Stretch-activated cation channel TRPV4 mediates hyposmotically induced prolactin release from prolactin cells of mozambique tilapia *Oreochromis mossambicus*

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Watanabe S, Seale AP, Grau EG, Kaneko T. Stretch-activated cation channel TRPV4 mediates hyposmotically induced prolactin release from prolactin cells of mozambique tilapia *Oreochromis mossambicus*. Am J Physiol Regul Integr Comp Physiol 302: R1004–R1011, 2012. First published February 29, 2012; doi:10.1152/ajpregu.00632.2011.—In teleost fish, prolactin (PRL) is an important hormone for hyperosmoregulation. The release of PRL from the pituitary of Mozambique tilapia is stimulated by a decrease in extracellular osmolality. Previous studies have shown that hyposmotically induced PRL release is linked with cell volume changes, and that stretch-activated Ca2+ channels are likely responsible for the initiation of the signal transduction for PRL release. In this study, we identified the stretch-activated Ca2+ channel transient receptor potential vanilloid 4 (TRPV4) from the rostral pars distalis (RPD) of tilapia acclimated to freshwater (FW). TRPV4 transcripts were ubiquitously expressed in tilapia; the level of expression in RPDs of FW-acclimated fish was lower than that found in RPDs of seawater (SW)-acclimated fish. Immunohistochemical analysis of the pituitary revealed that TRPV4 is localized in the cell membrane of PRL cells of both FW and SW tilapia. A functional assay with CHO-K1 cells showed that tilapia TRPV4 responded to a decrease in extracellular osmolality, and that its function was suppressed by ruthenium red (RR) and activated by 4a-phorbol 12,13-didecanoate (4aPDD). Exposure of dissociated PRL cells from FW-acclimated tilapia to RR blocked hyposmolality induced PRL release. PRL release, on the other hand, was stimulated by 4aPDD. These results indicate that PRL release in response to physiologically relevant changes in extracellular osmolality is mediated by the osmotically sensitive TRPV4 cation channel.

OSMOREGULATION is the essential function to maintain body fluid chemistry for every organism, especially vertebrates. Osmoregulation in teleost fishes is achieved to a large extent by integrated ion and water transport by osmoregulatory organs under the control of the endocrine systems (19). The osmoregulatory activities of those organs are optimized to internal fluid osmolality, which is a fundamental parameter to determine the direction and magnitude of ion and water transport. Recently, osmotic response factors (osmotic response element binding protein, osmotic stress transcription factor 1, p38 mitogen-activated protein kinase, and calcium-sensing receptor) have been reported in fish and are possibly involved in osmosensing and subsequent signal transduction pathways (10). However, little information is available on the osmoreceptor molecules that mediate osmoregulation in fish.

In teleost species, prolactin (PRL) is a multifunctional endocrine factor that is likely involved in many physiological events, and it is widely accepted that PRL is crucial for adaptation to freshwater (FW) and other hypotonic environments. The Mozambique tilapia possesses two PRL isoforms, PRL-188 and PRL177, both of which exert hyperosmoregulatory actions (18). There is abundant evidence that PRL acts on all major osmoregulatory organs in FW-acclimated fish, including the gills, skin, intestine, and kidney, to retain ions in body fluid and to reduce the water permeability of body surfaces (6, 18).

To coordinate hyperosmoregulatory functions in various tissues, osmosensitive mechanisms play an essential role in controlling PRL secretion. Although there are several reports that PRL release is under hypothalamic control, the relationship between these regulatory pathways and osmoregulation is still unclear (11, 15, 22, 30, 31, 40).

Previous studies have shown that PRL cells of tilapia are intrinsically responsive to changes in extracellular osmolality (12, 23, 32, 37). The release of PRL from dispersed PRL cells is stimulated by physiologically relevant decreases in extracellular osmolality (36), a response that is closely related to cell volume changes (33, 34, 45), and is consistent with the hyperosmoregulatory role of PRL. The maintenance of body fluid osmolality within a physiological range requires osmosensory mechanisms that are highly sensitive to changes in osmolality. Previous experiments using tilapia PRL cells indicate that the hyposmotic response of tilapia PRL cells is linked to cell volume changes that are driven by water influx across the cell membrane through the water channel aquaporin-3 (AQP3) (43, 45). There is firm evidence that PRL cells do not respond to changes in the concentration of specific extracellular ions but to changes in extracellular osmolality (45). An elevation of intracellular free [Ca2+] induces PRL release, and hyposmotically induced PRL release is dependent on the entry of extracellular Ca2+ (33). The hyposmotically induced rises in intracellular [Ca2+] and PRL release were suppressed by treatment with Gd3+, a known blocker of various mechanosensitive channels (34). Furthermore, this Ca2+-dependent signaling cascade is likely to be independent of both inositol triphosphate- and ryanodine-sensitive intracellular Ca2+ stores (35).

