A role for AQP5 in activation of TRPV4 by hypotonicity: concerted involvement of AQP5 and TRPV4 in regulation of cell volume recovery

Xibao Liu1, Bidhan Bandyopadhyay1, Tetsuji Nakamoto2, Brij Singh3, Wolfgang Liedtke4, James E. Melvin2, and Indu Ambudkar1

1Secretory Physiology Section, GTTB, NIDCR, NIH, Bethesda, MD; 2Center for Oral Biology, University of Rochester, Rochester, NY; 3Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, ND; 4Center for Translational Neuroscience, Duke University Medical Center, Durham, NC

Running Title: **TRPV4-AQP5 signaling complex regulates RVD**

Address correspondence to: Indu S. Ambudkar, Ph.D. Secretory Physiology Section, Gene Therapy and Therapeutics Branch, NIDCR, Building 10, Room 1N-113; NIH, Bethesda MD 20892. Phone: 301-496-1478, Fax: 301-402-1228, Email: indu.ambudkar@nih.gov

Regulation of cell volume in response to changes in osmolarity is critical for cell function and survival. However, the molecular basis of osmosensation and regulation of cell volume are not clearly understood. We have examined the mechanism of regulatory volume decrease (RVD) in salivary gland cells and report a novel association between osmosensing transient receptor potential vanilloid 4 (TRPV4) and aquaporin 5 (AQP5) which is required for regulating water permeability and cell volume. Exposure of salivary gland cells and acini to hypotonicity elicited increase in cell volume and activation of RVD. Hypotonicity also activated Ca\(^{2+}\) entry which was required for subsequent RVD. Ca\(^{2+}\) entry was associated with a distinct non-selective cation current that was activated by 4αPDD and inhibited by ruthenium red, suggesting involvement of TRPV4. Consistent with this, endogenous TRPV4 was detected in cells and in the apical region of acini along AQP5. Importantly, acinar cells from mice lacking either TRPV4 or AQP5 displayed greatly reduced Ca\(^{2+}\) entry and loss of RVD in response to hypotonicity although the extent of cell swelling was similar. Expression of N-terminal-deleted AQP5 suppressed TRPV4-activation and RVD, but not cell swelling. Furthermore, hypotonicity increased the association and surface expression of AQP5 and TRPV4. Both these effects, and RVD, were reduced by actin depolymerization. These data demonstrate that (i) activation of TRPV4 by hypotonicity depends on AQP5, not on cell swelling per se, and (ii) TRPV4 and AQP5 concertedly control regulatory volume decrease. These data suggest a potentially important role for TRPV4 in salivary gland function.

Introduction

The ability of cells to regulate their volume is essential for maintenance of cellular homeostasis under anisotonic environmental conditions (1-3). Changes in osmolarity of the extracellular medium induce water fluxes that result in swelling or shrinkage of cells depending on the osmotic gradient. Most cells respond to changes in tonicity and cell volume by initiating mechanisms which allow them to recover their original volume in the continued presence of the osmotic stress. Such regulatory volume changes depend on the activation of cation and anion permeabilities which reverse the osmotic gradient and direction of water flow (1-4). Emerging studies demonstrate that regulatory volume
changes are critical for cell survival and also for regulation of cellular processes such as gene transcription and proliferation (1). In addition, a variety of cells, such as exocrine gland cells, utilize the mechanism of regulatory volume change to drive fluid secretion (5). Several monovalent cation and anion channels as well as intracellular Ca\(^{2+}\) changes contribute to cell volume regulation. For example hypo-osmolarity-induced cell swelling has been associated with a rise in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in different cell types which is due to hypotonicity-activated Ca\(^{2+}\) entry pathways. It has also been clearly demonstrated that this Ca\(^{2+}\) entry is critical for regulating the ion fluxes which drive volume decrease (1-4). However, the underlying mechanism(s) that senses the change in osmolarity and /or cell volume to initiate volume regulation is poorly understood (5).

Regulation of transepithelial osmotic forces as well as cell volume critically impacts salivary gland fluid secretion induced by neurotransmitter-stimulation of the gland (5). The water channel, aquaporin 5 (AQP5) provides a regulated water permeability across the apical membrane of salivary gland acinar cells which is important not only for fluid secretion but also for regulatory volume changes (5-7). Aqp5-/- mouse salivary glands show decreased salivary secretion in response to muscarinic receptor stimulation. Additionally, dispersed salivary gland acini from these mice have reduced ability to control volume changes in response to hyper- or hyposmotic solutions (7). Thus fluid secretion and cell volume regulation converge at the level of AQP5, which mediates the final step in both processes, i.e. water efflux. However, it is not clear how cells sense the change in osmolarity and how this signal is transduced to achieve volume regulation.

As discussed above, hypotonic and hypertonic conditions induce [Ca\(^{2+}\)]\(_{i}\) increases in different cell types and Ca\(^{2+}\) entry has been reported to be critical for the volume recovery process (1-4). Neurotransmitter-stimulated Ca\(^{2+}\)-mobilization events, including intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry, as well as Ca\(^{2+}\)-dependent activation of cation and anion channels have been quite extensively studied in salivary and other exocrine gland cells (5,8). Further, increases in cytosolic Ca\(^{2+}\) due to Ca\(^{2+}\) entry have been correlated with ion channel activation and fluid secretion. However, the Ca\(^{2+}\) entry mechanism(s) involved in the cellular response to anisosmotic conditions has not yet been identified (5). The transient receptor potential vanilloid 4 (TRPV4), a member of the TRP super family of cation channels (9) has been shown to be activated by hypotonicity and a variety of other stimuli (10-15). This channel is expressed in several cell types and has been reported to be involved in RVD in airway epithelial and keratinocyte cell lines (16, 17). Trpv4-/- mice display impaired regulation of systemic tonicity (18) although exactly how TRPV4 activity leads to regulation of cell volume has not yet been directly demonstrated. The molecular mechanism by which tonicity changes regulate TRPV4 has also not yet been established (11, 12, 14, 16-18).

