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Expression of Aquaporin Water Channels in Rat Taste Buds

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Abstract

In order to gain insight into the molecular mechanisms that allow taste cells to respond to changes in their osmotic environment, we have used primarily immunocytochemical and molecular approaches to look for evidence of the presence of aquaporin-like water channels in taste cells. Labeling of isolated taste buds from the fungiform, foliate, and vallate papillae in rat tongue with antibodies against several of the aquaporins (AQPs) revealed the presence of AQP1, AQP2, and AQP5 in taste cells from these areas. AQP3 antibodies failed to label isolated taste buds from any of the papillae. There was an apparent difference in the regional localization of AQP labeling within the taste bud. Antibodies against AQP1 and AQP2 labeled predominantly the basolateral membrane, whereas the AQP5 label was clearly evident on both the apical and basolateral membranes of cells within the taste bud. Double labeling revealed that AQP1 and AQP2 labeled many, but not all, of the same taste cells. Similar double-labeling experiments with anti-AQP2 and anti-AQP5 clearly showed that AQP5 was expressed on or near the apical membranes whereas AQP2 was absent from this area. The presence of these 3 types of AQPs in taste buds but not in non–taste bud-containing epithelia was confirmed using reverse transcription–polymerase chain reaction. Experiments using patch clamp recording showed that the AQP inhibitor, tetraethylammonium, significantly reduced hypoosmotic-induced currents in rat taste cells. We hypothesize that the AQPs may play roles both in the water movement underlying compensatory mechanisms for changes in extracellular osmolarity and, in the case of AQP5 in particular, in the gustatory response to water.

Key words: aquaporin, immunocytochemistry, RT–PCR, taste, water

Introduction

Stimuli that humans recognize as salty, sour, sweet, bitter, and umami consist of a variety of molecules ranging from those that are small and ionic to complex organic molecules (Gilbertson and Kinnamon 1996). The context, such as the temperature and texture, in which these stimuli are presented has also been shown to have a significant influence on the gustatory signals generated during chemostimulation. Because taste stimuli may range from the very hypoosmotic to hyperosmotic compared with the interior tonicity of the taste cells (Feldman and Barnett 1995), it has been suggested that solution osmolarity may be an important variable to consider when determining the overall gustatory response. Consistent with this idea, 2 recent reports have demonstrated that the osmotic environment may directly affect taste receptor cell (TRC) activity and in that way influence the responses to sapid molecules.

Lyall et al. (1999) showed that hyperosmotic stimulation directly affected salt responses recorded in the chorda tympani. Increases in solution tonicity in this study lead to pronounced decreases in cell volume measured optically. These volume changes were accompanied by an enhancement of NaCl-induced activity in the chorda tympani nerve. Our laboratory (Gilbertson 2002) investigated the effects of hypoosmotic stimuli on taste cell activity using patch clamp recording on rat TRCs. Decreases in solution osmolarity lead to an increase in cell capacitance (i.e., surface area) and a concomitant activation of a Cl^- conductance. This Cl^- conductance had all the hallmarks (pharmacology, permeability properties) of the well-documented and ubiquitous swelling-activated Cl^- current ($I_{Cl,swell}$; Jentsch et al. 1999). It was hypothesized that this hypoosmotic-activated current, termed I_{HYPO-T} in TRCs might play roles in regulatory

volume decrease (RVD) or in the gustatory response to water (Gilbertson 2002). Taken together, these studies demonstrate the ability of TRCs to respond to changes in the extracellular osmotic environment that might affect peripheral taste processing.

The ability of taste cells to respond to water has been well documented (cf. Lindemann 1996; Gilbertson et al. 2006), and reports have shown that both gustatory and laryngeal afferent nerve fibers are stimulated by the application of dilute (e.g., hypoosmotic) stimuli (Cohen et al. 1955; Zotterman 1956; Shingai 1980; Sakaguchi et al. 1989). This response has been termed the water response, and it appears to be a common feature of many terrestrial vertebrates. Though the ability of water to activate gustatory nerve fibers has been known for decades, there is little or no information on the molecular mechanism underlying water movement in taste buds. In many cell types, the transmembrane movement of water is facilitated by a unique class of channels known as aquaporins (AQPs). The existence of these highly water permeable pathways was speculated long before their discovery owing to the fact that red blood cell membranes, membranes in renal collecting ducts, and other tissues were much more permeable to water than would be predicted by studies in simple bilayers (Murata et al. 2000). Functional AQP channels associate as tetramers of identical subunits that are structurally similar to many ion channel gene families like K^+ channels and cyclic nucleotide–gated (CNG) channels (Yool and Weinstein, 2002; Boassa et al. 2006). Water permeation in AQP channels is characterized by sensitivity to mercurial agents like $HgCl_2$ (Lee et al. 1997; Murata et al. 2000) and tetraethylammonium (TEA; Brooks et al. 2000; Yool et al. 2002; Detmers et al. 2006). To date there have been identified 13 different subtypes of mammalian AQPs (AQP0-12) that have differing tissue distributions that appear related to their functional roles in water-transporting epithelia (Agre et al. 2002; Yasui 2004; Verkman 2005a).

The importance of these channels in the movement of water is demonstrated by a number of diseases linked to mutations in several of the AQP family genes (Verkman 2005b). For example, transgenic knockout mice lacking AQP1 or AQP4 have impaired urinary concentrating ability (Ma et al. 1997, 1998), cerebral fluid balance (Manley et al. 2000), corneal fluid balance (Thiagarajah and Verkman 2002), hearing (Li and Verkman 2001), and water transport in the lungs (Bai et al. 1999; Song et al. 2000). Defective secretion in salivary and submucosal glands (Ma et al. 1999; Song and Verkman 2001) and altered lung fluid transport (Ma et al. 1999) have been shown in AQP5 knockout mice. Defects in AQP2 have been linked with nephrogenic diabetes insipidus (Canfield et al. 1997; Tamarappoo and Verkman 1998).

