Salivary Acinar Cells from Aquaporin 5-deficient Mice Have Decreased Membrane Water Permeability and Altered Cell Volume Regulation*

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Aquaporins (AQP5s) are channel proteins that regulate the movement of water through the plasma membrane of secretory and absorptive cells in response to osmotic gradients. In the salivary gland, AQP5 is the major aquaporin expressed on the apical membrane of acinar cells. Previous studies have shown that the volume of saliva secreted by AQP5-deficient mice is decreased, indicating a role for AQP5 in saliva secretion; however, the mechanism by which AQP5 regulates water transport in salivary acinar cells remains to be determined. Here we show that the decreased salivary flow rate and increased toxicity of the saliva secreted by Aqp5−/− mice in response to pilocarpine stimulation are not caused by changes in whole body fluid homeostasis, indicated by similar blood gas and electrolyte concentrations in urine and blood in wild-type and AQP5-deficient mice. In contrast, the water permeability in parotid and sublingual acinar cells isolated from Aqp5−/− mice is decreased significantly. Water permeability decreased by 65% in parotid and 77% in sublingual acinar cells from Aqp5−/− mice in response to hypertonicity-induced cell shrinkage and hypertonicity-induced cell swelling. These data show that AQP5 is the major pathway for regulating the water permeability in acinar cells, a critical property of the plasma membrane which determines the flow rate and ionic composition of secreted saliva.

The precise regulation of water and electrolyte transport in the acinar cells of the salivary gland is crucial for proper production of saliva. The fluid component of salivary secretions hydrates the oral cavity, aiding in the mastication and swallowing of food, in the neutralization of acids, and in protection against the invasion of potential pathogens. Clinically, salivary gland hypofunction commonly presents as xerostomia, a symptomatic complaint of dry mouth prevalent in the geriatric population (for review, see Ref. 1) which may result from either systemic or extrinsic causes (for review, see Refs. 1–3).

Saliva formation is a two-stage process (4, 5). First, the acinar cells secrete an isotonic plasma-like fluid, and second, ductal cells modify the acinar secretions primarily through the reabsorption of Na+ and Cl− so that the final saliva is hypertonic. This fluid secretion model predicts that saliva formation is primarily caused by transepithelial Cl− transport and that Cl− uptake is dependent on an inwardly directed Na+ chemical gradient across the basolateral plasma membrane. An increase in intracellular Ca2+, usually associated with muscarinic receptor stimulation, triggers fluid secretion by simultaneously activating apical Cl− channels and basolateral K+ channels. The efflux of Cl− and K+ across the apical and basolateral membranes, respectively, produces a transepithelial potential difference that is neutralized by paracellular Na+ transport across tight junctions. The resulting transepithelial osmotic gradient drives the movement of water, creating a plasma-like primary secretion.

In salivary gland acinar cells, secretion is associated with cell volume changes (6, 7). The shrinkage and swelling of salivary gland acinar cells following muscarinic and β-adrenergic stimulation, respectively, are thought to occur as a result of an imbalance between the influx and efflux of ions (specifically Cl−) between the luminal and basolateral membranes (8). The resulting change in tonicity requires a rapid and regulated change in acinar cell water permeability which is necessary for secretion and maintenance of cell volume following stimulation.

Aquaporin 5 (AQP5), a mercury-sensitive water channel, has been localized to the luminal surface of acinar cells in the salivary gland, the site of salivary secretion (9). Recently Ma et al. (10) showed that AQP5-deficient mice secrete a low volume of viscous hypertonic saliva after supramaximal pilocarpine stimulation. They hypothesized that AQP5 plays a role in regulating membrane water permeability and that it is also required for maintaining proper osmolality of the secreted saliva, although a mechanism by which this is accomplished was not examined. The results of this study are consistent with at least two potential mechanisms whereby hyposalivation might be induced in mice lacking AQP5. The simplest explanation is that AQP5 acts as the apical water pathway during stimulated fluid...
Here we directly measure membrane water permeability of isolated acinar cells from Aqp5+/+, Aqp5−/−, and Aqp5−/+ mice as well as flow rates and osmolality measurements of secreted saliva. Hyposalivation is not caused by changes in whole body fluid homeostasis, but instead, we demonstrate that the membrane permeability of salivary gland acinar cells is dramatically reduced in mice lacking AQP5. Our results are the first to provide a mechanism for AQP5 function during salivary secretion.