This study was directed towards defining the molecular basis of RVD. Towards this we have measured the response of salivary gland cell lines, primary cultures, and dispersed salivary gland acini to hypotonicity. These cells have been previously shown to display robust volume changes as well as efficient volume recovery in response to anisosmotic conditions (5, 7). The exact molecular events involved in these responses are not yet known. The data presented here demonstrate a novel association between TRPV4 and AQP5 which controls RVD in salivary gland cells. We show that AQP5 is required for the activation of TRPV4 by hypotonicity. Further, AQP5
and TRPV4 are concertedly involved in regulating recovery of cell volume.

**Experimental Procedures**

**Cell Culture and Transfection.** Cells were cultured in Dulbecco modified Eagle’s medium (DMEM, rat basophilic leukemia cells, RBL-2H3), Earle’s minimal essential medium (EMEM, HSG, human submandibular gland, and HSY, human parotid gland cells) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% penicillin/ streptomycin at 37°C in 5% CO2. HSG cells were transiently transfected with 1 µg of required plasmids and 0.2 µg GFP-encoding plasmid or only with GFP-plasmid using Lipofect-AMINE reagent 2000 (Invitrogen).

**Dispersed cell preparation from mouse parotid and submandibular gland cells.** All mice were maintained according to guidelines approved by the NIDCR, NIH Animal Care and Use Committee. Submandibular glands were removed, cleaned, minced and digested in standard external solution (SES) containing (mM): NaCl 145; KCl 5; MgCl2 1; CaCl2 1; Heps 10; Glucose 10; pH 7.4 (NaOH) with 0.02% soybean trypsin inhibitor and 0.1% bovine serum albumin containing collagenase P (2.5 mg/8 ml) (7). After 15 to 20 min incubation at 37°C the digest was washed twice with the normal external solution and resuspended in external solution.

**Electrophysiological Recording.** The patch pipette had resistances between 3–5 MΩ after filling with the standard intracellular solution that contained (mM): Cs methane-sulfonate 145; NaCl 8; MgCl2 10; HEPES 10; EGTA 10; pH 7.2 (CsOH). External solutions were composed as follows (mM). **Ca2+ & Na+ solution:** NaCl 145; CsCl 5; MgCl2 1; CaCl2 10; HEPES 10; glucose 10; pH 7.4 (NaOH). **NMDG solution:** NMDG 170; CsCl 5; MgCl2 1; HEPES 10; glucose 10; pH 7.4 (HCl). Osmolarity for all solutions was adjusted with d-mannitol to 305 ± 5 mmol/kg using Vapor Pressure Osmometer (Wescor). For measuring swelling-activated currents, an isotonic solution containing 75 mM NaCl, 6 mM CsCl, 5 mM CaCl2, 1 mM MgCl2, 10 mM Heps, 150 mM d-mannitol, and 10 mM glucose, pH 7.4 with NaOH (305 ± 5 mmol/kg) was used. Cell swelling was induced by omitting mannitol from this solution to reach proper osmolarity. In some experiments the reduction of osmolarity was achieved by reduction of NaCl from normal external solution where indicated.

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at room temperature (22–25°C) using Axopatch 200B amplifier (Axon Instrument) as described earlier (19, 20, 21). Voltage ramps ranging from –90 to 90 mV over a period of 1 s, were imposed every 4 s from a holding potential of 0 mV, and digitized at a rate of 1 kHz. A liquid-junction potential of less than 8 mV was not corrected, capacitative currents and series resistance were determined and minimized. For analysis, the first ramp was used for leak subtraction for the subsequent current records. There was no significant increase in the current under these conditions unless cells were exposed to HTS.

**Measurement of Intracellular Ca2+ Concentration.** Freshly isolated salivary gland cells were loaded with fura 2 for 45-60 min at 30°C and allowed to attach to glass bottom dish (Matek Corporation). Ducts and acinar cells were morphologically identified (7). Other cells were cultured overnight in Matek dishes. Fluorescence measurements were made using a Till Photonics- Polychrome IV spectrofluorimeter attached to a Olympus X51 microscope and Metafluor Imaging System, Universal Imaging Corporation (21). Fluorescence traces shown represent [Ca2+]i.
(values are averages from >50 cells and representative of results were obtained in at least 3-5 individual experiments).

**Measurement of Cell Volume.** Cells were loaded with the fluoroprobe calcein (Molecular Probes, Eugene, OR) and excited at 490 nm. Emitted fluorescence was measured at 510 nm. In situ calibration of the dye was performed. The relationship between dye fluorescence and the volume change was linear over a volume range from +35% to −35%. In some experiments, cell volume was estimated using an Olympus X51 microscope interfaced with Universal Imaging MetaMorph software. Data are presented as mean ±SEM. Origin 7.5 (OriginLab Northampton, MA) was used for data analysis and display. Significant difference between individual groups was tested by using ANOVA.

**Immunoprecipitation and Immunoblotting.** Immunoprecipitation was done using solubilized crude membranes or from cell lysates as previously described (21). Pre-cleared lysates were incubated with the required antibody (1:200 dilution of anti-TRPV4 or anti-AQP5). Interacting proteins bound to sepharose beads were separated, released with SDS-PAGE sample buffer and detected by Western blotting as described previously using anti-TRPV4 (1:500 dilution) or anti-AQP5 antibody (1:1000 dilution).

