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# Identification of aquaporin-5 and lipid rafts in human resting saliva and their release into cevimeline-stimulated saliva

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ABSTRACT

*Background:* It is unknown whether AQP5 and lipid rafts are released into human unstimulated (resting) saliva and saliva in response to secretagogues.
*Methods:* In order to quantitate the salivary concentration of AQP5, we produced a polyclonal antibody for human AQP5 and developed an enzyme-like immunosorbent assay (ELISA).
*Results:* AQP5 and lipid rafts were identified in human resting saliva. The amount of AQP5 in resting saliva showed a diurnal variation with high levels during waking hours, and an age-related decrease in AQP5 was coincident with the volume of resting saliva. Cevimeline, a muscarinic acetylcholine receptor (mAChR) agonist, induced the release of AQP5 with lipid rafts, amylase, mucin, and lysozyme. Changes in saliva AQP5 levels after cevimeline administration occurred simultaneously with changes in saliva flow rates. Confocal microscopy revealed that AQP5 was located in the apical plasma membrane and showed a diffuse pattern in parotid glands under resting conditions. Following cevimeline administration, AQP5 was predominantly associated with the APM and was localized in the lumen.
*General significance:* AQP5 and lipid rafts were released with salivary proteins from human salivary glands by the stimulation of $M_3$ mAChRs, and that changes in saliva AQP5 levels can be used as an indicator of salivary flow rate and also as a useful index of $M_3$ mAChR agonist's action on human salivary glands.


## 1. Introduction

The plasma membrane is a major barrier to water transport. The apical plasma membrane (APM) of the serous cells in the salivary glands is also impermeable to water which is the main component of saliva. It is widely accepted that aquaporins (AQPs), which are water channel proteins, are a family of membrane proteins which play a crucial role in the regulation of membrane permeability to water [1]. The permeability of the APM to water in salivary glands increases rapidly in response to $M_3$ muscarinic acetylcholine receptor (mAChR) agonists and $\alpha_1$-adrenoceptor (ADR) agonists. Several AQPs are expressed in salivary glands, and AQP5, a member of the AQP family, is highly expressed in parotid glands [2]. We previously reported that $M_3$ mAChR agonists and $\alpha_1$-ADR agonists caused salivary fluid secretion by the translocation of AQP5 and lipid rafts containing flotillin-2 and ganglioside GM1 to the APM of acinar and duct cells in rat parotid glands, and that the translocation of AQP5 with lipid rafts from the cytoplasm to the APM was followed by the dissociation of AQP5 to non-rafts in the APM by $Ca^{2+}$ signaling via their respective receptors [3–6]. Soluble guanylate cyclase (sGC)/cyclic guanosine 3′, 5′-monophosphate (cGMP) signal transduction plays a crucial role in $Ca^{2+}$ homeostasis with $M_3$ mAChR agonist- and $\alpha_1$-ADR agonist-induced increases in AQP5 levels in the rat parotid gland APM [7]. Cevimeline which acts at the $M_3$ mAChR in isolated rat parotid acinar cells, has been shown to induce a persistent oscillatory increase in $Ca^{2+}$ which led to an increase in the amount of AQP5 in the APM of these cells [8]. Recently, attention has been focused on the fate of AQP5 and lipid rafts after translocation to the APM in parotid glands by $M_3$ mAChR agonists and $\alpha_1$-ADR agonists.

It has been reported that AQP2, which is localized in the apical region of the collecting duct cells of the kidney, was excreted into

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urine in response to vasopressin in rats [9] and normal men [10], as well as in patients with nephrogenic diabetes insipidus [10] and those with congestive heart failure [11]. Changes in the amount of urinary AQP2 excretion were shown to be a useful index of vasopressin's action on the kidney [10].

However, it is not known whether AQP5 is released into saliva in response to secretagogues. In this study involving healthy subjects, we investigated whether AQP5 was released with lipid rafts and secretory proteins into resting saliva, and whether $M_3$ mAChR agonist-stimulated AQP5 release into saliva correlated with changes in salivary flow rate. Lipid rafts, cholesterol- and sphingolipid-enriched microdomains, were proposed to explain the vectorial transport of membrane proteins to the APM in polarized cells [12,13]. Co-assembly of flotillins into these microdomains induces the formation of membrane curvature and vesicle budding [14].

We prepared a polyclonal anti-human AQP5 antibody and found when using this antibody that AQP5 was released with lipid rafts and salivary proteins into human resting saliva. This release followed a circadian rhythm where levels were high and low during waking and sleeping hours, respectively. In addition, cevimeline, an $M_3$ mAChR agonist, stimulated the release of AQP5 into saliva, and the AQP5 levels in resting and stimulated saliva changed in proportion to flow rate. These findings show that the changes in human salivary AQP5 levels can be used to demonstrate the flow rates of resting and stimulated saliva from human salivary glands and to demonstrate the action of $M_3$ mAChR agonists on the salivary glands, and may also have diagnostic value in xerostomic conditions induced by stress, autoimmune diseases, and xerogenic drugs.

