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

Yan Pan [a,d], Fusako Iwata [b], Di Wang [a], Masahiro Muraguchi [b], Keiko Ooga [b], Yasukazu Ohmoto [b], Masaaki Takai [b], Gota Cho [a], Jinsen Kang [a], Masayuki Shono [c], Xue-jun Li [d], Ko Okamura [e], Toyoki Mori [b], Yasuko Ishikawa [a,*]

[a] Department of Pharmacology, The University of Tokushima Graduate School of Dentistry, 3-18-15, Kuramoto-cho, Tokushima, 770-8504, Japan
[b] Research Institute of Pharmacological and Therapeutical Development, Otsuka Pharmaceutical Co., Ltd., 463-10, Kagasuno Kawauchi-cho, Tokushima, 771-0192, Japan
[c] Support Center for Advanced Medical Sciences, The University of Tokushima, 3-18-15, Japan
[d] Department of Pharmacology, Health Science Center, Peking University, Beijing 100191, China
[e] Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan

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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 α_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 α_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 α_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 α_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

* Corresponding author. Tel.: +81 88 633 7332; fax: +81 88 632 0093.
E-mail address: isikawa@dent.tokushima-u.ac.jp (Y. Ishikawa).

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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).

2.6. Immunohistochemistry

Cevimeline (10.0 mg/kg) was injected into the rat tail vein. At 0 and 6 min after the injection, the parotid glands were quickly removed from the rats and embedded in Jung tissue freezing medium (Leica Instruments, Heidelberg, Germany) and rapidly frozen with liquid nitrogen. Following preparation of the sections, they were incubated with rabbit anti-human AQP5 antibody (OPP21702) and goat anti-flotillin-2 antibody (1:1000 dilution, Santa Cruz Biotechnology). The labeling was visualized with streptavidin-conjugated Alexa Fluor 488 or Alexa Fluor 568 (1:1000 dilution, Molecular Probes, Leiden, The Netherlands). To stain the nuclei, sections were incubated with 0.5 μg/ml of RNaseA then incubated with 50 μg/ml of propidium iodine (PI) for 1 h at 37 °C. Fluorescence images of sections excited at 488 nm or 588 nm and simultaneously at both wavelengths were captured with a confocal laser scanning microscope (Leica TCS NT) equipped with an Ar-Kr Laser and a ×40 dry objective (Leica Plan Apochromat), as described previously [5].

2.7. Other techniques

Saliva osmolality was measured using a vapor pressure osmometer (5520 Vapro, Wescor Inc. Logan, UT, USA).

2.8. Statistical analysis

Data are presented as means±standard error (S.E) and were analyzed for statistical significance using Student's *t*-test or analysis of variance at all points. Linear relationships between key variables were tested using Pearson's correlation coefficient. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of monoclonal antibody to recombinant human AQP5

The cDNA encoding human AQP5 was subcloned into the pET32b vector and was transformed into *E. coli*. The Trx-tagged recombinant protein was produced. A 27 kDa protein corresponding to the position of the monomer of human AQP5 was observed by SDS-PAGE (Fig. 1A). The inclusion body was solubilized and refolded. The recombinant protein was purified by Ni-NTA column chromatography (Fig. 1B). The amino acid sequence of the purified protein was analyzed and verified to be the predicted amino acid sequence of human AQP5. Rabbits were immunized using the recombinant protein. Specificity of this antibody was verified by Western blotting (Fig. 1C). Immunoblot analysis of human (Fig. 1D, lane 2), rat (Fig. 1D, lane 3), and wild-type mouse (Fig. 1D, lane 4) saliva after centrifugation at 1000 *g* for 10 min showed a clear solitary band with a mobility corresponding to a predicted molecular mass of 27 kDa. In AQP5-null mouse saliva (Fig. 1D, lane 5), no bands were detected, showing that rabbit anti-human AQP5 antibody (OPP21702) was able to cross-react with rat salivary AQP5 and mouse salivary AQP5.

3.2. Identification of AQP5 in human saliva

Salivary glands produce saliva and secrete it into the oral cavity throughout the 24-hour period. As shown in Table 1, human salivary glands secreted the fluid and proteins at a flow rate of 1.22±0.03 ml/min and 1.36±0.10 mg/min, respectively, at 9.00 a.m. Immunoblot analysis of human saliva using anti-human AQP5 antibody (OPP21702) showed a clear band at 9.00 a.m. (Fig. 2A). The band was fully ablated by the antibody preadsorbed with the excess immunizing peptide (data not shown). AQP5 was not recognized in the sediments obtained from the saliva by differential centrifugation at 550 g and then 1450 g for 10 min