**Immunocytochemistry.** Parotid glands were excised from the animals and fixed in 10% formalin solution, embedded in paraffin and used to prepare 5-10-µm sections (American Histolabs., Gaithersburg, MD). Sections were dewaxed, rehydrated, and permeabilized with 0.5 % Triton / PBS pH 7.5. Streptavidin-peroxidase reactions using DAB Histostain kit (Zymed Laboratories, San Francisco, CA) was used to detect specific proteins (anti-TRPV4 at 1: 70 and anti AQP5 at 1: 100 dilutions). In control sections rabbit IgG was used instead of primary antibody.

**Surface biotinylation.** Cells were treated as required and incubated with 0.5 mg/ml Sulfo-NHS-Biotin (Pierce) on ice (21), washed with buffer containing 0.1M glycine, and solubilized with 2 ml of cell lysis buffer containing NP-40, 0.1% SDS, and proteolytic inhibitors. Biotinylated proteins were pulled down with Neutr-Avidin-linked beads (Pierce). Bound fraction was washed and released with SDS-PAGE sample buffer and analyzed by western blotting.

**Results**

**Hypotonicity stimulates changes in cell volume and Ca\(^{2+}\) entry via a TRPV4-like channel in salivary gland cells.** Figure 1 A shows that hypotonic external solution (HTS) induced sustained increase in [Ca\(^{2+}\)], in HSG (human submandibular gland cell line) cells, which was dependent on the osmolarity. Average data representing peak increase in fluorescence from these experiments are shown in Figure 1B (significant increases above resting [Ca\(^{2+}\)], are indicated). Primary cultures of human submandibular gland cells (22) but not rat basophilic leukemia (RBL) cells, displayed a similar response to HTS (Figure 1C, 150 mmol/kg solution was used here and in subsequent experiments). The sustained [Ca\(^{2+}\)] increase in HSG cells was blocked by 100 µM but not 1 µM Gd\(^{3+}\) (Figure 1D), indicating that HTS-stimulated Ca\(^{2+}\) entry is unlike store-operated Ca\(^{2+}\) entry, which is blocked by 1 µM Gd\(^{3+}\) in these cells (19). This was further confirmed by whole cell current recordings. HTS increased HSG cell membrane conductance with a 25 to 40 s delay (Figure 1 E) and generated a weakly outwardly rectifying current which reversed at +6 ±3 mV (Figure 1F, HTS-stimulated [Ca\(^{2+}\)] increase and cation currents were seen in 91% (20/22) of cells). With NMDG as the cation in
the external solution (Figure 1G and H), inward current was greatly reduced, outward current remained relatively unchanged, and reversal potential showed a left-shift (this effect was seen in all the cells that displayed response to HTS). Together these data demonstrate that HTS stimulates a non-selective Ca$^{2+}$-permeable cation conductance that is distinct from the relatively inwardly rectifying store-operated Ca$^{2+}$ current I$\text{SOC}$ previously described in HSG cells (19, 20). However, the characteristics of the HTS-stimulated cation current in HSG cells are similar to HTS-activated TRPV4 currents in other cell types (14, 23, 24).

We therefore examined the possibility that TRPV4 contributes to HTS-induced Ca$^{2+}$ entry. 1µM Ruthenium red (RuR), a concentration used for maximum inhibition of TRPV4 currents (23, 25), substantially blocked both inward and outward currents stimulated by HTS (Figure 2A). Although the current decayed relatively fast in the control condition (see Figure 1E), the decay was faster when RuR was added to cells after the current developed (Figure 2A). Furthermore, washout of RuR induced a significant (p<0.05) recovery (41±6%, n=4) of the current to a value not significantly different from the current in control cells. This inhibitory effect of RuR can be more clearly seen in the I-V curves shown in Figure 2B (inhibition of inward (>90%) and outward (50-60%) currents, p<0.05, n=4). Although the outward current in RuR-treated cells was also decreased compared to that in the untreated cells, at membrane potentials >+70 mV, there was a relative increase in the current (Figure 2B). Thus, the outward current appears to be less sensitive to RuR, there was larger current at the positive membrane potentials relative to that at more negative potentials, consistent with the RuR block being voltage-dependent (23-25). Further 4α-PDD, an activator of TRPV4 (12, 14, 16, 23-25) induced a sustained increase in [Ca$^{2+}$], in Ca$^{2+}$ containing medium (Figure 2C), but not in Ca$^{2+}$-free medium (data not shown), which was attenuated by pre-exposure of the cells to HTS (Figure 2D). 4α-PDD-induced membrane conductance was similar to that induced by HTS (Figure 2E and F). In a number of previous studies 4αPDD activation of endogenous TRPV4 generated currents similar to those seen in HSG cells i.e., relatively more linear and somewhat outwardly rectifying (14, 23, 26-28). However, in other studies 4αPDD stimulation of exogenously expressed TRPV4 generated currents that displayed marked double rectification (12, 14, 23, 29). It should be noted that although HTS and 4α-PDD are efficient activators of TRPV4, they activate the channel via distinct mechanisms (12, 14). Consistent with HTS-activation of Ca$^{2+}$ entry, TRPV4 expression was detected in HSG cells but not in RBL cells (Figure 2G, membranes from brain and MDCK cells were used as controls). In aggregate, the data in Figure 2 suggest that TRPV4 might be involved in HTS induced Ca$^{2+}$ entry in salivary epithelial cells.