In the present study, we have attempted to identify the route for rapid water movement in TRCs by trying to localize several of the major AQPs in taste cells using immunocytochemistry and reverse transcription–polymerase chain reaction (RT–PCR). Because of similarities between salt/water transport pathways in lingual and kidney epithelia, we have initially focused our efforts on those AQPs that play major roles in kidney water transport (Nishimura and Fan 2003; Gade and Robinson 2006). Consistent with other salt- and water-transporting epithelia, TRCs contain several types of AQPs, and sequencing of PCR products revealed these taste AQPs to be virtually identical to those found in kidney or salivary gland. Interestingly, AQP1 and AQP2 were distributed over the basolateral membranes of the taste cells, whereas AQP5 appeared to have a more restricted cellular distribution that includes the apical membranes of the TRCs. We hypothesize that these water channels may play a role in water movement during hyper- or hypoosmotic stimulation and thus may be important both in the ability of taste cells to exhibit regulatory volume changes (RVD or RVI) and in the gustatory response to water.

Materials and Methods

Isolation of taste buds

Individual taste buds were isolated from the tongues of 2- to 5-month-old male Sprague–Dawley rats using techniques previously described (Béhé et al. 1990; Doolin and Gilbertson 1996). Briefly, tongues were removed and immediately immersed in ice-cold Tyrode's solution containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 10 glucose, and 10 Na^+ pyruvate (osmolarity ~310 mOsm). The pH was adjusted to 7.4 with NaOH. The anterior portion of the tongue containing the fungiform papillae was injected between the muscle layer and the lingual epithelium with approximately 1.0 ml of physiological saline (Tyrode) containing a mixture of collagenase I (0.5 mg/ml; Boehringer Mannheim, Indianapolis, IN), dispase (5 mg/ml; Boehringer Mannheim), and trypsin inhibitor (1 mg/ml; type I-S; Sigma Chemical Corp., St Louis, MO). Between 0.8 and 1.2 ml of the same enzyme solution was also used to inject the area surrounding the 2 foliate papillae and the circumvallate papilla. The injected tongue was incubated in a Ca^{2+}-Mg^{2+}–free Tyrode containing 2 mM BAPTA (Molecular Probes Inc., Eugene, OR) in place of the $CaCl_2$ and $MgCl_2$ and bubbled with O_2 for 25 min at room temperature (RT). Following incubation, the tongue was washed with saline and the epithelium was removed from the underlying muscle layer with forceps and pinned out in a Sylgard-lined petri dish. Individual taste buds were removed from the epithelium under low magnification (50×) with a suction pipette (~200-μm pore), washed several times in the Ca^{2+}-Mg^{2+}–free Tyrodes to remove connective tissue and epithelial cells, and plated onto a slide coated with Cell-Tak Tissue Adhesive (Boehringer Mannheim) for immunocytochemical experiments or into a 0.5-ml microfuge tube on ice for the RT–PCR assays.

Immunocytochemistry

The taste buds plated onto the Cell-Tak coated slides were immediately fixed with acid alcohol fixative (70% ethyl alcohol, 10% acetic acid in double-distilled water) for 10 min at RT. After washing 3 times with phosphate-buffered saline (PBS), cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min (RT). Taste buds were again washed 3 times with PBS, and nonspecific protein-binding sites were blocked by incubating in blocking solution (5% [w/v] nonfat dry milk, 150 mM NaCI, 20 mM Tris–HCI, 0.01% antifoam A, 0.02% sodium azide, and 0.1% Triton X-100) for 30 min at RT. Cells were incubated with anti-AQP1, anti-AQP2 polyclonal antibodies (Alomone Labs, Jerusalem, Israel), or anti-AQP5 polyclonal antibody (Alpha Diagnostic International, San Antonio, TX) for 72 h at 4 °C. All 3 antibodies were diluted 1:100 in blocking solution. After incubation with the primary antibody, cells were washed several times in blocking solution and subsequently incubated with biotin-conjugated goat anti-rabbit IgG (1:200) for 1–1.5 h at RT. After washing in blocking solution, cells were incubated with Texas Red- or Cy2-conjugated streptavidin (1:200) for 2.5 h at 4 °C. Cells were washed 3 times in PBS and mounted in glycerol supplemented with 0.25 M N-propyl-gallate. For negative controls, either the primary antibody was omitted or it was preincubated with 10× excess antigen for 2 h at RT. For double labeling involving both anti-AQP1 and anti-AQP2 or anti-AQP2 and anti-AQP5, cells were once again washed with blocking solution and were incubated with secondary antibody for another 48–72 h at 4 °C. Immunoreactive products were observed and photographed on a Zeiss confocal laser-scanning microscope.

To verify labeling of anti-AQP5 on the apical region of taste buds, we performed a series of experiments labeling slices of circumvallate papillae with anti-AQP5 antibodies. Rat tongues were removed and fixed in 4% paraformaldehyde for 2 h at RT. Slices of 50 μm were obtained from blocks of tissue containing circumvallate papillae using a vibrating microtome (Vibratome 3000, Vibratome Company, St Louis, MO). Standard immunocytochemical procedures were followed to visualize AQP5 labeling. Briefly, tissue sections were washed in PBS followed by incubation in 3% normal goat serum. Slices were then incubated in AQP5 polyclonal antibody (1:100; Alpha Diagnostic International) for 72 h at 4 °C. After several rinses in PBS, sections were incubated in biotin-conjugated anti-rabbit IgG (1:200) for 1 h. Slices were rinsed with PBS and then incubated with Alexa-fluor 594-conjugated avidin (1:200) for 2.5 h at 4 °C. The resulting labeling was visualized using a BioRad laser confocal microscope.

Reverse transcription–polymerase chain reaction

Specific forward and reverse primers used for PCR amplification of AQPs were as follows. For AQP1, the forward primer (5′-tcagtggtgctcacctcaac) and reverse primer (5′-gctgagccacctaagtctcg) corresponded to nucleotide sequences of 209–228 and 484–503 of the rat AQP1 (CHIP28) sequence (accession no.: X70257), respectively (Zhang et al. 1993). For AQP2, the forward primer (5′-tttgtcttctttggccttgg) and reverse primer (5′-ttgtggagagcattgacagc) corresponded to nucleotide sequences of 151–170 and 433–452 of the rat AQP2 (vasopressin-responsive water channel) sequence (accession no.: NM012909), respectively (Deen et al. 1994). For AQP5, the forward primer (5′-ccctctcactgggtcttctg) and reverse primer (5′-ccttttctccagtggtccag) corresponded to nucleotide sequences of 710–729 and 940–959 of the rat AQP5 sequence (accession no.: NM012779), respectively (Raina et al. 1995). The sizes of expected PCR products were 295, 302, and 250 bp for AQP1, AQP2, and AQP5, respectively.

