a molecular mass of 68 kDa. (B)

697   The asterisks indicated significant differences of VHA A abundance between

698   the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions.

699   The pound signs indicated significant differences of VHA A abundance

700   between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW

701   or FW tilapia gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, $P < 0.05$).

702   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.
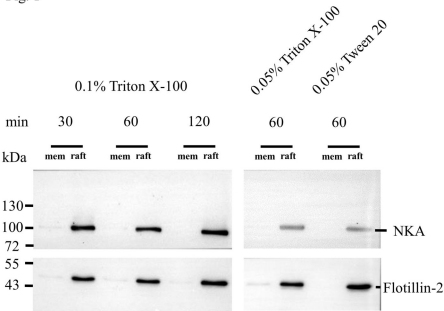
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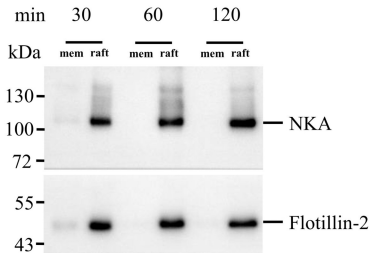
Fig. 1

Fig. 1

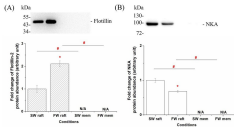- 0.1% Triton X-100
- 0.05% Triton X-100
- 0.05% Tween 20

min   30   60   120   60   60

kDa   mem raft mem raft mem raft mem raft mem raft

130 —
100 —   ← NKA
72 —

55 —
43 —   ← Flotillin-2

Fig. 2

0.1% Triton X-100

Fig. 3

(A)

kDa
55-
43-
34-

— Flotillin

Fold change of Flotillin-1
protein abundance (arbitrary unit)

Conditions

SW raft   FW raft   SW mem   N/A
                                FW mem

(B)

kDa
130-
100-
72-

— NKA

Fold change of NKA
protein abundance (arbitrary unit)

Conditions

SW raft   FW raft   SW mem   N/A
                                FW mem

Fig. 4

(A)

kDa
55-
43- — Flotillin
34-

Fold change of Flotillin-2 protein abundance (arbitrary unit)

SW raft   FW raft   SW mem   FW mem
Conditions

N/A   N/A
#

(B)

kDa
130-
100- — NKA
72-

Fold change of NKA protein abundance (arbitrary unit)

SW raft   FW raft   SW mem   FW mem
Conditions
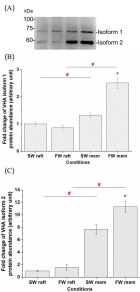
*
*

N/A   N/A

Fig. 5

(A)

(B)

Fig. 6

Tilapia (*Oreochromis mossambicus*)   Milkfish (*Chanos chanos*)

Fresh water   vs   Seawater (35‰)
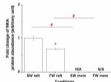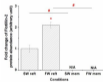
Gills

Establishing lipid raft (LR) isolation method

Flotillin-2 (LR marker)   Na⁺, K⁺-ATPase (NKA)   V-type H⁺-ATPase (VHA)

Tilapia

Milkfish