### Ca$^{2+}$ entry is required for regulatory volume decrease.

The role of Ca$^{2+}$ entry in the cellular response to HTS was further determined by measuring volume changes. HTS induced an immediate relatively slow increase in cell volume which was maintained for over a minute and then decreased to a steady state volume similar to that in resting cells (Figure 3A, the data calculated based on the area of the cell). Comparable results were obtained using calcein fluorescence measurements (Figure 3B). The decrease in cell volume but not the initial increase was attenuated by removal of external Ca$^{2+}$ and or addition of 100 µM Gd$^{3+}$ (Figure 3C and D, respectively). These data strongly suggest that Ca$^{2+}$ entry triggered by HTS regulates RVD. Cell volume regulation is driven by osmotic gradients generated via activation of
K+ and Cl- channels (3, 5). In HSG cells, HTS stimulated a charybdotoxin-sensitive KCa channel and an as yet unidentified Cl- channel that was blocked by niflumic acid, a relatively non-specific blocker of Cl- channels (Online Supplemental Figure 1). We suggest that the K+ channel is activated via TRPV4-mediated Ca2+ entry and contributes to RVD. The regulation of the Cl- channel is yet to be determined. Additionally, HTS-induced TRPV4 currents were not altered by inclusion of niflumic acid in the bath suggesting that there is minimal contribution of the Cl- current to the TRPV4 current. Note that K+ currents are abolished under the conditions used to measure TRPV4 currents.

**Involvement of TRPV4 in hypotonicity-stimulated Ca2+ entry and RVD.** To conclusively establish the involvement of TRPV4 in salivary cell volume regulation, we used dispersed submandibular gland acinar cells from *TrpV4+*/+ and *TrpV4−*/− mice (18). Robust [Ca2+]i increase was stimulated by HTS in cells from control mice (Figure 4A). This HTS-induced [Ca2+]i increase was >70% lower in cells isolated from TRPV4 null animals (Figure 4 B and 4C for average values). Similar loss of 4α-PDD-induced TRPV4 activity in cells from *TrpV4−*/− mice has been previously shown (14, 23). HTS-stimulated cation current was also inhibited >70% (Figure 4 D, E and F). Thus, TRPV4 contributes to HTS- and 4α-PDD-induced [Ca2+]i increase and cation currents in submandibular gland acinar cells. Importantly, cells lacking TRPV4 displayed lack of RVD response in hypotonic conditions, note that cell swelling was not altered (Figure 4G). A key molecular component required for the RVD response to HTS in salivary acinar cells is AQP5 (7). Figure 4H shows that TRPV4 expression is lost in submandibular glands from *TrpV4−*/− mice but that of AQP5 is not altered. These data provide strong evidence that TRPV4-mediated Ca2+ entry is required for AQP5-dependent RVD. The relatively low level of HTS-stimulated, non-TRPV4 dependent, Ca2+ entry detected in *TrpV4−*/− cells does not support RVD.

**Requirement of AQP5 for activation of TRPV4 by hypotonicity.** The functional association between TRPV4 and AQP5 was further assessed by examining the response of salivary gland acinar cells from *Aqp5−*/− and *Aqp5+*/+ mice to HTS (7). A major, and somewhat unexpected, finding was that HTS-stimulated Ca2+ entry was significantly decreased in submandibular gland acini and almost completely abolished in parotid acini isolated from *Aqp5−*/− mice (Figure 5A and B, respectively; see Figure 5C for average data obtained from parotid gland cells). 4α-PDD induced Ca2+ entry was about 50% lower than HTS-induced Ca2+ entry in *Aqp5+*/+ cells and was not significantly different from 4α-PDD induced Ca2+ entry in *Aqp5−*/− cells (data from parotid glands are shown in Figure 5C). Consistent with these findings, HTS-stimulated current was almost completely abolished in parotid acini (Figure 5D and E, >90% decrease at −80 and +80 mV, respectively, n=4). AQP5 was expressed only in glands from *Aqp5+*/+ mice while TRPV4 expression was similar in control and *Aqp5* null mice (Figure 5F). Thus, changes in TRPV4 expression do not account for the loss of HTS-stimulated Ca2+ entry. Previous studies with parotid and sublingual acini from *Aqp5−*/− mice showed that the magnitude of HTS-induced swelling was similar to that in *Aqp5+*/+ cells, although the rate was slower. Importantly, RVD was almost fully inhibited in these cells (7). We noted a similar decrease in the rate (Figure 5 G) but not in the magnitude of swelling (Figure 5H) in
submandibular gland cells from Aqp5-/- mice. Importantly, RVD was significantly decreased in cells lacking AQP5 (Figure 5I, >80% decrease, p<0.01). The results of cell volume measurements with parotid cells, were similar to those reported earlier (7) and are not shown here. Together these findings suggest that TRPV4-mediated Ca$^{2+}$ entry regulates RVD in salivary gland acinar cells. More interestingly these novel findings demonstrate that AQP5 is required for activation of TRPV4 by hypotonicity rather than swelling per se.

**Association of TRPV4 and AQP5 in salivary gland cells.** Since AQP5 appeared to determine TRPV4 function we examined the localization of AQP5 and TRPV4 in mouse submandibular gland. TRPV4 (Figure 6A right panel) was localized in the apical region of mouse submandibular gland acinar cells, as was AQP5 (Figure 6A, left panel, also reported previously, see references 30-33). Further, immunoprecipitation with anti-AQP5 antibody pulled down AQP5 with TRPV4 and vice-versa (Figure 6B). AQP3 was present in HSG cells but did not co-immunoprecipitate with TRPV4 (AQP1 and AQP8 were not detected in HSG cells, data not shown). Neither conventional (assessing N and C-terminal interactions between the two proteins) nor split-ubiquitin membrane-based (using the full-length proteins) yeast two hybrid analysis techniques revealed significant interaction between the two proteins (data not shown). Thus, although TRPV4 and AQP5 are associated with each other, it is likely that they do not directly interact with each other. Alternatively, other regulatory mechanisms govern their interaction under hyposomotic conditions.