Total RNA was prepared from isolated taste buds of the fungiform, circumvallate, and foliate papillae of male Sprague–Dawley rat by guanidium thiocyanate–phenol–chloroform extraction (Sigma Chemical Corp.). As in our previous reports (Gilbertson et al. 2005; Liu et al. 2005), care was taken to remove nonadherent cells from the taste buds prior to RNA extraction to reduce the likelihood of the inclusion of non–taste cells in the preparation. Non–taste epithelium (NTE) was excised from the lingual epithelium just anterior to the intermolar eminence of 4 male Sprague–Dawley rats, and RNA was isolated from each of these samples using the RNeasy Mini Kit in accordance with manufacturer's instructions (QIAGEN Inc., Valencia, CA). To exclude genomic DNA contamination, total RNA was treated with DNase I (RNase-free, Gibco, Grand Island, NY) for 20 min at 25 °C. After denaturing DNase I, first-strand cDNA templates were synthesized by priming total RNAs with random hexamers and reverse transcribed with SuperScript II reverse transcriptase (Gibco) or using the Omniscript RT kit (QIAGEN Inc.) according to the manufacturer's recommended procedures. Complementary DNA was amplified by 35 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min) of PCR using Red *Taq* polymerase (Sigma Chemical Corp.) or *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). PCR fragments were resolved by electrophoresis through a 2% agarose gel, then stained with ethidium bromide or SYBR Safe (Invitrogen, Carlsbad, CA). Single-banded PCR products of each AQP were purified by using the QIAquick PCR purification kit (QIAGEN Inc.). The sequences of purified cDNAs were determined by a PE Biosystem 377 automated DNA sequencer. Partial sequences for each of the AQPs were examined by using the BLAST 2.0 search engine (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/).

Patch clamp recording

Briefly, we followed the protocols used in our earlier report characterizing hypoosmotic currents in rat TRCs (Gilbertson 2002). Fungiform taste cells were isolated in the manner described above and plated onto Cell-Tak coated microscope slides fitted with a Sylgard O-ring that acted as a recording

chamber in normal physiological saline (Tyrodes). During these patch clamp recordings, an intracellular solution that replaced the K^+ with Cs^+ and mannitol was used in the whole-cell–recording mode. This solution contained (in mM) 90 mM CaCl, 100 mannitol, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 11 ethylene glycol-bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 3 adenosine triphosphate. The pH was adjusted to 7.2 with CsOH (~310 mOsm). This CsCl intracellular solution helped to eliminate most of the voltage-activated outward K^+ current, which facilitated the analysis of the hypoosmotic-induced current and set the Cl^- equilibrium potential (E_{Cl}) to near zero.

To record hypoosmotic-activated currents, extracellular solutions were used that varied only in osmolarity while holding all other ions constant. The control solution consisted of (in mM) 90 NaCl, 100 mannitol, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 10 Na^+ pyruvate. The pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted as necessary to 310 ± 3 mOsm and measured with a vapor pressure osmometer (Wescor, Logan, UT). To create the hypoosmotic solution, the mannitol concentration was decreased to 40 mM, which yielded a solution with an osmolarity of 255 ± 3 mOsm. This solution represented the intermediate hypoosmotic solution used in our previous study (Gilbertson 2002). To determine the sensitivity of hypoosmotic-activated currents to the AQP inhibitor TEA (Brooks et al. 2000; Yool et al. 2002; Detmers et al. 2006), TEA (25 mM) was added to all extracellular solutions with a concomitant reduction in mannitol to maintain solution osmolarity. We chose this concentration to ensure maximal block of water entry through AQP channels into these native mammalian cells. To control for any effect of this concentration of TEA on potassium channels and currents carried through these channels, we blocked them with cesium in the intracellular solution.

Whole-cell currents were recorded from individual fungiform TRCs either isolated individually or maintained in the taste bud by using patch clamp methods. Patch pipettes were pulled to a resistance of 5–10 MΩ when filled with intracellular solution. Series resistance and cell capacitance were compensated optimally before the recording. The holding potential in all experiments was –110 mV. Ramp protocols from –110 mV to +50 mV (duration: 480 ms; 0.333 V/s) were used to generate instantaneous current–voltage (I–V) relationships in the various test solutions. Command potentials were delivered, and current data were recorded with pCLAMP software (version 9) interfaced to an AxoPatch 200B amplifier with a Digidata 1322A A/D board (Molecular Devices, Foster City, CA). Data were collected at 5 kHz and filtered online at 1 kHz. No records were leak subtracted in the present study.

Results

We have used immunocytochemical and molecular biological approaches to identify AQP water channels in taste receptors. Our previous results demonstrated that taste cells were capable of responding rapidly to decreases in solution tonicity (Gilbertson 2002), and those by Lyall et al. (1999) that showed hyperosmotic-induced volume changes in taste cells suggested that TRCs must have a route that permits rapid water movement across their plasma membrane. Verification of a potential role for AQPs in the rapid water movement that generated the hypoosmotic response came from a series of patch clamp experiments that examined the sensitivity of hypoosmotic-induced currents to the AQP inhibitor, TEA.

Immunocytochemistry

Because of their importance as water transport pathways in kidney epithelial cells (Lee et al. 1997; Agre et al. 2002), we began by using antibodies against a variety of different AQP channels, including AQP1, AQP2, AQP3, and AQP5. Of these, only AQP3 antibodies failed to label isolated taste buds from the fungiform, foliate, and circumvallate papillae (data not shown) and will not be discussed further. The remaining 3 antibodies did label TRCs within each of these 3 classes of lingual taste buds (see below).

AQP1

Isolated taste buds from each of the 3 papillae in the tongue labeled with an anti-AQP1 antibody (Figure 1). Labeling of isolated taste buds with anti-AQP1 antibody was typically seen as a diffuse staining over the majority of the individual taste cell surface when viewed using confocal microscopy. In taste buds from the fungiform papillae, the AQP1 label was clearly evident on the basolateral surface of the taste bud in both lateral (Figure 1A) and transverse orientations (Figure 1C). Whether there was labeling on the apical membrane remains equivocal in the single-label studies. AQP1 labeling in the foliate (Figure 1D,E) and vallate (Figure 1F,G) taste buds, however, was primarily, if not exclusively, on their basolateral membranes. There was little or no apparent label evident on their apical membranes.