Hyposmotically induced PRL release also appears to be independent of voltage-gated Ca2+ channel mediation (33, 34), which has been reported to play a role in PRL release suppression by cortisol (13). Despite the lack of information on the molecular basis of PRL cell osmoreception, these studies suggest that stretch-activated ion channels that are permeable...
to calcium are responsible for the initiation of the signal transduction pathway that triggers PRL release. Recently, an increase in PRL release in response to a decrease in extracellular osmolality was also reported in silver sea bream Sparus sarba (17), suggesting that osmoreception in PRL cells may be widespread among teleost species. Therefore, clarification of the precise mechanism underlying the intrinsic osmosensitivity of PRL cells is important to a better understanding of osmoregulatory control of PRL in teleosts and may provide insight into the mechanisms governing osmoreception generally.

In recent years, transient receptor potential (TRP) family proteins have been identified in various species. TRP channels commonly contain six transmembrane domains and a highly conserved pore loop between transmembrane domains 5 and 6 (25, 41). Among TRP channel family members, TRP vanilloid 4 (TRPV4) has been reported to be an osmosensory cation channel activated by osmotic cell volume increase, being likely involved in various physiological events, including thermo-sensing, nociception, mechanosensation, and osmosensing (24). Although Ca\(^{2+}\) influx occurs in response to supraphysiological reductions in extracellular osmolality in most cells studied (20, 27, 39), intracellular Ca\(^{2+}\) increase is induced by smaller osmolality decrease in the cells expressing TRPV4 (14). These findings suggest that TRPV4 is an important component of osmosensing systems that monitor body fluid osmolality. In mammalian species, TRPV4 presumably mediates ion and water transport in the kidney, especially in the ascending limb of the loop of Henle (8). A gene knock-out study in mice showed that the lack of TRPV4 led to impaired secretion of arginine vasopressin, an antidiuretic hormone (17a, 21).

In the present study we report the molecular characterization of TRPV4 from Mozambique tilapia and examine the function of TRPV4 in tilapia PRL cells. Our results show that hypsomotically induced PRL release is mediated by TRPV4 in tilapia PRL cells.

**MATERIALS AND METHODS**

Euryhaline tilapia (Oreochromis mossambicus) were maintained in tanks supplied with either recirculated FW or full-strength seawater (SW, 31–33 ppt) at 25–27°C. Fish were fed once a day on commercial tilapia pellets “Tilapia 41M” (Nishi-Nihon Kumiai Shiryo, Tokushima, Japan). Fish were anesthetized with 0.1% 2-phenoxyethanol and decapitated before removal of tissues. Experiments were conducted according to the principles and procedures approved by the Institutional Animal Care and Use Committee of University of Tokyo and University of Hawaii.

**Molecular cloning of tilapia TRPV4 cDNA.** FW-adapted tilapia was used for total RNA preparation. After anesthesia, the pituitary was extracted and the rostral pars distalis (RPD) was dissected from the other part of the pituitary. The tilapia PRL cells are isolated as a nearly homogenous tissue in the RPD (26). Total RNA was extracted from the RPD with RNA extraction solution (ISOGEN, Nippon Gene, Tokyo, Japan). Total RNA was treated with DNase I (Life Technologies, Carlsbad, CA) at room temperature for 15 min, and the resulting total RNA (700 ng) was reverse transcribed using SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions.