**Effect of N or C-terminal deletion of AQP5 on hypotonicity-induced TRPV4 activation and RVD.** To further characterize the regulation of TRPV4 by AQP5, we utilized constructs of AQP5 in which either the C- or N-terminus of AQP5 has been deleted (34). Expression of N-terminal deleted AQP5 (Figure 6D-F) but not wild type AQP5 or C-terminal deleted AQP5 (Figure 6C) blocked HTS-induced, but not 4α-PDD- or thapsigargin-induced (data not shown), Ca$^{2+}$ entry and HTS-stimulated cation currents. Importantly, as seen in cells lacking AQP5, the extent of HTS-induced swelling was not altered although RVD was significantly reduced (Figure 6D). C-terminal deletion of AQP5 has been shown to disrupt its trafficking to the apical membrane, resulting in retention of the protein in the cell while N-terminal deletion does not affect its intracellular targeting (34). Based on the effects of these mutant AQP5 proteins on the response of cells to HTS, it seems likely that AQP5 lacking the C-terminus does not exert any effect since it does not interact with endogenous AQP5 or reach the apical membrane. The N-terminal deleted AQP5, on the other hand, induces a dominant negative effect. We suggest that this protein can interact with the endogenous AQP5, but does not form a functional channel. This further indicates that the N-terminus of AQP5 might not be involved in homomeric interactions of AQP5. Additionally, the AQP5 N-terminus might be involved in the HTS-regulation of TRPV4. While the mechanism by which the AQP mutant suppresses HTS-responses has to be further established, these data strongly suggest that TRPV4 and AQP5 are associated functionally and physically (although likely via indirect interactions). These data also reveal a novel regulation of TRPV4 i.e. that channel activation by hypotonicity is dependent on the presence of functional AQP5. These data are also consistent with previous suggestions that TRPV4 is not activated by cell swelling per se (16).

**Involvement of cytoskeleton in activation of TRPV4 and regulatory volume decrease.** AQP5-mediated regulation of cellular volume
during agonist-stimulation of salivary gland cells has been associated with translocation of the water channel protein to the plasma membrane (31, 32). This recruitment depends on \([\text{Ca}^{2+}]_i\) increase and is blocked by depolymerization of actin with cytochalasin D (cyto-D). Additionally TRPV4 has also been shown to interact with the cytoskeleton (35). Thus, we examined the effect of depolymerization of the cytoskeleton on the responses of HSG cells to HTS. HTS- but not 4α-PDD-stimulated \(\text{Ca}^{2+}\) entry in HSG cells was blocked by actin depolymerization (Figure 7A). Interestingly, 4α-PDD induced a transient activation of \(\text{Ca}^{2+}\) entry (compare Figure 7A with Figure 2C) and cation currents (compare Figure 7B with Figure 2E). Furthermore, HTS-induced swelling of HSG cells was not affected by cyto-D but RVD was blocked (Figure 7C) and addition of 4α-PDD to the swollen cells induced a small transient decrease in cell volume consistent with the transient \(\text{Ca}^{2+}\) entry.

**Effect of hypotonicity on cell surface expression and association of TRPV4 and AQP5.** Figures 7D-F show the effect of HTS on the surface expression of TRPV4 and AQP5 in control and cyto-D treated HSG cells. HTS induced a marked increase in the level of AQP5 and TRPV4 in the biotinylated fraction, which was attenuated in cells treated with cyto-D (Figure 7D, plasma membrane \(\text{Ca}^{2+}\) pump in this fraction was not changed, Supplemental Figure 2). Additionally, HTS increased co-immunoprecipitation of TRPV4 with AQP5 which was also blocked by cyto-D pretreatment of cells (Figure 7E, left upper blots). Since anti-AQP5 was used to pull down AQP5, the level of AQP5 is similar in both samples (right panels, which also indicates similar protein load, note that AQP5 and TRPV4 were detected using the same blot). Input levels of TRPV4 and AQP5 in lysates of control and cyto-D-treated cells are shown in Figure 7F (note that these lysates were used for IP with either avidin or anti-AQP5, i.e. blots shown in Figures 7D and E, and thus serve as a control for both). The increased association of TRPV4 and AQP5 was verified using dispersed mouse submandibular gland cells. HTS induced a similar increase in the co-immunoprecipitation of TRPV4 with AQP5 in these cells (Figure 7G, IP was done using anti-AQP5 antibody. Blots show TRPV4 and AQP5 in IP samples in the top panels and cell lysates, input, in the lower panels). An increase was seen only in the levels of TRPV4 in HTS-treated cells. Thus, the effects of HTS and cyto-D on the association of TRPV4 with AQP5 are not due to differences in protein load. In aggregate, these data suggest that HTS (i) increases the association between TRPV4 and AQP5, and (ii) regulates plasma membrane trafficking/insertion of these channels. Cytoskeletal rearrangements appear to be involved in regulating both these events. It is important to note that both AQP5 and TRPV4 have been suggested to be regulated via interactions with the cytoskeleton (31, 32, 34, 35). Further studies are required to determine whether the two proteins associate with each other prior to translocation to the plasma membrane or after they are individually trafficked to the surface membrane.