## 2. Experimental procedures

### 2.1. Participant selection and collection of human saliva

This study enrolled 42 healthy adults (20 men and 22 women), ranging in age from 20 to 80 years. The participants were all volunteers who were provided with written and oral information regarding the risks and benefits of the study procedures. Each participant signed a written consent form. This study received ethical clearance and approval by the appropriate Committees of Tokushima University. In general, saliva was collected at 2:00 p.m. In some experiments, saliva was collected at 4-hourly intervals for 24 h, starting at 9:00 a.m. Participants gently chewed on a Salivette (Sarstedt, Nummbrecht, Germany) swab for 1 min and the saliva collected was used as the control (resting) saliva. In another experiment, saliva was collected at 10-minute intervals for 3 h after a single oral dose of cevimeline (30 mg/60 kg body weight) starting at 10 a.m. These samples were used as the cevimeline-stimulated (stimulated) saliva. All samples were centrifuged at 1000 g for 10 min to remove cellular elements before use.

### 2.2. Collection of animal saliva

Cevimeline (10.0 mg/kg) was injected intraperitoneally into rats (12-week-old, male Wistar rats), wild-type mice (12-week-old male mice, C57BL6/G) and AQP5-null mice (which were provided by Dr. Alan S. Verkman) [15]. During the first 30 min after cevimeline administration, saliva was collected by pipette.

### 2.3. Cloning of human AQP5 and antibody preparation

Polyclonal anti-human AQP5 antibody against human AQP5 cloned from human saliva was prepared as follows: in brief, nested, degenerated oligonucleotide primers were designed to correspond to the deduced C-terminal region (amino acids 159–290) of human AQP5. The sense primer was 5′-AAGAATTCGGAGCTGATTCT-GACCTTCCA-3′, and the antisense primer was 5′-AACTCGAGT-CAGCGGGTGGTCAGCTCCA-3′, where GAATTC and CTCGAG were EcoR I and Zho I sites, respectively. The cDNA was amplified by PCR (35 cycles: 94 °C, 15 s; 60 °C, 30 s; 68 °C, 60 s) using salivary gland quick-clone cDNA. The purified cDNA was ligated into the PCR-Blunt vector at the EcoR I/Zho I site. The ligation mixture was transformed into *E. coli*. The plasmid was extracted using the Miniprep kit (Quigen, Mississauga, Canada) and was verified using the DNA Analysis System (BECKMAN COUNTER CEQ 2000). The sequenced AQP5 cDNA corresponded to Gen BankTM accession number NM001651. The cDNA encoding human AQP5 (amino acids 159–290) in the plasmid, epitope-tagged with thioredoxin (Trx), was subcloned into the pET32b vector (Novagen Inc, Darmstadt, Germany) and transformed into *E. coli,* which was grown in LB medium then solubilized with 1% Triton X-100 and sonicated. After centrifugation at 10,000 rpm for 20 min, the pellet was washed using the same procedure. The pellet was solubilized with 50 mM Tris–HCl (pH 7.4) containing 7 M guanidine. A portion of the pellet was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue (CBB). The solubilized protein was applied to a Ni-NTA column (Quigen, GmbH, Germany) and then eluted with 50 mM Tris–HCl (pH 7.4) containing 7 M guanidine. After refolding with 50 mM Tris–HCl (pH 7.4) containing 8 M urea, the refolded solution was dialyzed with 50 mM Tris–HCl (pH 7.4). Each eluted fraction was subjected to SDS-PAGE and stained with CBB. Rabbit polyclonal antibody was raised against this recombinant AQP5 (OPP21703). The polyclonal antibodies were purified via the Ni-NTA column as described above. Specificity of this antibody (OPP21702) was verified by Western blotting.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

A 96-well plate was incubated with 5 μg/ml of anti-human AQP5 rabbit IgG (OPP21702) overnight at 4 °C and blocked with Tris–HCl buffer saline (pH 8.0) containing 0.1% bovine serum albumin and 0.05% sodium azide. The defrosted saliva was applied to each well of the antibody-coated plate and incubated overnight at 4 °C. Recombinant AQP5 was used as the standard. The wells were washed three times with Tris–HCl wash buffer (pH 8.0) containing 0.05% Tween 20 and 0.9% NaCl, followed by 100 μl of a final 1:1000 dilution of biotinylated-OPP21703. The plate was incubated for 2 h at room temperature. Each well was then washed three times with the wash buffer described above. The binding of OPP21703 was determined using the horseradish peroxidase streptavidin method.

### 2.5. Western blot analysis

Defrosted saliva samples were treated with solubilizing buffer [16] and the samples with 15 μg of protein or 15 μl were subjected to SDS-PAGE in 12.5% linear PAG. Following this procedure, the separated proteins were transferred to a nitrocellulose transfer membrane (Hybond ECL; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using Trans Blot apparatus (Bio-Rad, Hercules, CA, USA). Western blot analysis was then performed as described previously [5]. In brief, the blots were probed with rabbit anti-human AQP5 (OPP21702) (1:1500 dilution), rabbit anti-amylase (1:1000 dilution), rabbit anti-mucin MUG7 (1:1000 dilution), rabbit anti-lysozyme (1:1000 dilution), goat anti-flotillin-2 (1:1500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ganglioside GM1 (1:1500 dilution, Calbiochem-Novabiochem, Darmstadt, Germany), or with these antibodies preadsorbed with the excess synthetic peptides used to raise the respective polyclonal antibodies, as described previously [5]. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunodetection was performed according to the ECL method (Amersham). Chemiluminescence was measured using Chemi Doc apparatus (Bio-Rad) and analyzed using Quantity One software (Bio-Rad).