For cloning of partial-length cDNA, degenerate primers were designed based on the available information in vertebrate species. The PCR was carried out using sense (TRPV4-df, GAYTGGGCTAAYG-GNCCNGT) and antisense (TRPV4-dr, TGICCNACNTYTCNC-CCAT) primers in a reaction mixture for rTaq DNA polymerase (Takara, Shiga, Japan). To check the expression of other TRPVs, the degenerate primer set for other molecules were also designed as follows: For TRPV1 and 2, ACNGARTGGGYNTAYGG and CAT-ICDDATYGDAYWTTRA; for TRPV3, TRPV4-df and ATNACYTETYGDATCATNAC; and for TRPV5/6, TAYGAYYTIACN-GARATHGA and GGICCIACATYTSRAANCC. The PCR products were analyzed on a 2% agarose gel and ligated into pBluescript SK (–) (Stratagene, La Jolla, CA). The plasmid DNA was purified, and both strands of the DNA were sequenced using a DNA sequencer ABI PRISM 310 (Life Technologies). Sequence data were analyzed with Sequencher software version 3.1.1 (Hitachi, Tokyo, Japan).

After determination of the partial cDNA sequence, 3’ rapid amplification of cDNA end (RACE) was carried out to extend sequence information at the 3’ end. The 3’ end of tilapia TRPV4 cDNA was amplified with a gene-specific primer (TRPV4-3’-r1, TCAC-CTTTCTGTGCTGTACTCTC), and an adaptor primer (NUP, CTA-ATACGACTCACTATAGGG) in a reaction mixture for LA Taq DNA polymerase (Takara). For 5’-RACE, instead of adaptor primer and a gene-specific primer (TRPV4-5’-r1, GTATCCAGTGAAGACAGATC) were used. The clone obtained was subcloned and sequenced as described above.

The tentative motifs and transmembrane domains of tilapia TRPV4 were predicted by online software available at ExPASy’s website (InterPro Scan; http://www.ebi.ac.uk/Tools/InterProScan/). SOSUI; http://bp.nuap.nagoya-u.ac.jp/sosui/). The amino acid identities of tilapia TRPV4 and other TRPV4s were analyzed by conducting a BLAST search.

**Functional analysis for tilapia TRPV4.** The open reading frame (ORF) of tilapia TRPV4 was amplified with primers TRPV4-BglII-f (TGTAGATCGAGGGAATGAG) and TRPV4-BglII-r (TCAA-GATCTGGGGAATCAG) in a reaction mixture of KOD plus DNA polymerase (Toyobo, Tokyo, Japan) and ligated into pBluescript II SK(–). The TRPV4 ORF was digested by BglIII (Takara) and subcloned into the BglIII digestion site of an expression vector, pIRE2- DsRed Express (Clontech). The resulting construct (pIRE2- OmTRPV4) was sequenced to confirm that appropriate manipulations were made. pIRE2-OmTRPV4 was transfected into CHO-K1 cells (RIKEN BRC: RCB0285), a known endogenous TRPV4-free cell line (4), with lipofectamine LTX (Life Technologies) according to the manufacturer’s instructions. The irresponsiveness to extracellular hyposmolality in CHO-K1 cells was further confirmed before the experiment. The efficiency of transfection was checked by the observation of DsRed Express fluorescence. Effects of an inhibitor, ruthenium red (RR, Sigma, St. Louis, MO) and an activator 4a-phorbol 12,13-di-cenoate (4aPDD, Sigma), of TRPV4 were examined by adding these reagents to the experimental media. Concentrations of RR and 4a-PDD in the experimental media used in this experiment were 10 μM and 1 μM, respectively. pIRE2-TRPV4-transfected cells were incubated with 5 μM of Fluo-8-AM (ABD Bioquest, Sunnyvale, CA), a fluorescent Ca\(^{2+}\) indicator, in Hank’s balanced salt solution (HBSS) containing 0.04% Pluronic F-127 at 25°C for 30 min. Osmolality of HBSS was adjusted to 290 mosM, which was isosomotic to the culture media for CHO-K1, for the avoidance of unexpected osmotic responses in the transfected cells. The Ca\(^{2+}\) indicator-loaded cells were washed with isosomotic HBSS at 25°C, and then experimental media (hyposmotic HBSS with or without RR, hypsosmotic HBSS without Ca\(^{2+}\), and isosomotic HBSS with 4aPDD) were loaded to the culture wells. In experiments assessing RR effectiveness, washing medium containing 10 μM RR was used to rinse the cells. Final osmolality of the hypsosmotic experimental media was set to 230 mosM (20% decrease). The osmolality of experimental media was adjusted by varying the concentration of NaCl and verified by using a vapor pressure osmometer (Wescor 5100C, Logan, UT). Temporal changes in fluorescence intensity were recorded by an inverted fluorescence microscope (Ti-S, Nikon, Tokyo, Japan), and the data were analyzed using the imaging software Nis element BR (Nikon).