**Discussion**

The data presented above provide evidence that TRPV4 and AQP5 concertedly control RVD in salivary epithelial cells. Hypotonic conditions induce cell swelling and activation of \(\text{Ca}^{2+}\) entry via TRPV4. Subsequent activation of RVD is dependent on this \(\text{Ca}^{2+}\) entry as well AQP5, which regulates water permeability in these cells. Importantly, we demonstrate that TRPV4 activation by HTS is dependent on the presence of functional AQP5. TRPV4 is not activated by HTS in cells lacking AQP5 even though cells undergo similar magnitude swelling, albeit at a slower
rate, as in control cells. The slower rate of swelling likely reflects lack of contribution by AQP5 to plasma membrane water permeability. Further, we also show that HTS stimulates the association and plasma membrane trafficking of both these channels. Conditions which block these processes also block RVD. Thus, our data reveal for the first time an important functional link between TRPV4 and the water channel AQP5. Despite a number of recent studies which demonstrate the involvement of TRPV4 in cell volume regulation and mechanosensation (10-18), the molecular basis for the regulation of TRPV4 has not yet been established. Further, the exact mechanisms by which AQP5 is regulated is also not clear (5). Our data provide evidence of a novel association between TRPV4 and AQP5 that is involved in activation of TRPV4 by hypotonicity and regulation of cellular response to the osmotic stress. We suggest that TRPV4 and AQP5 are assembled in a signaling complex that responds to anisosmotic conditions and coordinates cellular volume regulation.

Our data also suggest that TRPV4 can have a critical role in salivary gland fluid secretion. A fundamental role for AQP5 in regulation of salivary gland fluid secretion has been shown earlier (7). Aqp5-/- mice display pronounced decreases in salivary fluid secretion and salivary gland cells from these mice exhibit diminished RVD response to HTS (5,7). Thus, it was suggested that AQP5 regulates salivary secretion by controlling the water permeability across salivary acinar cell apical membrane. However, the molecular basis of regulation RVD in salivary gland and other cell types is not yet clear. Although it has been long recognized that regulatory volume changes are determined via regulation of [Ca²⁺], the mechanisms involved in sensing and transducing signals related to changes in the osmolarity of the cell medium have not yet been determined. Our data clearly demonstrate that, like AQP5, TRPV4 has a central role in regulating RVD in salivary epithelial cells. TRPV4 channels are activated in response to hypotonicity in freshly dispersed salivary gland cells and salivary cell lines. Further, the Ca²⁺ entry via TRPV4 is required for activation of a Ca²⁺-activated K⁺ channel and subsequent RVD. Importantly, we show that dispersed salivary gland cells from TrpV4-/- mice (18) display decreased responses to HTS; Ca²⁺ entry is greatly reduced and the ability to regulate cell volume after swelling is lost. These data demonstrate that TRPV4 contributes to HTS-activated Ca²⁺ entry and regulates RVD via activation of K⁺ channel. Together with an as yet unknown Cl⁻ channel, these K⁺ and Cl⁻ fluxes generate the osmotic gradient that drives water flow, via AQP5, to induce shrinkage of cells to their original volume. Thus, TRPV4 has a critical role in the regulation of RVD. Since regulation of transepithelial osmotic forces as well as cell volume critically impacts salivary gland fluid secretion TRPV4 is likely to have a central role in regulating salivary gland function. This remains to be confirmed since salivary secretion could not be measured reliably in TrpV4-/- mice due to the sensitivity of the mice to general anesthesia (Liedtke, unpublished observations).

A pivotal finding of the present study is that hypotonicity-induced activation of TRPV4 is dependent on the presence of functional AQP5 and not on the osmolarity of the medium or magnitude of cell swelling per se. Further, we show that the association between AQP5 and TRPV4 as well as their surface expression are increased upon exposure of the cells to hypotonic conditions. Although the molecular scaffold mediating these events is not yet known, based on our data, we suggest that sustained RVD is mediated by a cytoskeleton-dependent increase in the association and cell surface expression of AQP5 and TRPV4. This suggestion does not exclude the
possibility that other HTS-activated signals such as arachidonic acid metabolites or protein kinase (12, 36) could contribute to this channel regulation. Consistent with our findings, Arniges et al. previously reported that reduced cell swelling does not account for lack of TRPV4 activation and loss of RVD in airway epithelial cells exogonously expressing a mutant cystic fibrosis membrane regulator, CFTR (16). CFTR has been known to interact with several other ion transport proteins and regulate fluid secretion. Cystic fibrosis airway epithelia have defective swelling-activated K\(^+\) and Cl\(^-\), channel activities and therefore display loss of RVD response (16). Although the role of AQP5 in CFTR-dependent regulation of cell volume has not yet been described, it has been suggested that alveolar AQPs might be more involved in cell volume regulation than transepithelial fluid flux (6). In this regard, it is interesting that AQP5 and CFTR appear to be colocalized apically in pancreatic ductal cells and salivary epithelial cells (37). TRPV4 is also found in the apical region of salivary gland cells (30-33). Recently, TRPV4 has been reported to form a functional signaling complex with large conductance Ca\(^{2+}\)-activated K\(^+\) channels that are involved in vasodilation (27). Additionally, and consistent with our data, TRPV4 has been reported to interact with MAP-7 interacts via its C-terminus, the interaction links the channel to the cytoskeleton and regulates surface expression of the channel (35). Together our present data and these previous studies suggest that TRPV4 and AQP5 are components of a larger signaling platform that can not only sense osmotic and mechanical signals but also transduce these signals and coordinate the regulation of key ion channels that are involved in controlling cell shape and volume recovery. It is also interesting that TRPV4, like TRPV1 and TRPV2, is translocated to the membrane in response to the stimulus. Thus, regulated trafficking appears to be a common mechanism underlying regulation of TRPV4 channels by specific sensory signals (38-41).