AQP2

Isolated taste buds from the 3 lingual papillae were also labeled with antibodies against AQP2, another of the predominant water channels found primarily in the collecting ducts of the kidney (Nielsen et al. 1993; Knepper and Inoue 1997; Loffing et al. 2000). Qualitatively, all 3 taste bud types displayed a similar pattern of labeling with the majority of the AQP2 staining found over the basolateral surface (Figure 2). There was little or no AQP2 found in the regions of the apical membranes in taste buds isolated from the fungiform (Figure 2A,D), foliate (Figure 2B,E), or vallate papillae (Figure 2C,F).

AQP5

AQP5 is a water channel found in a variety of areas including the salivary glands, lacrimal glands, alveolar epithelial cells,

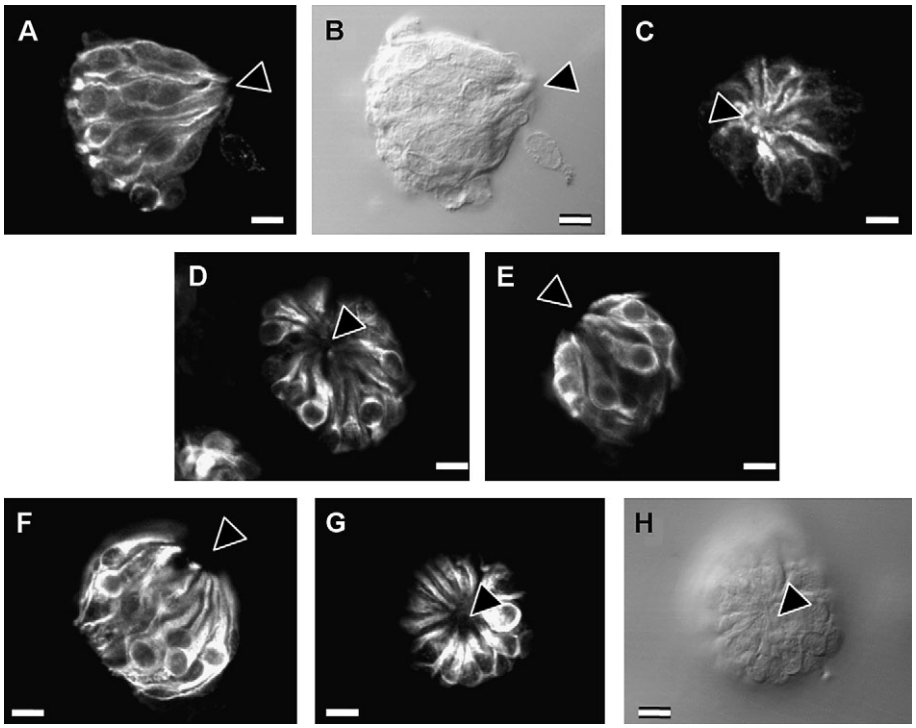

Figure 1 Labeling of isolated taste buds with an anti-AQP1 antibody. Confocal images of individual taste buds from the fungiform **(A–C)**, foliate **(D, E)**, and vallate **(F–H)** papillae in rat tongue labeled with the anti-AQP1 antibody. (B) and (H) show DIC images of the taste buds shown in (A) and (G), respectively. Arrowheads point toward the location of the apical membranes of the cells. Scale bar, 10 μm.

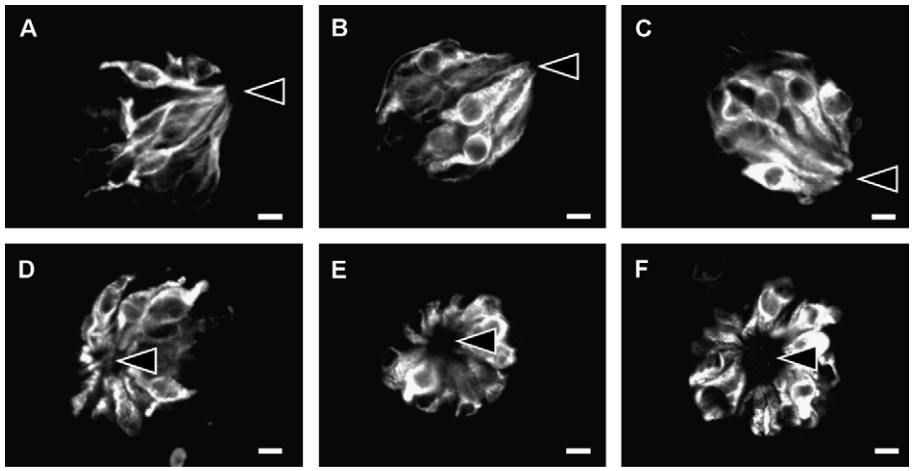

Figure 2 Labeling of isolated taste buds with an anti-AQP2 antibody. Confocal images of labeled taste buds focused at the plane of the apical membrane in fungiform **(A, D)**, foliate **(B, E)**, and vallate **(C, F)** taste buds. In all 3 taste bud types, the AQP2 label appeared to be more concentrated in the basolateral regions of the cells. Arrowheads point toward the location of the apical membranes of the cells. Scale bar, 10 μm.

and corneal epithelial cells in mammals (Ishida et al. 1997; Nielsen et al. 1997; Funaki et al. 1998; Hamann et al. 1998). In these areas, its expression appears to be limited to the apical membranes of cells. Labeling of lingual taste buds with anti-AQP5 revealed a much different pattern than that found for either AQP1 or AQP2. Figure 3 shows the staining pattern for AQP5 protein in foliate (Figure 3A), vallate (Figure 3C,E), and fungiform (Figure 3G) taste buds with their corresponding differential interference contrast (DIC) images. Although in isolated taste buds it appeared that AQP5 was not apically "restricted" per se, it is clear that AQP5 could be found on portions of taste cells that contained the apical membranes in all 3 taste bud types we examined. Sections through the circumvallate papillae labeled with the