**Expression analyses for TRPV4 transcript.** For tissue distribution analysis of TRPV4, total RNA was extracted from the brain, pituitary, heart, kidney, liver, spleen, anterior and posterior part of intestine,
muscle, gill, and skin of FW and SW tilapia as described above. Resulting RNA was reverse transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies) according to manufacturer’s instructions. The cDNA of TRPV4 was amplified with a primer pair TRPV4-qf (AGTGGAGCCCATCAATGAG) and TRPV4-qr (TGTTGATGTGCGATCAGG) in a reaction mixture for Ex Taq DNA polymerase (Takara).

For quantitative real-time PCR (qRT-PCR) analysis of TRPV4 expression in the RPD of FW and SW fish, total RNA, extracted with Tri-reagent according to the manufacturer’s protocols (MRC, Cincinnati, OH), was reverse transcribed using a high-capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturers’ instructions. qRT-PCR for TRPV4 was performed using the StepOnePlus real-time PCR system (Life Technologies), and reactions were setup as previously described (28a). All quantitative data were expressed in arbitrary units after normalization to the expression levels of elongation factor 1α (EFL1a), after it was confirmed that there was no salinity effect upon its expression. The primers used for for EFL1a detection have been reported by Breves and coworkers (6).

Antibody. A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of the COOH-terminal region of tilapia TRPV4 (amino acid residues 849 – 858, ELSKGSQSHD). The antigen conjugated with keyhole limpet hemocyanin (KLH) was emulsified with complete Freund’s adjuvant, and immunization was performed in a New Zealand White rabbit. The antiserum was obtained after several booster injections, and the specific antibody was affinity purified with the antigen peptide.

Specificity confirmation of the antibody. The specificity of the antibody raised against the synthetic peptide was confirmed by immunocytochemistry in tilapia TRPV4-expressing CHO-K1 cells and Western blot analysis. Immunocytochemistry was done according to the procedures described previously (44) except for the antibody (anti-tilapia TRPV4 diluted 1:2,000). For Western blot analysis, the pituitary was isolated from FW-adapted tilapia and homogenized in 200 μl lysis buffer [pH 6.8; 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 tablet of Complete-Mini (Roche Diagnostics) per 10 ml lysis buffer] using a Polytron homogenizer (PT-1200E; Lucerne, Switzerland) and left on ice for 30 min to lyse cells completely. The lysate was centrifuged at 4,000 g for 5 min at 4°C to remove debris, and the supernatant was collected for Western blot analysis. The resulting protein sample was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the protein was transferred from the gel to a polyvinyl difluoride membrane (Immobilon-P Transfer membrane; Millipore, Billerica, MA) with CAPS transfer buffer (10 mM N-cyclohexyl-3-aminopropanesulfonic acid containing 0.05% SDS, pH 11). After incubation with blocking buffer [5% skim milk in 20 mM Tris-buffered saline containing 0.1% Tween-20 (TBST)] for 30 min at room temperature, the membrane was incubated with anti-tilapia TRPV4 diluted 1:2,000 with blocking buffer overnight at 4°C. After being rinsed with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) diluted 1:5,000 with blocking buffer for 3 h at room temperature. The immunoreactive band was detected using Immobilon Western chemiluminescent HRP substrate (Millipore).

Immunohistochemistry. The pituitaries were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h at 4°C. After fixation, the pituitaries were immersed in 70% ethanol overnight. Fixed pituitaries were rehydrated in phosphate-buffered saline (PBS, pH 7.4), immersed in 30% sucrose in PBS, and embedded in OCT compound (Miles, Elkhart, IN) for cryosection at −80°C. Cryosections (20 μm) were cut and mounted on MAS-coated slides (Matsunami, Osaka, Japan). After being blocked with NB-PBS (PBS containing 2% normal goat serum, 0.1% bovine serum albumin, 0.02% KLH, and 0.01% sodium azide) for 30 min, the sections were incubated with antitilapia TRPV4 diluted 1:1,000 with NB-PBS overnight at 4°C, and then with goat antirabbit IgG labeled with Alexa Fluor 488 (Life Technologies) diluted 1:1,000 with PBS overnight at 4°C. After a brief rinse in PBS, the sections were incubated with 1 μg/ml of 4′,6-diamino-2-phenylindole (DAPI) in deionized water for 30 min. The specimens were observed under a confocal laser scanning microscope (CL, Nikon).