In conclusion, our data demonstrate a role for AQP5 in the activation of TRPV4 by hypotonicity in salivary gland cells. Further, we suggest that AQP5 and TRPV4 are concertedly involved in regulation of cell volume and salivary gland fluid secretion. AQP5 also mediates fluid secretion in mucosal glands and epithelial cells in the lung as well as in cells from pancreas and sweat glands (6, 7). TRPV4 has been suggested to regulate RVD in airway epithelial cells, although the functional interaction between TRPV4 and AQP5-mediated volume changes in these tissues remains to be clarified. Thus, based on the role of TRPV4 in osmosensation and cell volume regulation demonstrated here and previously (16-18, 27) it can be suggested that TRPV4 can function as a transducer of osmotic stimuli and in concert with AQP5 contribute to the regulation of cellular volume. Further studies are required to determine the critical molecular components that are involved in detection of these signals and coordination of the cellular responses.

**Footnotes:**

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**Abbreviations:** AQP5, aquaporin 5; TRPV4, transient receptor potential vanilloid 4; RVD, regulatory volume decrease; HTS, hypotonic solution; NMDG, N-methyl D glucamine, 4\(\alpha\)PDD, 4\{alpha\}-phorbol 12, 13-didecanoate; SMG, submandibular gland; IP,
immunoprecipitation; cyt D., cytochalasin D.

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18. Liedtke, W., and Friedman, J. M. (20030 PNAS 100, 13698-13703
Figure 1. HTS induces volume changes and Ca\(^{2+}\) entry in human salivary gland cells. HTS-induced [Ca\(^{2+}\)]\(_i\) increase (increase in 340/380 fluorescence ratio) measured in fura2-loaded HSG cells (A, B). Effect of varying osmolarity are shown in A, average data from these experiments are presented in B (* indicates values that are significantly different from those in the control condition, p<0.01, n=100-200). HTS at 150 mmol/kg was used in all subsequent experiments. C. Effect of HTS on [Ca\(^{2+}\)]\(_i\) in human primary submandibular gland (SMG) and rat basophilic leukemia (RBL) cells to HTS. D. Gd\(^{3+}\) sensitivity of HTS-induced sustained [Ca\(^{2+}\)]\(_i\) increase in HSG cells. 1 µM or 100 µM Gd\(^{3+}\) was added to the external solution where indicated by arrows and a control trace is shown by the dashed line. The decrease in fluorescence in the presence of 1 µM Gd\(^{3+}\) was not different from that in control cells, 100 µM Gd\(^{3+}\) rapidly decreased fluorescence to that in resting cells. E. Activation of a non-selective cation conductance by HTS in HSG cells. Time course of the current measured at 80 and -80 mV in cells perfused with Ca\(^{2+}\)+Na\(^{+}\)-HTS are shown (see Materials and Methods for details). F. I-V relationship of the current at the time indicated by arrow in E. G. Cation permeability of HTS-stimulated channel in HSG cells. External Ca\(^{2+}\)+Na\(^{+}\)-HTS was replaced by NMDG-HTS solution for the period indicated. H. I-V curves of the current at the time points indicated in G. Current traces are representative of results obtained with a minimum of 3 cells in each case.

Figure 2: Possible role of TRPV4 in hypotonicity-activated Ca\(^{2+}\) entry. A. Effect of ruthenium red (included where indicated in A) on the HTS-induced cation current measured at 80 and -80 mV. I-V curves of the current at the indicated time points are shown in B. Amplitude of the current in the presence of RuR was significantly lower (50-60 % lower for outward and >90% for inward currents) than in control cells (p<0.01, n=4). All current recordings are representative of results obtained from at least 4 different cells. 4α-PDD activated [Ca\(^{2+}\)]\(_i\) increases (C, D) and cation current as HTS in HSG cells (E, F). G. Expression of TRPV4 in HSG and RBL cells, crude membranes from brain and MDCK cells were was used as a control. The bands around 97 kDa represent TRPV4.

Figure 3. RVD depends on HTS-stimulated Ca\(^{2+}\) entry. A. HTS-induced HSG cell volume changes in HSG cells (measured using Universal Imaging MetaMorph software, representative images acquired at the times indicated by arrows are shown). Other conditions were as described in Figure 1. B-D. Dependence of RVD on HTS-stimulated Ca\(^{2+}\) entry (volume was determined based on measurements of calcine fluorescence). Traces represent average values obtained from >80 cells, similar results were obtained in 3-4 separate experiments. Additions and other details are indicated in the figure.

Figure 4. Loss of HTS-induced Ca\(^{2+}\) entry and RVD response in salivary gland cells from TRPV4 null mice. Dispersed salivary gland cells were prepared as described in Experimental Procedures. HTS- and 4α-PDD-induced [Ca\(^{2+}\)]\(_i\) increases in fura-2 loaded submandibular gland acinar cells isolated from Trpv4\(^{+/+}\) and Trpv4\(^{-/-}\) mice (A and B, respectively, each trace is an average of at least 20-30 cells, average values from 3-4 separate cell preparations shown in C). D and E. Membrane current recorded at -80 mV and representative I-V plots of the maximum current generated in each case. F: average current densities. G. HTS-induced cell volume changes. H. Expression of TRPV4 (upper blot) and AQP5 (lower blot) in submandibular glands.
from TRPV4-/− and TRPV4+/+ mice. ** indicates values significantly different from control values in each set (p<0.01, n=4-6 for whole cell current measurements, n=80-100 for Ca²⁺ imaging experiments).