**Experimental Procedures**

**Generation of Aqp5 Replacement Targeting Construct—Mouse**

Genomic clones containing the Aqp5 locus were isolated from a 129SvJ mouse strain and characterized as described (13). Two fragments of the Aqp5 locus, an 1.8-kb KpnI left arm fragment and a 0.8-kb HindIII/BamHI right arm fragment, were inserted to flank the 3′- and 5′-ends of the PKG-neomycin resistance minigene (pPKG-NEO), respectively. A herpes simplex virus-thymidine kinase expression cassette (pMC1-TK) was positioned outside of the homologous segment, 5′ of the left arm in the 3′-5′ orientation. The replacement construct deletes 55 bp of intron 2, the entire exon 3 (84 bp), and 467 bp of intron three and replaces them with a 1.6-kb PKG-neomycin minigene cassette, resulting in a net addition of 1 kb. A 931-bp 5′-screening probe outside of the homologous region is indicated. UTR, untranslated region. Panel B, genotypic analysis of an F2 litter from sibling matings of Aqp5 heterozygous F1 founders. Mouse tail DNA was isolated and digested with BamHI. Southern hybridization was performed using a 931-bp 5′-screening probe. All three genotypes are represented in this litter: +/+ (4.5 kb), +/+ (4.5 kb/5.5 kb), and −/− (5.5 kb).

**Panel C** Northern hybridization of total RNA from Aqp5−/+ and Aqp5−/− parotid glands. A mouse AQP5 cDNA clone containing the entire open reading frame and 3′-untranslated region was a-32P labeled and used to hybridize 20 μg of adult mouse parotid gland total RNA from Aqp5−/+ and Aqp5−/− mice (n = 2 each genotype). A 1.8-kb transcript is indicated with an arrow. The 28 S and 18 S RNA bands were visualized by UV illumination of the ethidium bromide-stained agarose gel and are shown to demonstrate RNA quality and loading. Panel D, Western analysis of total membrane preparations from Aqp5−/+, Aqp5−/−, and Aqp5−/+ parotid glands. An AQP5 rabbit polyclonal antibody (LL639; 0.5 μg/ml) generated against a C-terminal peptide specific to mouse AQP5 sequence was used in immunoblotting experiments against 20 μg of total membrane proteins isolated from the parotid glands of Aqp5−/+, Aqp5−/−, and Aqp5−/+ mice. Two immunoreactive bands at 27 and 29 kDa are indicated in the Aqp5−/+ and Aqp5−/+ lanes by arrows but are absent in the Aqp5−/+ lanes (n = 2 each genotype). Protein isolation and Western blotting were performed as described (see “Experimental Procedures”).

**Fig. 1. Mouse Aqp5 gene targeting construct, Southern, Northern, and Western analyses.** Panel A, generation of an Aqp5 locus-specific replacement type targeting vector. A schematic of the genomic organization and partial restriction map of the wild-type mouse Aqp5 locus based on analysis of a 129SvJ genomic subclone (11) is shown. B, BamHI; H, HindIII; K, KpnI. Exons are shown as indicated. Two fragments of the Aqp5 locus, a 1.8-kb KpnI left arm fragment and a 0.8-kb HindIII/BamHI right arm fragment, were inserted to flank the 3′- and 5′-ends of the PKG-neomycin resistance minigene (pPKG-NEO), respectively. A herpes simplex virus-thymidine kinase expression cassette (pMC1-TK) was positioned outside of the homologous segment, 5′ of the left arm in the 3′-5′ orientation. The replacement construct deletes 55 bp of intron 2, the entire exon 3 (84 bp), and 467 bp of intron three and replaces them with a 1.6-kb PKG-neomycin minigene cassette, resulting in a net addition of 1 kb.