Primary incubation of PRL cells. Dispersed PRL cell incubations were carried out as previously described (36) with slight modifications. After removal of individual pituitaries, the RPD was placed in isosmotic PBS (330 mosM), pooled, diced into fragments, and treated with 0.125% trypsin (Sigma) in PBS for 20 min. After termination of trypsin treatment by adding trypsin inhibitor (Sigma, 0.125%), cells were harvested and resuspended in hyperosmotic culture medium (355 mosM, modified Krebs bicarbonate-Ringer solution, containing 10 mM HEPES, 500 mg/l glucose, 300 mg/l glutamine, 10,000 U/l penicillin, 10 mg/l streptomycin, and Eagle’s minimum essential medium). The resulting cells were incubated at 26–28°C in a 96-well Primaria plate (Becton Dickinson, Franklin Lake, NJ) with saturated humidity. Cells were preincubated for 1 h in hyperosmotic medium before exposure to hyposmotic medium (300 mosM) and pharmacological treatment (10 μM R in hyperosmotic media and 1 μM 4a-PDD in hyperosmotic media). For the constancy of the experimental designs in the previous reports (33–35), the same hyposmotic condition (355 to 300 mosM, ~15% decrease) was also adopted in this study. The cells subjected to treatment with 10 μM R in hyposmotic medium were preincubated for an additional 10 min with 10 μM R in hyperosmotic medium, whereas cells in the other groups were washed with hyposmotic medium alone. After incubation for 20 min, medium was collected and stored at −80°C for radioimmunoassay. Medium osmolality was adjusted by varying the concentration of NaCl and verified using the vapor pressure osmometer (Wescor 5100C).

Radioimmunoassay (RIA). PRL188 levels in culture medium were measured by homologous RIA (3, 47). For dispersed cell cultures, values obtained as nanograms per milliliter were first converted to nanograms per 106 cells and then converted to percent change from hyperosmotic (355 mosM) treatment.

Statistical analysis. Statistical differences were determined by one-way analysis of variance, followed by the Bonferroni’s method, Dunn’s test, or Student’s t-test. Differences at P < 0.05 were considered significant.

RESULTS

Molecular cloning of tilapia TRPV4. We identified 3461 bp of a full-length tilapia TRPV4 cDNA consisting of 312 bp of a 5’ untranslated region (UTR), 2610 bp of an open reading frame, and 539 bp of a 3’-UTR except for poly(A)+ tail (DBJ/EMBL/GenBank accession no. AB648937). This cDNA encodes a protein with 870 amino acid residues. Figure 1 shows the alignments of the deduced amino acid sequences of TRPV4s from tilapia, zebrafish Danio rerio (DQ858167), chicken Gallus gallus (AF261883), and human Homo sapiens (BC143315). The tilapia TRPV4 amino acid sequence shares high identity with those from zebrafish (75%), chicken (72%), and human (70%). Hydropathy and motif analyses showed six putative transmembrane domains, cytoplasmic NH2- and COOH-terminal tails and an extracellular long connecting loop between 5th and 6th membrane-spanning domains in tilapia TRPV4, all of which are characteristic of the TRP family. Moreover, tilapia TRPV4 contains putative ankyrin domains in the NH2-terminal tail and a pore-loop domain located in the long connecting loop, which are highly conserved among proteins of the TRPV family (Fig. 1). No positive amplification for other TRPVs was obtained from the cDNA of FW tilapia RPD (data not shown).
Functional assay for tilapia TRPV4. Intracellular calcium mobilization assay with CHO-K1 cells transiently expressing tilapia TRPV4 showed that intracellular Ca\(^{2+}\) concentration of TRPV4-expressing cells was significantly increased in response to extracellular osmolality decrease, when compared with intracellular Ca\(^{2+}\) changes observed in isosmotic control and Ca\(^{2+}\)-deleted hyposmotic media (Fig. 2). Hyposmotically induced intracellular Ca\(^{2+}\) increase in TRPV4-expressing cells was suppressed by 10 \(\mu\)M RR. An increase in intracellular Ca\(^{2+}\) was also observed when TRPV4-expressing cells were treated with isosmotic medium containing 1 \(\mu\)M 4aPDD, a specific TRPV4 activator; this effect was abolished when Ca\(^{2+}\) was absent from the incubation media (Fig. 2).

