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

Soichi Watanabe,1 Andre P. Seale,2 E. Gordon Grau,2 and Toyoji Kaneko1

1Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo, Tokyo, Japan; and 2Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, Hawaii

Submitted 17 November 2011; accepted in final form 25 February 2012

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 4α-phorbol 12,13-didecanoate (4αPDD). 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 4αPDD. These results indicate that PRL release in response to physiologically relevant changes in extracellular osmolality is mediated by the osmotically sensitive TRPV4 cation channel.

stretch-activated cation channel TRPV4; osmoregulation; 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 hyposmotic environments. The Mozambique tilapia possesses two PRL isoforms, PRL188 and PRL177, both of which exert hypomembranitory 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 hypomembranitory 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 hypomembranitory 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 hypomembranitory 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 hypomembranitory 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 trisphosphate- 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 hyposmotically 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 homogeneous tissue in the RPD (26). Total RNA was extracted from the RPD with RNA extraction solution (ISOGEN, Nippon Gene, Tokyo, 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.

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, GAYTGGGCITAYG-GNCCNGT) and antisense (TRPV4-dr, TGICCNACTYTCNC-CCAT) primers in a reaction mixture for Taq 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, ACNGARTGGYNTAYGG and CAT-ICCDATGYDWAYTTRA; for TRPV3, TRPV4-df and ATNACYTTYGDTACATNC; and for TRPV5/6, TAYGAYYTIACN- GARATHGA and GCICCICATRYTSRAANCC. 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 Sequerencer 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, TCACCTGGTGCTGCTACTC), and an adaptor primer (NUP, CTAATACGACTCACTATAGG) 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, GTATCCAGTGAAGAGTGATG) 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 (TGGAGATCTGGGGATATGAG) and TRPV4-BglII-r (TCAAGATCTGGGGAATCG) in a reaction mixture of KOD plus DNA polymerase (Toyobo, Tokyo, Japan) and ligated into pBluescript II SK(−). The TRPV4 ORF was digested by BglII (Takara) and subcloned into the BglII digestion site of an expression vector, pRES2-DsRed Express (Clontech). The resulting construct (pRES2-DsTRPV4) was sequenced to confirm that appropriate manipulations were made. pRES2-DsTRPV4 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 experiments. The efficacy 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-didecanoate (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. pRES2-TRPV4-transfected cells were incubated with 5 μM of Fluo-8AM (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 isosmotic 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 isosmotic HBSS at 25°C, and then experimental media (hyposmotic HBSS with or without RR, hyposmotic HBSS without Ca$^{2+}$, and isosmotic 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 hyposmotic 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,
molecule, 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 (AGTGGACCCATCAATGAG) and TRPV4-qr (TGGGTATGTGGGTATGGAG) 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 manufacturer’s instructions. qRT-PCR for TRPV4 was performed using the StepOnePlus real-time PCR system (Life Technologies), and reactions were set up as previously described (28a). All quantitative data were expressed in arbitrary units after normalization to the expression levels of elongation factor 1α (EF1a), after it was confirmed that there was no salinity effect upon its expression. The primers used for for EF1a 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, ELSKGQSQSDH). 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 8.6; 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 14,000 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 from the gel to a polyvinyl difluoride membrane (Immobilon-P (SDS-PAGE). After electrophoresis, the protein was transferred to 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 observed from the cDNA of FW tilapia RPD (data not shown).

**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 (DDBJ/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 pIRES2-OmTRPV4 (Fig. 4B–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. 5A 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. 5C and D). In the PI, immunoreaction of antitilapia TRPV4 was mainly observed in the cytoplasmic area (Fig. 5E 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 N-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). 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 shares 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
TRPV4 are homologous to those of mammalian TRPV4. These interact and activate, respectively, mammalian TRPV4, our results indicated by 4aPDD. Since RR and 4aPDD are known to inhibit activity of tilapia TRPV4 was completely blocked by RR and stimulation by increasing intracellular Ca\(^{2+}\). The function of tilapia TRPV4 during osmoreception, a finding that 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.

### Western Blot Analysis

Previous studies have indicated that the cells expressing alternatively spliced TRPV4 show unresponsiveness to hypertonic 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 indicated that the antibody we obtained specifically recognized TRPV4 in the tilapia 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-
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 recruitment. Although there is no clear evidence for the relationship among alternative splicing, glycosylation, and TRPV4 recruitment, posttranscriptional TRPV4 regulation forming loop in murine TRPV4 is involved in its membrane localization. Xu and coworkers (46), N-linked glycosylation at the pore-forming loop in murine TRPV4 is involved in its membrane recruitment. Although there is no clear evidence for the relationship among alternative splicing, glycosylation, and TRPV4 recruitment, posttranscriptional TRPV4 regulation might be important and needs to be examined in future studies. The pharmacological assay with dispersed PRL cells from FW fish clearly showed that hyposmotically induced PRL release was blocked by treatment with an inhibitor of TRPVs. Furthermore, PRL release was increased by a TRPV4 activator independently of medium osmolality. These results obtained from PRL cell cultures in vitro are consistent with a previous study that reports lower osmosensitivity of 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 telencephalon.

**ACKNOWLEDGMENTS**

We are grateful to Prof. Tetsuya Hirano for invaluable advise and discussions. Benjamin Moorman and Jacob Stagg provided helpful assistance during the study.

**GRANTS**

This work was supported by Grants-in-Aid for Scientific Research nos. 21780174 and 22248021 from Japan Society for the Promotion of Science, and grants from the National Science Foundation (IOB05-17769, OISE08-52518, and IOS-1119693).

**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. drafted manuscript; 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**