Experimental Stem Cell Targeting—Isogenic ES cells derived from the 129SvJ mouse strain were electroporated with 50 μg of linearized targeting vector. After electroporation, the cells were subjected to selection with G418 and gancyclovir as described (15). A 931-bp diagnostic probe 5′ to the homologous region indicated in the targeting vector was used to screen 187 ES cell clones by Southern analysis. The probe was polymerase chain reaction amplified from an Aqp5 genomic clone (13) using 10 pmol of each primer (forward primer (4.5 Seq) 5′-CCG-CGAGAACAACAGACTC-T3′; reverse primer (Pri Ext 3) 5′-CCGATCTCTTCGGCTCAGCGC-3′); 0.25 mM each dNTP, 2.0 mM MgCl2, 60 mM Tris-HCl at pH 9.0, 12.5 mM (NH4)2 SO4, 10 ng of 2.1/4.5 plasmid DNA (11), 0.1 unit of Taq polymerase (Life Technologies, Inc.) in a total reaction volume of 20 μl. Polymerase chain reaction was performed in an MJ PTC-100 Thermocycler device (Watertown, MA) with the following conditions: 94 °C for 2 min; (92 °C for 30 s−57 °C for 1 min−72 °C for 1 min) 35 cycles; −72 °C for 7 min. Products were separated by gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained agarose gel and are shown to demonstrate RNA quality and loading.

**Embryonic Stem Cell Targeting—Isogenic ES cells derived from the 129SvJ mouse strain were electroporated with 50 μg of linearized targeting vector. After electroporation, the cells were subjected to selection with G418 and gancyclovir as described (15). A 931-bp diagnostic probe 5′ to the homologous region indicated in the targeting vector was used to screen 187 ES cell clones by Southern analysis. The probe was polymerase chain reaction amplified from an Aqp5 genomic clone (13) using 10 pmol of each primer (forward primer (4.5 Seq) 5′-CCG-CGAGAACAACAGACTC-T3′; reverse primer (Pri Ext 3) 5′-CCGATCTCTTCGGCTCAGCGC-3′); 0.25 mM each dNTP, 2.0 mM MgCl2, 60 mM Tris-HCl at pH 9.0, 12.5 mM (NH4)2 SO4, 10 ng of 2.1/4.5 plasmid DNA (11), 0.1 unit of Taq polymerase (Life Technologies, Inc.) in a total reaction volume of 20 μl. Polymerase chain reaction was performed in an MJ PTC-100 Thermocycler device (Watertown, MA) with the following conditions: 94 °C for 2 min; (92 °C for 30 s−57 °C for 1 min−72 °C for 1 min) 35 cycles; −72 °C for 7 min. Products were separated by gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained agarose gel and are shown to demonstrate RNA quality and loading.
electrophoresis on a 1% low melting point agarose (Life Technologies, Inc.) in 1 x TAE buffer and gel extracted (Qiagen gel extraction kit, Valencia, CA). The replacement construct deletes 55 bp of intron 2, the targeted allele resulting from homologous recombination were identified by agarose gel electrophoresis on a 1% low melting point agarose (Life Technologies, Inc.) with calcein-AM (Molecular Probes, Eugene, OR).

Cell volume was estimated by confocal microscopy, as described (18). Cells were allowed to adhere to the base of a superfusion chamber mounted on an Olympus FMT2 fluorescence microscope interfaced with an Ultima™ confocal microscope (Genomic Solutions, Ann Arbor, MI). Intracellular dye was excited with 488 nm band of an argon laser and emitted fluorescence measured at 530 nm. Changes in cell volume were monitored by measuring the fluorescence intensity of calcein within a defined intracellular volume. In combination with an Olympus Dplapo 40× objective, a 225-μm confocal pinhole produces an ~4-μm-thick optical section in the z direction. Using Ultima™ software, an x-y area of the two-dimensional image was circumscribed within individual acini. In some experiments, cell volume was estimated using a Nikon Diaphot 200 microscope interfaced with an Axon Imaging Workbench system (Foster City, CA). Cells were excited at 490 nm, and emitted fluorescence was measured at 530 nm. The initial linear rate of cell volume change was used as an index of acinar cell water permeability. Cell volume was correlated with fluorescence by in situ calibration of the dye performed using solutions of different osmolalities. The relationship between dye fluorescence and the volume change was linear over a volume range from +30% to −30%, which is within the physiological range of cell volume changes observed in acinar cells. Cell volume was expressed in arbitrary units as 1/normalized calcein fluorescence.