Expression analyses of TRPV4. Tissue distribution analysis in FW and SW tilapia by RT-PCR revealed that TRPV4 was expressed in all tissues examined, including the brain, and pituitary and osmoregulatory organs such as the kidney, intestine, and gills (Fig. 3A). Quantitative RT-PCR in the RPD showed significantly higher TRPV4 mRNA levels in SW tilapia compared with levels in the RPD of FW fish (Fig. 3B).

Specificity confirmation of the antibody. Western blot analysis on the proteins from the pituitary of FW fish showed that the antibody raised against the synthetic peptide of partial tilapia TRPV4 residues specifically recognized a 98-kDa protein, whose molecular mass was identical to the predicted
molecular mass of tilapia TRPV4 (Fig. 4A). Immunosignals of antitilapia TRPV4 were observed only in CHO-K1 cells expressing DsRed Express, the indicator for successful transfection of pRES2-OmTRPV4 (Fig. 4, B–D).

**Cellular localization of TRPV4.** Immunohistochemistry on the pituitaries of FW and SW tilapia detected TRPV4 in the RPD, pars intermedia (PI) and neurohypophysis (NH) projecting into the RPD and PI (Fig. 5, A and B). Immunosignals for TRPV4 in the RPD, which is mainly composed of PRL cells, were predominantly observed on the cellular membrane, with no apparent difference in TRPV4 signal intensity between FW and SW fish (Fig. 5, C and D). In the PI, immunoreaction of antitilapia TRPV4 was mainly observed in the cytoplasmic area (Fig. 5, E and F).

**PRL release assay.** Prolactin release from dispersed PRL cells was stimulated in response to hyposmotic (300 mosM) medium; hyposmotically induced PRL release was completely suppressed by treatment with 10 μM RR (Fig. 6). PRL release in hyperosmotic medium, on the other hand, was increased after incubation with 1 μM of 4aPDD.

**DISCUSSION**

In the current study, we have identified the full length of tilapia TRPV4 cDNA sequence from the cDNA library obtained from FW tilapia RPDs. We have also searched for the expression of other TRPVs in the RPD of FW tilapia but only found expression of TRPV4. Topology prediction and motif analysis indicated that tilapia TRPV4 shared known characteristic features of group 1 TRP subfamilies (41). Among these features, ankyrin domains in the NH2-terminal tail and a pore domain located in the long connecting extracellular loop, between the 5th and 6th transmembrane domains, are essential for functional TRPV4 (1, 7, 24). Aspartic acid and methionine residues in the pore domain, important for TRPV4 ion permeability characteristics (42), are found in tilapia TRPV4. In mammalian TRPV4, at least three ankyrin domains exist in the N-tail, and these are involved in oligomerization and membrane recruitment of TRPV4 (1, 2, 9). According to motif search, tilapia TRPV4 contains at least five predicted ankyrin domains in the N-tail, and all domains at important positions in mammalian homologues are highly conserved. These results suggest that tilapia TRPV4 is a functional cation channel in organismic conditions and works properly in mammalian cell.

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**Fig. 4.** Specificity confirmation of the antibody. A: Western blot analysis with an antibody raised against a synthetic peptide corresponding to a part of the COOH-terminus of tilapia TRPV4. The protein sample from the pituitary of FW tilapia was separated by SDS-PAGE and transferred to a membrane. Positions for molecular mass markers are shown on the left. A single protein band with molecular mass of 98 kDa was detected. B–D: Immunocytochemical detection of tilapia TRPV4 in the CHO-K1 cells transiently expressing DsRed Express. A, phase contrast; B, DsRed-positive cells (arrowheads); D, TRPV4 immunosignals were observed only in the DsRed-positive (arrowheads) cells (D). Bar: 10 μm.