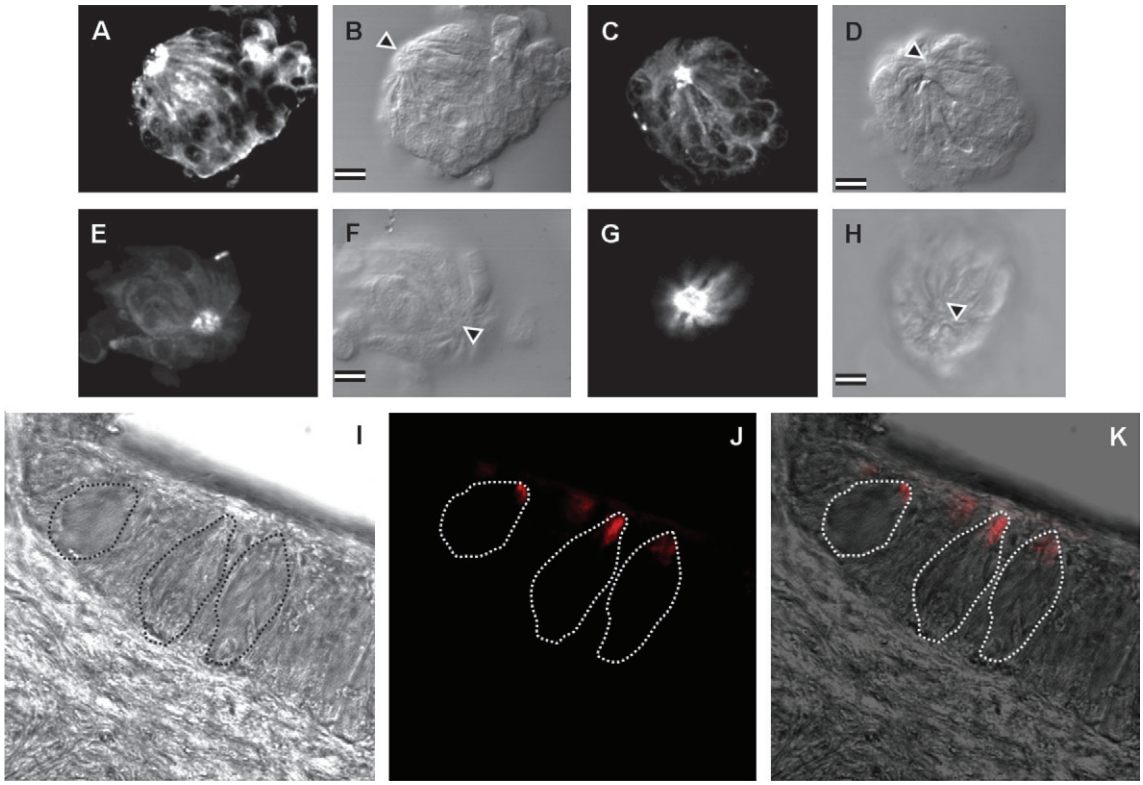

Figure 3 Labeling of isolated taste buds with an anti-AQP5 antibody demonstrates apical localization of AQP5. Confocal images of taste buds from the foliate **(A, B)**, vallate **(C–F)**, and fungiform **(G, H)** papillae in rat tongue labeled with the anti-AQP5 antibody. Left image of each pair is the immunofluorescent image of anti-AQP5 staining and the right side is its corresponding DIC image. Arrowheads in DIC images point toward the region of the apical membranes of the taste cells. Scale bars (A–H), 10 μm. Anti-AQP5–labeled DIC **(I)**, fluorescent **(J),** and overlay **(K)** images of a 50-μm slice through the vallate papilla are consistent with apical expression of AQP5.

anti-AQP5 antibody are consistent with protein expression of AQP5 at the area corresponding to the apical membranes (Figure 3I–K).

Double labeling

In order to try and examine the differences in the distribution patterns of AQP1, AQP2, and AQP5 in greater detail, we used double labeling of AQP1/AQP2 and AQP2/AQP5 is isolated taste buds. In all taste buds examined, there was a similar distribution of AQP1 and AQP2 at the cellular level (Figure 4A–D) with the predominant labeling being toward the basolateral regions of the taste buds. That is, in each of the 3 types of lingual taste buds, AQP1 and AQP2 labeled the same cells within the taste bud with few exceptions.

Similarly, double labeling of taste buds with anti-AQP2 and anti-AQP5 showed that many of the same cells expressed both types of AQPs (Figure 4E–G). However, these double-labeling experiments also highlighted the differences in regional expression of AQP2 (or AQP1) and AQP5 proteins in taste cells. Although AQP5 is apparently not apically restricted, there is clear labeling with anti-AQP5 antibodies at the apical end of the taste bud (Figure 4F,G) and this region is devoid of staining with AQP2 (Figure 4E,G). Given this level of analysis, it is clear that there are differences in the expression patterns for various subtypes of AQP water channels that are consistent with the expression of these AQPs in other tissues.

Reverse transcription–polymerase chain reaction

To verify the expression of AQP1, AQP2, and AQP5 in taste buds, we have isolated RNA from each of the 3 lingual papillae and probed with specific primers for each of the 3 subtypes of AQPs. Consistent with our immunocytochemical results showing expression of AQP1, AQP2, and AQP5 proteins, RT–PCR revealed that the message for each of these water channels could be isolated from taste buds pooled from the fungiform, foliate, and vallate papillae (Figure 5A). This was verified in 3 separate sets of experiments. Controls omitting reverse transcriptase from the reaction (Figure 5A) or those with no template (data not shown) did not produce any detectable signal. Positive controls for AQP2 (kidney) and for AQP5 (salivary gland) produced PCR products of the expected sizes (Figure 5A). Subsequent experiments confirmed the expression of each of the 3 AQP channels independently in the 3 taste bud types by RT–PCR (data not shown). All PCR products were sequenced using a PE Biosystem 377 automated DNA sequencer, and rat taste bud AQP1 showed a >99% homology with the published sequence for rat kidney