Mercury-sensitive Water Permeability—The initial linear rate of cell volume change was used as an index of acinar cell water permeability. Aqp5+/+ and Aqp5−/− parotid and sublingual acinar cells were enzymatically dispersed and loaded with fluorescent dye as described above and subsequently equilibrated in an isosmotic (~300 mosm), intracellular-like solution to eliminate ion gradients. The solution contained 15 mM NaCl, 50 mM KCl, 75 mM potassium gluconate, 0.4 mM KH2PO4, 0.33 mM NaH2PO4, 20 mM HEPES, 10 mM glucose, 0.8 mM MgSO4, and 1.2 mM CaCl2. A 30% hypertonic shock was induced by perfusion of sacin in the above solution containing 90 mM sucrose, and the rate of cell volume change was determined ("control" rate). Sucrose was then removed to permit the cell volume to reequilibrate before exposure to 1 mM HgCl2 for 5 min. The same cells were then exposed to a second hypertonic shock in the presence of HgCl2. The rate of volume change in the presence of 1 mM HgCl2 was used to calculate the mercury-sensitive water permeability of acinar cells.

**TABLE I**

| Osmolality | Agonist concentration |
|------------|-----------------------|
| Aqp5+/+    | Aqp5−/−               |
| 2 mg pilocarpine/kg body weight | 2 mg pilocarpine/kg body weight |
| 5 min      | 165.8 ± 6.5           | 225.8 ± 16.8*               |
| 10 min     | 174.0 ± 11.1          | 229.7 ± 18.4*               |
| 15 min     | 173.0 ± 10.7          | 246.5 ± 17.0*               |
| 10 mg pilocarpine/kg body weight | 10 mg pilocarpine/kg body weight |
| 5 min      | 196.5 ± 4.1           | 257.0 ± 9.8*                |
| 10 min     | 216.5 ± 10.1          | 271.8 ± 14.6*               |
| 15 min     | 221.5 ± 6.9           | 272.3 ± 10.1*               |

*Statistically significant values are indicated (*) (p < 0.05).
Aqp5 generated by replacing 600 bp of the mouse expressed as vary, nasal, and tracheal glands. Saliva was collected from the lower sublingual secretions, with a very minor component from minor salivary, nasal, and tracheal glands. saliva was collected from the lower cheek pouch by a suction device at intervals of 5, 10, and 15 min and expressed as μl/min. The osmolality of the saliva was measured using a vapor pressure osmometer (Wescor 5500, Logan, UT).

Water Intake and Urine Output Analysis—Adult Aqp5+/+ and Aqp5−/− littersmates (n = 12 each genotype; n = 6 male, n = 6 female per genotype) were housed one per metabolic cage and acclimated for 48 h prior to urine collection. Mice had free access to drinking water and standard 1% NaCl mouse chow diet throughout the experiment. Baseline urine samples were collected over a period of 24 h for 3 consecutive days, and the volume, electrolyte composition, and osmolality were recorded. Aliquots of urine samples were centrifuged at 10,000 × g for 5 min to remove any suspended material, and the supernatants were determined using a Labconco Digital Chloridometer (Kansas City, MO).

Blood gas and plasma electrolyte measurements of Aqp5+/+ and Aqp5−/− mice

Blood gas/electrolyte

| pH | pCO₂ | pO₂ | Na⁺ | K⁺ | Cl⁻ | HCO₃⁻ |
|----|------|-----|-----|-----|-----|-------|
| 7.436 ± 0.011 | 31.950 ± 1.994 | 83.833 ± 5.703 | 149.333 ± 0.422 | 5.945 ± 0.252 | 113.000 ± 0.856 | 21.240 ± 0.823 |
| 7.437 ± 0.015 | 35.160 ± 0.829 | 82.460 ± 3.795 | 150.8 ± 0.583 | 5.968 ± 0.288 | 113.000 ± 0.837 | 22.775 ± 0.641 |