Figure 5. Loss of HTS-induced Ca²⁺ entry and RVD response in salivary gland cells from AQP5 null mice. HTS-induced [Ca²⁺], changes in submandibular (A) and parotid acinar cells (B) from Aqp/-− and Aqp+/+ mice (average data are shown in C). The number of cells tested is indicated in the figure. D. HTS-stimulated membrane currents in Aqp5/-− and Aqp5+/+ parotid acinar cells recorded at -80 mV, IV curves of the maximum current is shown in E. The current recordings represent results obtained with at least 4 cells in each case. F. Expression of TRPV4 (upper blot) and AQP5 (lower blot) in submandibular (SG) and parotid (P) glands from Aqp5/-− and Aqp5+/+ mice. Changes in cell volume in response to HTS was measured in calcein-loaded submandibular gland cells from AQP5/-− and Aqp+/+ mice. G. t₁/₂ of cell swelling (time required for ½-maximal swelling); H. % increase in cell volume; and I. extent of cell volume recovery. ** indicates values that are significantly different from unmarked values (p<0.05. n= 29-34 for imaging experiments).

Figure 6. Association and functional interaction between TRPV4 with AQP5. A. Localization of AQP5 (left image) and TRPV4 (right image, magnified image included) in serial sections of mouse submandibular glands (reactivity was not detected in absence of primary antibody, image not shown). B. IP with anti-TRPV4 antibody (second panel, IB antibodies indicated below the blots) or AQP5 antibody (third panel, inputs shown in first panel). IP with TRPV4 does not pull down AQP3 (IP fraction, first lane, unbound fraction, second lane). HTS-induced increase [Ca²⁺], in HSG cells transiently expressing AQP5-wt or AQP5-ΔC (C) or AQP5-ΔN (D). Effect of AQP5-ΔN expression on HTS-activated cation current (E) and HTS-induced volume changes, average trace measured in 20-30 cells (F). 4α-PDD was included in the external solution where indicated.

Figure 7. HTS-induced, cytoskeleton-dependent, association and surface expression of TRPV4 and AQP5 regulates RVD. Effect of cytochalasin D treatment (CytoD, 1 µM for 30 min) on HTS-induced [Ca²⁺], increase (A), cation current (B) and regulation of cell volume (C). Changes in the external medium and time (horizontal bar) are indicated in the figure. D-G. Effect of HTS on surface expression and association of TRPV4 and AQP5 in HSG (D-F) and freshly dispersed submandibular gland cells (G). Cells were treated with cytochalasin D as described above. Control and cyto-D cells were then exposed to HTS and biotinylated on ice. Cell lysates from control and HTS-treated cells were incubated with either avidin (D) or anti-AQP5 (E) and the blots were probed with anti-TRPV4 or anti-AQP5 antibodies as indicated, TRPV4 and AQP5 in HSG cell lysates (F). G. Effect of HTS on co-IP of TRPV4 and AQP5 from dispersed mouse submandibular glands. Cells were prepared as described in Experimental procedures. Cyto-D treatment and other experimental conditions were similar to those used for HSG cells. Data are representative of results obtained in three independent experiments.
FIGURE 1

A

B

C

D

E

F

G

H
FIGURE 2

A

RR (1 μM)  
α  
PDD  
HTS

B

HTS  
HTS  
RR  
HTS + RR

C

pA/pF  
4αPDD

D

pA/pF  
340/380

E

pA/pF  
4αPDD

F

pA/pF  
340/380

G

kDa

97  
52

IB: TRPV4

HSG  RBL  MDCK Brain
FIGURE 3

A

Relative Volume

1.0
1.2
1.4

HTS

60 s

B

Ca\(^{2+}\) (1 mM)

Relative volume (%)

100
110
120
130

HTS

60 s

C

Ca\(^{2+}\) free

HTS

60 s

D

Gd\(^{3+}\) (100 µM)

HTS

60 s
**FIGURE 5**

A

- AQP5-/- (n=36)
- AQP5+/+ (n=32)

B

- AQP5 +/+ in Ca²⁺ free
- AQP5 -/-
- AQP5 +/+ in Ca²⁺ free

C

- HTS
- 4αPDD

D

- AQP5 -/-
- AQP5 +/+ in Ca²⁺ free

E

- TRPV4
- 97kDa
- 28kDa

F

- P-/- P+/+
- SG-/- SG+/+
- AQP5

G

- t₁/₂ of cell swelling (s)

H

- Cell Swelling (%)

I

- RVD (%)
FIGURE 7

A. CytD

B. CytD

C. Relative Cell Volume (%)

D. IP: Avidin

E. IP: AQP5

F. Input

G. IP: anti-AQP5

HSG cells.
Myelin basic protein-primed T cells induce neurotrophins in glial cells via αvβ3 integrin.
Avik Roy, Xiaojuan Liu, and Kalipada Pahan

In the entire article, we have mistaken αv as α5. Therefore, we request the readers to consider α5β3 as αvβ3 and the title of the article as “Myelin Basic Protein-primed T Cells Induce Neurotrophins in Glial Cells via αvβ3 Integrin.”

We used anti-αv antibody (clone RMV-7) from BD Biosciences (catalog no. 552299) and eBioscience (catalog no. 16-0512) for fluorescence-activated cell sorting analysis (Fig. 5) and neutralization experiments (Fig. 6 and supplemental Figs. 1–3). The following primers were used for reverse transcription-PCR analysis of αv (Fig. 5C): 5′-GTT GGG AGA TTA GAC AGA GGA-3′ (sense) and 5′-CAA AAC AGC CAG TAG CAA CAA-3′ (antisense). The labeling sequence for Fig. 5 (A and B, right panels) should be itgaL, itgaM, itgaV, itgaX, itgb1, etc.
A role for AQP5 in activation of TRPV4 by hypotonicity: concerted involvement of AQP5 and TRPV4 in regulation of cell volume recovery
Xibao Liu, Bidhan B. Bandyopadhyay, Tetsuji Nakamoto, Brij B. Singh, Wolfgang Liedtke, James E. Melvin and Indu S. Ambudkar

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