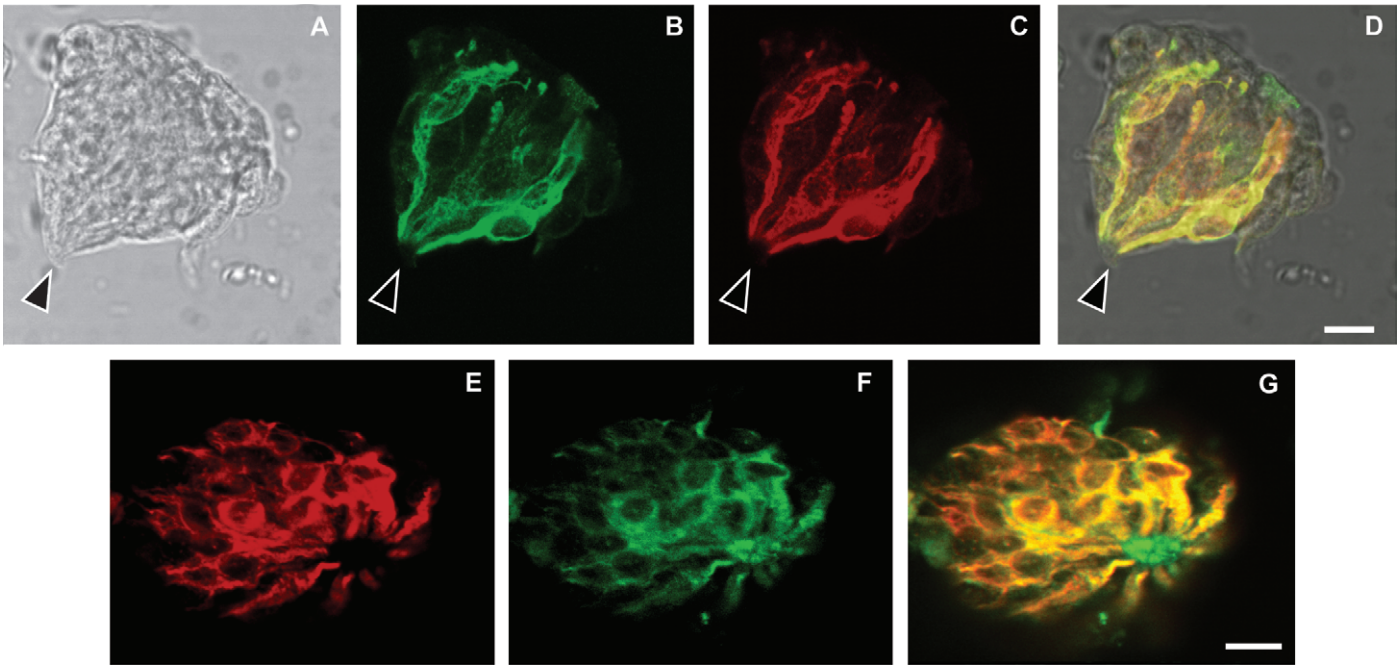

Figure 4 Double labeling of vallate taste buds with anti-AQP antibodies. DIC image of a vallate taste bud **(A)** and the same taste bud labeled with an anti-AQP1 (Cy2; green, **B**) and an anti-AQP2 (Texas red, **C**). Overlay of panels A, B, and C is shown in **(D)**, highlighting the lack of apical labeling in these taste buds (arrowhead). **(E**, **F)** show confocal images of a vallate taste bud labeled with an anti-AQP2 antibody (Texas red) and an anti-AQP5 antibody (Cy2), respectively. **(G)** Overlay of images in (E) and (F). Scale bars, 10 μm.

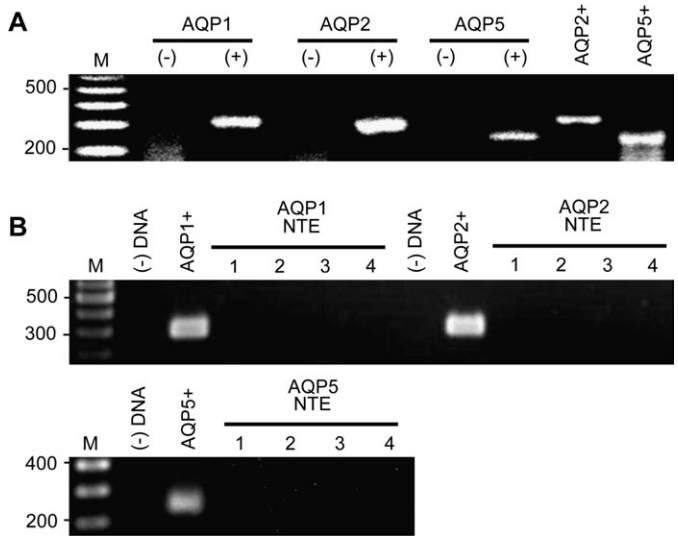

Figure 5 PCR products reveal the presence of AQP1, AQP2, and AQP5 RNA in taste buds but not in NTE. **(A)** Primers for AQP1, AQP2, and AQP5 amplify ethidium bromide–stained PCR products of expected sizes (AQP1, 302 bp; AQP2, 295 bp; AQP5, 250 bp). Lanes marked (–) represent controls for AQP1, AQP2, and AQP5, respectively, in which reverse transcriptase was omitted from the PCR reaction. Positive controls are shown for AQP2 with rat kidney RNA (AQP2+) and for AQP5 with rat salivary gland RNA (AQP5+). M, 100-bp ladder. **(B)** Primers for AQP1 and AQP2 (top) and AQP5 (bottom) amplify products for positive controls (rat lung for AQP1 and AQP5; rat kidney for AQP2) but not for NTE obtained from 4 different rats (labeled 1–4).

AQP1 (rAQP1), rat taste AQP2 was 98% homologous with rat kidney collecting duct AQP2 (rAQP2), and rat taste AQP5 sequence was >99% homologous with rat salivary gland AQP5 (rAQP5).

To determine whether these AQP channels were expressed specifically in TRCs, we examined expression of AQP1, AQP2, and AQP5 in RNA isolated from NTE of 4 Sprague–Dawley rats using RT–PCR. None of these AQP channels were expressed in NTE samples (Figure 5B). Positive controls showed products of expected size and (–)DNA, no template controls, showed no amplification (Figure 5B). Therefore, AQP1, AQP2, and AQP5 appear to be expressed in TRCs but not in the surrounding NTE of the tongue.