RESULTS

Generation of Aqp5−/− Mice—The Aqp5 targeted allele was generated by replacing 600 bp of the mouse Aqp5 gene, which includes a portion of intron 2, all of exon 3, and a portion of intron 3, with the neomycin resistance gene (Fig. 1A). The targeted replacement results in the deletion of extracellular loop E of the mouse AQP5 protein, which contains the highly conserved Asn-Pro-Ala (NPA) motif (20) and the mercury-sensitive cysteine residue at position 182 (13). Alterations in the NPA motifs in either the B or E loops have been shown to disrupt water permeability in aquaporin family members (21). Six independently derived Aqp5 targeted ES cell lines were identified by the presence of both 5.5-kb and 4.5-kb BamHI fragments by Southern analysis corresponding to the targeted and wild-type alleles respectively (not shown). Chimeras were generated through blastocyst injection of two ES cell lines, and germline transmission was obtained. A representative Southern blot from an F1 heterozygous mating is shown in Fig. 1B. We observed a birth genotypic ratio of 1 (+/+); 2 (+−); 0.5 (−−), suggesting a role for Aqp5 in prenatal survival. Adult Aqp5−/− mice weighed ~90% of their Aqp5+/+ and Aqp5−/− age- and sex-matched littersmates (not shown). No difference in morbidity, mortality, or longevity was observed among the three genotypes from birth to >1 year of age (not shown). Submandibular, sublingual, and parotid glands from 12–16-week-old Aqp5+/+, Aqp5−/−, and Aqp5−/− littersmates were histologically normal as revealed by light microscopic analysis of hematoxylin and eosin-stained tissue sections (not shown).

Northern Analysis of Salivary Gland Total RNA—Northern analysis was performed on total RNA isolated from parotid glands from Aqp5+/+, Aqp5−/−, and Aqp5−/− littersmates using a mouse AQP5 cDNA probe containing the entire open reading frame and the 3′-untranslated region. A 1.5-kb band corresponding to the mouse AQP5 transcript was observed in RNA from +/+ and +− mice but was not present in the RNA from Aqp5−/− glands (Fig. 1C). Thus, the targeted replacement of the Aqp5 locus results in the absence of AQP5 mRNA in Aqp5−/− mouse salivary glands. Identical results were obtained with total RNA from the sublingual and submandibular glands (not shown). Western Analysis of Salivary Gland Total Membrane Preparations—Immunoblotting of total membrane fractions prepared from parotid glands from Aqp5+/+, Aqp5−/−, and Aqp5−/− mice with an anti-AQP5 polyclonal antibody identified both the 27-kDa and 29-kDa AQP5 immunoreactive bands in Aqp5+/+ and Aqp5−/− mice, which were reported previously in mouse salivary glands (13). Neither the 27-kDa nor the 29-kDa bands were present in Aqp5−/− glands (Fig. 1D). Therefore, the targeted replacement of the Aqp5 locus ablates AQP5 protein production and results in AQP5 null mice. Identical results were obtained with total membrane fractions from the sublingual and submandibular glands (not shown).

Salivary Flow Rate—A previous study has shown that Aqp5−/− deficient salivary glands produce less saliva in response to a supramaximal concentration of a cholinergic agonist (80 mg/kg pilocarpine), and the saliva was hypotonic (420 mosM) (10) rather than hypotonic as in wild-type mice. In the present study, two different physiological concentrations of agonist (2 and 10 mg of pilocarpine HCl/kg of body weight, injected intraperitoneally) were used to stimulate salivary secretion. The
induced Aqp5
S.E.; in the continued presence of HgCl₂. Changes in cell volume are ex-
shock by the addition of 60 mM sucrose during the time indicated by the
Parotid acinar cells from wild-type mice were exposed to a hypertonic
crease as cell volume decreases in response to hypertonic shock.
the effect of HgCl₂. HgCl₂ inhibits the rate of
induced rate of shrinkage of parotid acinar cell from wild-type mice and
all three intervals with both pilocarpine concentrations was
ments, the osmolality of stimulated salivary secretions was
determined for three 5-min intervals over a 15-min duration (Table I). These
results indicate that the final osmotic composition of stimulated saliva
is affected by AQP5 expression.