**Fig. 5.** Immunohistochemical detection for TRPV4 in the pituitary of FW- and SW-adapted tilapia. A and B: immunoreaction for TRPV4 in the pituitary from FW (A) and SW (B) tilapia. TRPV4 immunoreaction was observed in the rostral pars distalis (RPD), neurohypophysis (NH), and pars intermedia (PI), whereas almost no immunosignal was detected in the proximal pars distalis (PPD). Bar, 100 μm. C and D: magnified images for TRPV4 immunoreaction in the RPD of FW (C) and SW (D) fish. The TRPV4 signals were constant with environmental salinity and were localized in the cell membrane region of the RPD. Arrowheads indicate representative membrane-localized TRPV4 immunosignals. Bar, 5 μm. E and F: magnified images for TRPV4 localization in the PI of FW (E) and SW (F) fish. Immunoreaction for TRPV4 was observed in the intracellular area of PI. Bar, 10 μm.

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culture systems. Using a functional assay for tilapia TRPV4 expressed in CHO-K1 cells, we revealed that tilapia TRPV4 enables cells to respond to a decrease in extracellular osmolality by increasing intracellular Ca\(^{2+}\). In this study, the function of tilapia TRPV4 was completely blocked by RR and stimulated by 4aPDD. Since RR and 4aPDD are known to inhibit the Ca\(^{2+}\)-dependency of 4aPDD-induced increases in intracellular Ca\(^{2+}\). Our findings indicate that tilapia TRPV4 is a Ca\(^{2+}\) channel that transports extracellular Ca\(^{2+}\) into the cell in response to a decrease in extracellular osmolality.

The tissue distribution analysis revealed that TRPV4 expression was ubiquitously present in tilapia acclimated to FW and SW. Similarly, in mammalian species, TRPV4 is expressed in a broad range of tissues (24). The TRPV4 is considered to be a multifunctional sensor for osmolality, chemicals, pressure, and temperature (24, 25). Auer-Grumbach et al. (2) reported that mutations in the TRPV4 gene cause a disorder of the peripheral nervous system. Taken together, these findings suggest that tilapia TRPV4 is involved in the transduction of stimuli caused by various environmental inputs, not only in the central nervous system but also in peripheral organs. TRPV4 expression was also observed in the osmoregulatory organs of tilapia. Recently, TRPV4 was identified in European sea bass (Dicentrarchus labrax), and it was reported that its expression levels in the gills were increased after transfer from SW to FW (5). Although we did not quantify TRPV4 expression levels in the gills of FW- and SW-acclimated tilapia, it is suggested that TRPV4 is involved in a branchial function, presumably FW adaptation, through its mechanosensing properties. In the mammalian kidney, TRPV4 is likely to mediate sensing of tubular flow and osmosensing of the ascending loop of Henle’s interstitium and collecting duct (8, 16). Despite limited information available on the three-dimensional, spatial structure of the renal tubule in teleosts (38), TRPV4 in fish may also play a role in kidney osmoregulation. The function of TRPV4 in fish peripheral osmoregulatory organs needs to be clarified in future studies.

Further examination of TRPV4 transcripts in the RPD by quantitative RT-PCR showed that the expression of TRPV4 in RPDs of SW tilapia was approximately twofold higher than that in RPDs of FW fish, whereas PRL expression in the RPD is remarkably lower in SW fish than in FW fish (43). Whereas those responses appear to be contradictory, lower TRPV4 expression in the FW RPD could be explained by the relationship between intracellular cation homeostasis, especially Ca\(^{2+}\) fluxes, and cell death. It is known that intracellular Ca\(^{2+}\) overload caused by receptor overstimulation and cytotoxic agents induces cell death (28). Recently, it has been reported that TRPV4 is likely involved in Ca\(^{2+}\) overload-induced cell death caused by chronic intraocular pressure increase (29). Moreover, osmosensitivity of FW PRL cells is enhanced by abundant AQP3 compared with SW PRL cells (43). These findings suggest that overabundant TRPV4 expression in RPL cells of FW tilapia might lead to cell death by excessive Ca\(^{2+}\) influx following chronic hypomotic stress. Conversely, in SW, when PRL cells are infrequently exposed to hypomotic extracellular environments, Ca\(^{2+}\) influx by TRPV4 activation would rarely occur. Although we cannot fully account for the high expression levels of TRPV4 in the RPD of SW fish, our findings imply that there is a regulatory mechanism for TRPV4 expression based on the frequency of Ca\(^{2+}\) influx in PRL cells which in turn may be directly sensitive to extracellular osmolality. Whether TRPV4 expression is directly sensitive to Ca\(^{2+}\) influx and changes in extracellular osmolality, however, remains to be determined.