Hypoosmotic-induced currents are inhibited by the AQP inhibitor TEA

To test for evidence of a functional role for AQPs in water movement in taste cells, we performed whole-cell patch clamp recording on taste buds from the fungiform papillae in the presence of the AQP1 and AQP2 inhibitor, TEA (Brooks et al. 2000; Yool et al. 2002; Detmers et al. 2006). As shown in Figure 6A, hypoosmotic currents were rapidly activated in roughly 70% (16/23) of TRCs by switching from an isoosmotic solution (“control,” 310 mOsm) to a hypoosmotic solution (“hypo,” 255 mOsm) similar to the protocol used in our earlier study (Gilbertson 2002) where we identified this current as being carried by Cl^- ions. The net hypoosmotic current was calculated by subtracting the currents in

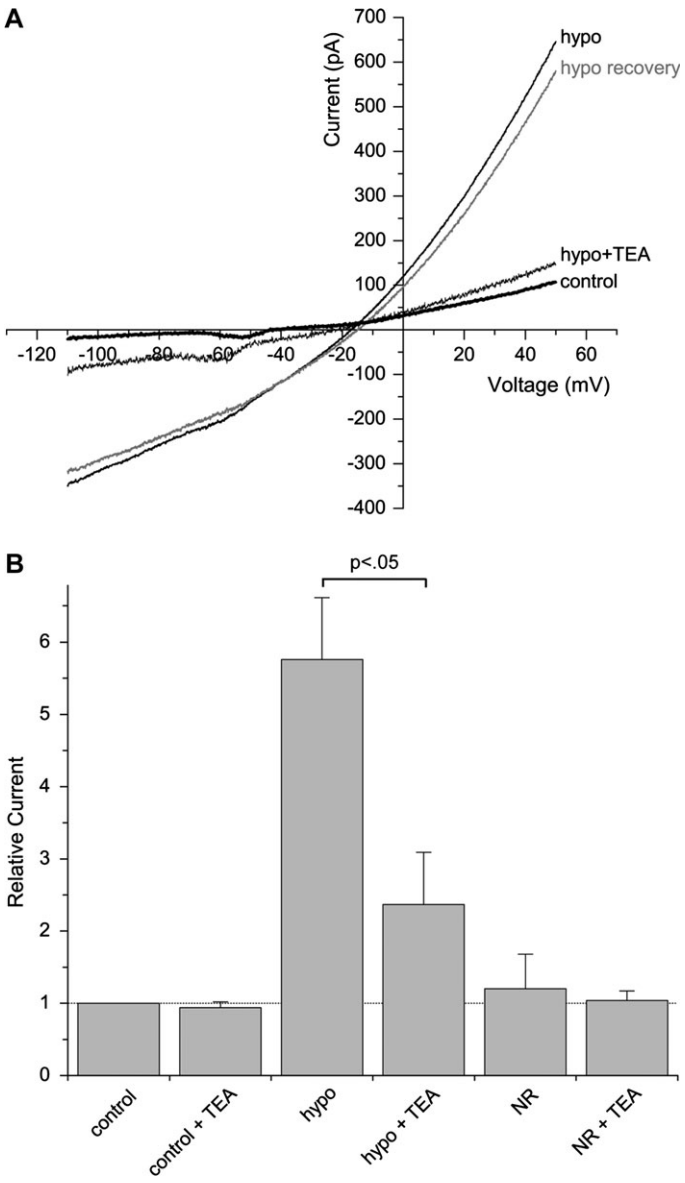

Figure 6 Hypoosmotic-induced currents are reduced by the AQP inhibitor TEA. **(A)** Instantaneous current–voltage curves generated by applying ramps of voltage (−110 to +50 mV). In this cell, application of a hypoosmotic stimulus (255 mOsm, "hypo") caused a large increase in conductance that was reversibly inhibited by TEA (25 mM). Control refers to the currents generated in the 310 mOsm (isoosmotic) solution. **(B)** Mean relative currents ± standard deviation measured at +50 mV in the various solutions. Currents were normalized relative to the magnitude of the current in the control (310 mOsm) solution. TEA significantly inhibited the hypoosmotic-induced current (hypo) but did not affect the control currents or those currents during hypoosmotic stimulation in nonresponsive (NR) cells. Each bar represents the average from 6–10 cells.

the control solution from those in the hypoosmotic solution (cf. Gilbertson 2002). Typically, these hypoosmotic currents ranged in magnitude from ~400–800 pA (current densities from 32–70 pA/pF). Cells that showed hypoosmotic-activated currents less than 100 pA (<10 pA/pF) greater than those obtained in the control (isoosmotic) solution were considered nonresponsive. In hypoosmotic responsive TRCs, the AQP inhibitor TEA (25 mM) significantly inhibited the hypoosmotic-induced current (Student's *t*-test, $P < 0.05$; Figure 6B). TEA had no effect, however, on the currents in the control solution or on currents in the nonresponding cells.

Discussion

Because of similarities between salt and water transport in the taste system and kidney, we have initially focused on the identification of several AQPs that are known to play important roles in water transport in the kidney. In the present study, we have demonstrated the expression of several types of AQP water channels in mammalian taste buds and, based upon our electrophysiological assays, hypothesize that they play a role in the rapid water movement in TRCs during non-isoosmotic stimulation. The presence of a swelling-activated chloride conductance was demonstrated in isolated rat TRCs, which was suggested to be involved in the mechanism of RVD and in the gustatory response to water (Gilbertson 2002). In addition, it has been shown that the peripheral gustatory system responds to hyperosmotic stimulation with a sustained volume decrease concomitant with an alteration in TRC activity (Lyall et al. 1999). These 2 reports are consistent with mammalian TRCs being capable of rapid water movement across their cell membranes, a property generally attributed to AQPs. Using both immunocytochemistry and RT–PCR, we have identified 3 subtypes of AQPs in TRCs that may provide the molecular route for water movement under both hypoosmotic and hyperosmotic conditions. The relative cellular distributions of the AQPs are consistent with TRCs being able to sense both changes in interstitial osmolarity via basolateral AQPs and changes in the osmolarity of the oral cavity (i.e., "water taste") presumably via apically localized AQP proteins.

The regional expression of AQP5, where it appears expressed on the apical membranes of the taste bud, would be predicted to be important in the ability of TRCs to allow rapid transcellular movement of water under changing osmotic conditions in the oral cavity. Although AQP5 labeling was not apically restricted (Figures 3 and 4), it clearly was predominant toward the apical pole of the taste buds. The case was less clear, however, for AQP1 that appeared to have some overlap with the apical regions of the fungiform taste buds only (Figure 1). Indeed, the oral cavity is exposed to a variety of solution tonicities, ranging from extremely hypoosmotic (distilled water rinses) to high-salt solutions that may be an order of magnitude greater than normal salivary ion concentrations (Feldman and Barnett 1995). Clearly, the ability of TRCs to respond to changes in solution osmolarity has been well established. Afferent nerve recordings from the chorda tympani, glossopharyngeal, and laryngeal nerves have shown that water (e.g., decreased osmolarity) is an effective gustatory stimulus (Cohen et al. 1955; Zotterman

1956; Shingai 1980; Sakaguchi et al. 1989). Though in the present study we have limited our investigation to the lingual taste buds, our previous electrophysiological studies demonstrating rapid hypoosmotic responses in taste buds from the soft palate, nasopharynx, and epiglottis (Gilbertson 2002) suggest that similar water transport proteins may be present in these areas as well. Future studies are needed to address this issue directly.