Fluid Intake and Urine Output—To determine whether the
absence of AQP5 affects whole animal fluid homeostasis, water
intake and urinary volume output were monitored in adult
Aqp5−/− and Aqp5+/- mice (Table II). Interestingly, there were
no significant differences in the volume of water intake or urine
excreted by Aqp5−/− mice compared with their age- and sex-
matched littermates. In addition, urine osmolality, potassium,
sodium, and chloride concentrations did not differ between
wild-type and AQP5 knockout mice (Table II).

Blood Gas and Electrolyte Analysis—To examine the role of
AQP5 in the maintenance of blood gas and plasma electrolyte
homeostasis, blood samples from awake adult Aqp5+/+ and
Aqp5−/− mice were collected and analyzed for plasma elec-
lytes, blood pH, and blood gas levels. No significant
differences were observed in these parameters when comparing the AQP5
knockout mice with wild-type littersmates (Table III). Taken
together, the results shown in Tables II and III demonstrate that the
hyposalivation observed in Aqp5−/− mice is not caused
by changes in whole animal fluid and electrolyte homeostasis.

AQP5-dependent and Mercury-sensitive Acinar Cell
Water Permeability—AQP5 was initially identified and cloned from
the rat submandibular gland and was shown to be a mercury-
sensitive water channel (22). To examine whether AQP5 is
pilocarpine-stimulated salivary flow rate was determined for
Aqp5+/+ and Aqp5−/− mice at three 5-min intervals over the
course of 15 min (see Fig. 2). The flow rate for Aqp5−/− mice at all
three intervals with both pilocarpine concentrations was
inhibited significantly compared with the rate observed for
Aqp5+/+ mice (range = 45–80% of the control rate; mean ±
S.E. = 64.7 ± 6.8% inhibition). Thus, AQP5 deficiency results
in a sustained −65% decrease in the rate of pilocarpine-stim-
ulated saliva flow regardless of the agonist concentration used.
These data demonstrate that AQP5 is critically important to
salivation, independent of the magnitude of receptor
activation.

Salivary Osmolality—In addition to flow rate measure-
ments, the osmolality of stimulated salivary secretions was
determined for three 5-min intervals over a 15-min duration
after stimulation with 2 or 10 mg of pilocarpine/kg of body
weight (Table I). Osmolality was increased significantly (p < 0.01) in the saliva from Aqp5−/− mice compared with their
wild-type littermates, and it remained increased over the col-
clection period at all three intervals (Table I). These results
indicate that the final osmotic composition of stimulated saliva
is affected by AQP5 expression.

FIG. 3. Cell volume changes: mercury-sensitive hypertonicity-
induced parotid acinar cell shrinkage. Panel A, hypertonicity-
induced rate of shrinkage of parotid acinar cell from wild-type mice and
the effect of HgCl₂. HgCl₂ inhibits the rate of Aqp5−/− parotid acinar

cell volume changes induced by hypertonic shock. The cell volume
of mouse parotid acinar cells was estimated by confocal microscopy using
the intracellular fluorescent dye calcein. Acinar cells were loaded with
the fluoroprobe by incubation for 15 min with 2 μM calcein-AM, and the
fluorescence intensity emitted from within a defined intracellular vol-
ume was monitored. The normalized fluorescence intensity (Fn) in-
creases as cell volume decreases in response to hypertonic shock.
Parotid acinar cells from wild-type mice were exposed to a hypertonic
shock by the addition of 60 mM sucrose during the time indicated by the
x-axis (followed by the break in the x axis) followed by exposure to a second hypertonic shock in the
continued presence of HgCl₂. Changes in cell volume are ex-
pressed as 1/Fn. Panel B, summaries of the rates of hypertonicity-
induced Aqp5+/+ and Aqp5−/− parotid acinar cell shrinkage in the
absence and presence (stippled bar) of 1 mM HgCl₂. The asterisks (*)
indicate a significant difference in the rate of cell shrinkage of Aqp5+/+
(filled bar) in the presence of mercury (50%; p < 0.0001, n ≥ 36), and a
−65% decrease in Aqp5−/− (open bar) compared with the intrinsic
Aqp5+/+ rate. The membrane permeability of Aqp5−/− acinar cells was
enhanced (** p < 0.0001; n ≥ 36) in the presence of mercury compared with
untreated Aqp5−/− cells.
involved in acinar cell water permeability, the rate of volume change was determined in wild-type cells in response to a hypertonic stress in the presence and absence of HgCl₂. Water movement was osmotically driven by introducing a 30% hypertonic solution. Mercury did not inhibit water movement in parotid acinar cells but actually enhanced the water permeability of acinar cells. Mercury did not inhibit water movement in Aqp5⁻/⁻ acinar cells but actually enhanced the water permeability of both parotid and sublingual Aqp5⁻/⁻ acinar cells by an unknown mechanism (Figs. 3B and 4B; \( p < 0.0001; n \geq 36 \)).