Previous studies have indicated that the cells expressing alternatively spliced TRPV4 show unresponsiveness to hypotonic stimuli due to a lack of its multimerization and membrane localization (1). To determine the subcellular localization of TRPV4 in PRL cells, a specific antibody was raised against tilapia TRPV4. The result of Western blot analysis with the antibody showed a band of 98 kDa, which corresponds to the predicted mass of tilapia TRPV4, suggesting that the majority of TRPV4 in the pituitary of FW tilapia was not glycosylated. We have also confirmed the specificity of the antibody by immunocytochemistry in tilapia TRPV4-expressing mammalian cells used for the functional assay. All our findings indicate that the antibody we obtained specifically recognized TRPV4 in the pituitary. Our immunohistochemical study further revealed that TRPV4 was localized in the RPD, NH, and PI of the tilapia pituitary. Intense immunofluorescence was observed in NH projections into the RPD and PI regions in both FW and SW fish, suggesting that there could be unknown environmental stimulus-induced controls to these areas via neuroendocrine factors, such as prolactin-releasing peptide. Almost no TRPV4 immunosignal was observed in the PPD, which consists of growth hormone (GH)- and gonadotropin-producing cells. The absence of TRPV4 in the PPD is consis-

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**Fig. 6.** Effects of hyposmotic (300 mosM) media, RR, and 4aPDD on PRL release from dispersed PRL cells of FW tilapia. Values are standardized by the mean of hyposmotic (355 mosM) medium control group (hyper). An increase in PRL release was observed in hyposmotic medium (hypo) and hyperosmotic medium containing 1 μM 4aPDD (hyper+4aPDD). Hyposmolality induced PRL increase was blocked by 10 μM RR treatment (hyper+RR). Data are expressed as means ± SE; n = 10–12. Different letters indicate significance of difference at P < 0.05 by Dunn’s test.
tent with a previous study that reports lower osmosensitivity of GH release from dispersed PPD cells (36). Further observations showed that TRPV4 was localized in the cell membrane area of the RPD cells and in the cytoplasm of the PI cells. Membrane trafficking of TRPV4 is one of the most important factors for its responsiveness to a decrease in extracellular osmolality (1, 2, 9, 46). Therefore, the existence of membrane-localized TRPV4 in the RPD provides strong evidence of its role in PRL cell osmoreception as the mediator of hyposmotically induced intracellular Ca\(^{2+}\) increase. Conversely, cytoplasmic TRPV4 observed in the PI, presumably produced due to alternative splicing, is unlikely to be involved in osmoreception-related physiological events. According to a report by Xu and coworkers (46), N-linked glycosylation at the pore-forming loop in murine TRPV4 is involved in its membrane translocation in tilapia, posttranscriptional TRPV4 regulation might be important and needs to be examined in future studies. These results obtained from PRL cell cultures in vitro are consistent with the molecular characteristics and subcellular localization of tilapia TRPV4.

**Perspectives and Significance**

According to previous and present results, we conclude that TRPV4 is the molecule that links the hyposmotically induced increase in cell volume to a rise in intracellular Ca\(^{2+}\) levels, which ultimately leads to a rise in PRL release. At the organismal level, the TRPV4-dependent mechanism for osmoreception present in tilapia PRL cells is most likely to provide the basis for the environmental control of PRL secretion, which in turn is essential to maintain osmotic homeostasis in FW. The cellular osmoreponsiveness mediated by TRPV4, ubiquitously expressed in tilapia, may be a versatile osmosensing mechanism in fish, and further studies in the central nervous system will provide significant information for clarifying osmosensory neurons that primarily govern the osmoregulatory functions in the teleosts.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: S.W. conception and design of research; S.W. and A.P.S. performed experiments; S.W. and A.P.S. analyzed data; S.W., A.P.S., E.G.G., and T.K. interpreted results of experiments; S.W. prepared figures; S.W., A.P.S., E.G.G., and T.K. edited and revised manuscript; S.W., A.P.S., E.G.G., and T.K. approved final version of manuscript.

**REFERENCES**