AQP5 channels have been identified in a number of peripheral tissues including salivary and lacrimal glands, lung, trachea, and the eye (Ishida et al. 1997; Nielsen et al. 1997; Funaki et al. 1998; Hamann et al. 1998). Like our results in taste buds, AQP5 is typically localized to the apical membranes of these cells, many of which actively secrete water. Functionally, AQP5, like AQP1, is a constitutively open channel that permits rapid water movement but has no apparent glycerol permeability characteristic of other AQP subtypes, such as AQP3, 7, 9, and 10 (Agre et al. 2002; Yasui 2004). Transgenic mice lacking AQP5 exhibit decreased salivary secretion and increased salivary osmolarity (Ma et al. 1999), which has been shown to be a result of decreased water permeability in salivary acinar cells (Krane et al. 2001). In addition, these cells had a reduced ability to respond to hypertonic and hypotonic solutions effectively altering their ability to regulate cell volume. Based upon both our previous electrophysiological study (Gilbertson 2002) and the apical localization of AQP5 demonstrated in the present work, we would hypothesize that AQP5 channels may play a similar regulatory role in the oral cavity, enabling TRCs to respond to changing mucosal osmolarity and to permit the rapid water entry during water stimulation. No previous studies using AQP5 knockout mice have looked for direct effects on the gustatory system. However, because of the importance of saliva composition to taste cell function (Rehnberg et al. 1992), it might be predicted that alterations of the salivary composition in the AQP5 knockout mice alone (Ma et al. 1999; Krane et al. 2001) may yield changes in peripheral gustatory function.

The 2 additional AQPs we have identified in the present study, AQP1 and AQP2, were localized primarily to the basolateral membranes of the TRCs with little significant apical labeling in the posterior tongue. This distribution is consistent with the hypothesis that AQP1 and AQP2 could facilitate the water movement that enables the taste cells to regulate their volumes during changes in interstitial fluid or plasma tonicity. The ability of TEA, an effective inhibitor of water movement through AQP1 and AQP2 (Brooks et al. 2000; Yool et al. 2002; Detmers et al. 2006), to decrease the magnitude of hypoosmotic-induced currents in TRCs in the present study (Figure 6) is consistent with a functional role for one or both of these AQPs. Recent experiments have demonstrated that mammalian TRCs regulate their volume according to the osmolarity of the extracellular environment (Lyall et al. 1999; Gilbertson 2002) and that TRCs are capable of RVD during prolonged hypoosmotic stimulation (Gilbertson 2002). In our previous experiments, TRCs were perfused with hypoosmotic solutions over their entire surface, so it is impossible to determine if water movement through apical and/or basolateral AQPs was important for the rapid water entry and subsequent RVD. Clearly, our electrophysiological assays in this study suffered from the limitation of applying the AQP inhibitor, TEA, over the entire TRC surface. However, in our electrophysiological experiments using an intact lingual epithelium in a bipartitioned Ussing-style chamber that functionally separated the apical and basolateral membranes (Gilbertson et al. 2001), perfusion of water (after saline) onto the apical membranes of TRCs resulted in transient changes in whole-cell conductances consistent with apical water entry in approximately one-third of cells tested (Gilbertson TA, Boughter JD, Zhang H, Smith DV, unpublished data). Clearly, given these expression data, it would be reasonable to revisit these experiments using this modified Ussing chamber in order to attempt to determine the relative contributions of apical and basolateral water channels to hypoosmotic and hyperosmotic responses.

Though the immunocytochemical and molecular biological experiments do not shed insight into the specific functions of AQP1 and AQP2 in the taste system, the importance of these 2 AQPs has been demonstrated in a variety of other tissues. In addition to serving as pathways for water movement, these 2 AQPs have unique properties that may have implications for gustatory system function. AQP1 has been shown to function as a cGMP-gated cation channel (Yool and Weinstein 2002; Boassa et al. 2006). AQP1 channels and CNG channels share significant sequence similarity in their carboxyl termini (Anthony et al. 2000), the region in CNG channels that mutagenesis studies have shown to be important in binding cyclic nucleotides (Goulding et al. 1994; Varnum et al. 1995). Although it is unclear if AQP1 fulfils this role in the peripheral taste system, a cGMP-gated channel has been identified in mammalian TRCs (Misaka et al. 1997); however, this was of the CNG A3 subtype also found in cone photoreceptors and a subset of olfactory receptor neurons (Kaupp and Seifert 2002). To date, there has been no demonstration of a functional cGMP-activated conductance in TRCs. Transduction pathways for certain bitter tastants have been shown to result in an increase in intracellular cGMP concentrations in TRCs (Rosenzweig et al. 1999) though the downstream effect of cGMP, if any, remains elusive (cf. Gilbertson et al. 2000; Lindemann 2001).

AQP2 channels are the vasopressin-stimulated water channels of the kidney that are necessary for normal water conservation (Knepper and Inoue 1997; Sasaki et al. 1998). The majority of AQP2 channels are normally localized to intracellular vesicles, and upon vasopressin-mediated activation of type V2 receptors, they are rapidly inserted into the apical membranes to increase water transport. This is similar to the effects of vasopressin on epithelial sodium channel (ENaC) expression, where vasopressin, acting through V2-type receptors and the second messenger cGMP, leads to an

increase in functional ENaCs in the kidney (Ecelbarger et al. 2000). It has been previously shown that vasopressin stimulates amiloride-sensitive Na^+ currents (presumably via ENaC-like channels) in rat taste cells, an effect that was mimicked by membrane-permeant analogs of cGMP indicative of V2 receptor activation (Gilbertson et al. 1993). The effects of vasopressin on water transport and hypoosmotic or hyperosmotic responses in taste receptors cells have not been examined. Interestingly, AQP2 protein was absent from the area of the apical membranes in the present study, and we could not reliably determine if the labeling that was observed was primarily intracellular or membrane bound. Nonetheless, it will be interesting to follow these initial observations by determining if vasopressin is able to alter the normal distribution of AQP2 in TRCs, which may be indicative of the ability of the taste system (e.g., water taste) to be regulated by natriferic hormones.

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