**Fig. 6.** Targeted disruption of the Aqp5 gene inhibits hypertonicity-induced cell swelling and the associated RVD sublingual acinar cells. The role of AQP5 in the RVD response was examined in sublingual acinar cells loaded with calcine as described under “Experimental Procedures.” Panel A, sublingual acini isolated from Aqp5⁺/⁺ (solid line) and Aqp5⁻/⁻ (dotted line) were perfused in an isotonic solution, and then hypertonic cell swelling was induced by switching the perfusate to a second medium diluted with 30% water during the time interval indicated by the cross-hatched rectangle. Changes in cell volume are represented as values of 1/Fₚ, Panels B and C, summaries of the relative rates of swelling (panel B) and RVD (panel C), respectively, in Aqp5⁺/⁺ (filled bar) and Aqp5⁻/⁻ (open bar) of parotid acinar cells. Values represent mean ± S.E. of \( n \approx 36 \) cells from three different experiments. Significant differences from the control are indicated by asterisks (*) (\( p < 0.05 \)).
regulate their cell volume subsequent to swelling ~2.7-fold faster than sublingual acini from wild-type mice. Because the water permeabilities of parotid and sublingual acini were comparable in response to anisosmotic challenges (see Figs. 5B and 6B), the differences in the rates of RVD were probably caused by differences in the membrane permeability of ions in these two cell types.

DISCUSSION

Defects in water channel protein expression and/or function have been implicated in the pathogenesis of inherited and acquired forms of diseases of fluid imbalance (23, 24). To understand the molecular mechanisms by which the aquaporins regulate water balance in mammals, targeted disruption of individual aquaporins in mice has been actively investigated (AQP1 (25), AQP3 (26), AQP4 (27), AQP5 (10)).

We used AQP5-deficient mice to dissect the mechanisms by which AQP5 functions in the regulation of acinar cell volume and in the stimulation of salivary secretion. Northern and Western analyses show that mice homozygous for the targeted allele produce no full-length AQP5 mRNA and are null for AQP5 protein, respectively. Phenotypically, Aqp5−/− mice are 10% smaller in body weight compared with wild-type littermates. Birth genotypic ratios were 1:2:0.5 and deviated from the expected 1:2:1 Mendelian ratio, suggesting a role for AQP5 in prenatal development. The ratios observed by us also differ from a previously published report by Ma et al. (10), indicating an observed 1 (Aqp5+/+) : 1 (Aqp5+/−) : 0.4 (Aqp5−/−) ratio of F2 litters in an independently generated AQP5-deficient mouse strain. It is possible that the difference observed between our results (1 (Aqp5+/+) : 2 (Aqp5+/−) : 0.5 (Aqp5−/−)) and the ratios reported by Ma et al. (10) is attributable to a difference in the genetic backgrounds of the two Aqp5-deficient strains.

Functionally, AQP5 deficiency results in dramatically reduced saliva production during pilocarpine stimulation. This result suggests two potential mechanisms whereby Aqp5 disruption might induce hyposalivation. The simplest explanation is that AQP5 is required for transcellular water movement. Alternatively, targeted disruption of the Aqp5 gene may alter whole animal water and electrolyte balance, resulting in a state of dehydration, a condition known to inhibit salivation (11, 12). To test this latter hypothesis, we measured multiple parameters related to whole animal water and electrolyte homeostasis. Loss of AQP5 function did not alter serum electrolyte and gas levels in AQP5 knockout mice. Likewise, urine osmolality and electrolyte composition, and urine output and water intake were not significantly different between wild-type and knockout mice, suggesting that AQP5 deficiency does not alter whole animal fluid homeostasis under normal physiological conditions.

Thus, decreased saliva production by mice lacking AQP5 cannot be explained easily by an indirect effect of water and electrolyte imbalance. This conclusion strongly suggests that the secretion defect observed in Aqp5−/− mice is caused by a loss of a critical transcellular water movement pathway. In fact, AQP5 deficiency results in a large decrease in mercury-sensitive, acinar cell water permeability as well as decreased ability of acinar cells to regulate cell volume under anisosmotic conditions. The regulation of acinar cell volume during salivary secretion is a dynamic process influenced by muscarinic and β-adrenergic stimulation, resulting in cell shrinkage and swelling, respectively. Mechanistically, changes in transepithelial osmotic forces drive fluid movement into the lumen and correlate with changes in acinar cell volume. Our data suggest that AQP5 is responsible for mediating the bulk of the acinar cell water permeability under anisosmotic conditions (≥65%). Interestingly, we observed that the addition of mercury to isolated Aqp5−/− parotid and sublingual acinar cells resulted in a relatively small but significant increase in water permeability. A recent study by Yasui et al. (30) reported a similar effect of mercury on the osmotic membrane permeability of oocytes expressing AQP6, a related water channel. It is therefore possible that an AQP6-like molecule is expressed in salivary gland acinar cells which is enhanced by the presence of mercury. It is also possible that mercury works nonspecifically to affect other membrane proteins, thereby affecting membrane permeability. Consistent with this latter possibility, mercury was also shown to mimic the effects of low pH on the activation of ion conductance in AQP6-expressing oocytes (30).

The Aqp5 knockout mouse has also allowed us to evaluate the importance of this water channel in the context of whole animal physiology. The in vivo analysis of pilocarpine-stimulated salivary secretion and osmolality revealed that AQP5 is critically important in determining both saliva flow rates and final ionic composition. Aqp5 null mice secrete saliva at an ~65% slower rate than wild-type mice, consistent with the ≥65% reduction of water permeability of acinar cells in the knockout mice (Fig. 2). In our studies, the average osmolality during 15 min of saliva collection from wild-type mice was 171 mosM and 212 mosM during 2-mg and 10-mg pilocarpine stimulation, respectively. Our results are consistent with the observation that mammalian saliva, including that of the mouse saliv (29), is generally hypotonic (28). It is possible, but unlikely, that the difference in osmolality seen in our study and that reported by Ma et al. (10) is caused by the supramaximal concentration of pilocarpine used by Ma et al. (80 mg/kg of body weight), as our measurements of the average osmolality of the saliva collected from wild-type mice was 202 ± 2.6 mosM when 80 mg of pilocarpine was used (data not shown, n = 6). Thus we also performed experiments using lower concentrations of pilocarpine which are likely closer to the physiological range of agonist concentrations (see Table I). Genetic background differences may explain the variation we observed in the osmolality between our Aqp5 knockout mice and the Aqp5 strain examined by Ma and colleagues (420 mosM).

Taken together, the cell volume measurements and in vivo measurements of saliva flow rates and composition reveal the critical mechanism by which fluid secretion is accomplished in the salivary gland. The cell volume measurements directly show that AQP5 regulates salivary secretion by increasing the membrane water permeability of acinar cells and that AQP5 regulates the cell volume of individual acinar cells. To date, this is the first reported evidence that deficiency in a water channel dramatically affects the regulation of cell volume in a native tissue. Based on the significant effect of AQP5 ablation on fluid secretion in the salivary gland, it is likely that other members of the mammalian AQP family which are involved in secretory or absorption may also be involved in controlling cell volume. The AQP5-deficient mouse may thus prove to be a useful animal model to investigate pathophysiological mechanisms of salivary gland dysfunction in humans.

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