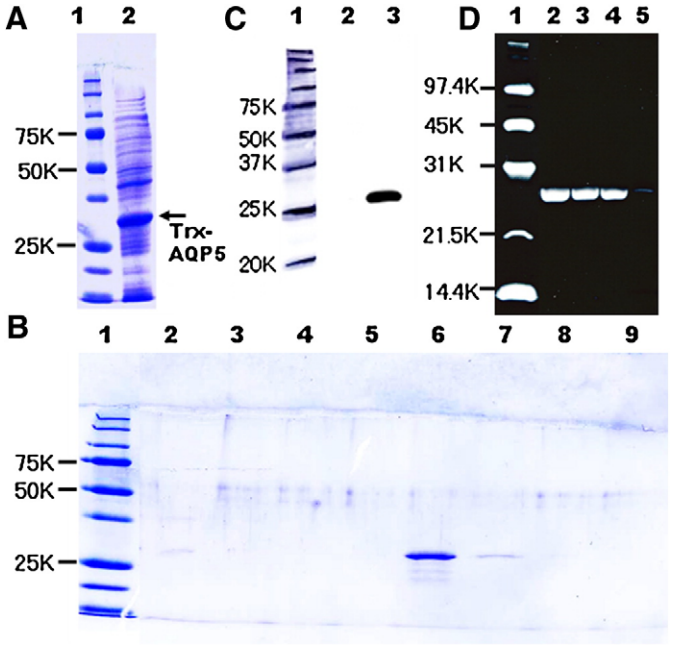

Fig. 1. Cloning of human AQP5 and characteristics of anti-human AQP5 antibody. (A) Human AQP5 cDNA was subcloned into the pET32b vector and the recombinant protein was expressed in *E. coli*. The sediment of *E. coli* lysate (lane 2) and protein marker (lane 1) was subjected to SDS-PAGE and stained with Coomassie brilliant blue (CBB). (B)The *E. coli* lysate was applied to a Ni-NTA column. Washing fractions (lanes 2 and 3) and eluted fractions (lanes 4 to 9) were subjected to SDS-PAGE and stained with CBB. (C) The recombinant human AQP5 (OPP21703) (0.5 μg of protein) (lane 3) and vehicle (lane 2) were subjected to SDS-PAGE for Western blotting with anti-human AQP5 (OPP21702). Protein marker (lane 1) was subjected to SDS-PAGE and stained with CBB. (D) After injection of cevimeline (10 mg/kg) intraperitoneally, saliva was collected from rats (lane 3), wild-type mice (lane 4), and AQP5-null mice (lane 5) for 30 min. Saliva was also collected from healthy men (lane 2). After centrifugation at 1000 g for 10 min, supernatants (15 μg of protein) were subjected to SDS-PAGE for Western blotting with anti-human AQP5 (OPP21702).

(data not shown). These findings showed that intact AQP5 was present in human saliva and that cells, zymogen granules and their fragments were not the source of AQP5 in the saliva. In human saliva, GM1 and flotillin-2 were also recognized along with AQP5, amylase, mucin, and lysozyme (Fig. 2A). GM1, a member of the raft-associated gangliosides, and flotillin-2, a raft-associated protein, were used as markers of lipid rafts [17]. Lipid rafts have been implicated in a number of cell functions, such as protein sorting to the APM and trafficking [12,13,18], as well as receptor signaling [19,20]. Amylase, mucin, and lysozyme are major components of saliva [21] and are markers of stress [22], non-immune host defense system [23], and heart disease [24], respectively. It has been reported in rat parotid gland cells, that cevimeline induced the translocation of AQP5 and lipid rafts from the cytoplasm to the APM, followed by the dissociation of AQP5 to non-rafts in the APM [5]. These findings show that AQP5 and lipid rafts are released along with amylase, mucin, and lysozyme into the saliva from human salivary glands.

3.3. Circadian rhythm of AQP5 secretion into human saliva

Healthy participants aged 20 to 39 years, who ate meals at 7:00 a.m., 12:00 a.m. and 7:00 p.m., and who slept from 11.00 p.m. to 6.00 a.m., showed a circadian rhythm in resting saliva AQP5 levels. As shown in Fig.2B, the AQP5 level in saliva collected at 9:00 a.m. was 1.5±0.1 ng/ml. This level was maintained until 9:00 p.m. (1.6±0.3 ng/ml), then decreased markedly to 0.8±0.1 ng/ml at 1:00 a.m. and to 0.5±0.2 ng/ml at 5:00 a.m. These changes in AQP5 levels in resting saliva showed a circadian variation which had one peak; the AQP5 concentrations were high and low during waking and sleeping hours, respectively. The flow rate of resting saliva (Table 1) showed a similar circadian variation to that

Table 1
Diurnal variation in volume, protein concentration, ratio, AQP5, lipid rafts, amylase, mucin and lysozyme in human saliva

Time of day	9 a.m.	1 p.m.	5 p.m.	9 p.m.	1 a.m.	5 a.m.
Salivary volume (ml/min)	1.22±0.03	1.38±0.03	1.51±0.08	1.51±0.08	0.50±0.05	0.63±0.03
Protein concentration in saliva (mg/ml)	1.36±0.10	1.50±0.06	1.49±0.01	1.50±0.18	0.92±0.11	1.03±0.07
Ratio	0.92±0.04	0.90±0.06	0.97±0.03	0.94±0.02	0.83±0.04	0.95±0.09
AQP5 (%)	100	127±12	118±9	123±9	45*±5	56*±6
GM1 (%)	100	124±12	109±10	101±8	59**±8	52*±8
Flotillin-2 (%)	100	144**±11	129±9	138±9	43*±5	44*±6
Mucin (%)	100	133±6	128±6	95±8	46*±5	40*±5
Lysozyme (%)	100	106±8	109±8	111±8	44*±6	31*±5
Amylase (%)	100	128±9	119±9	122±11	64**±6	40*±5

The ratio was expressed as the flow rate of saliva to the amount of AQP5. Chemiluminescence in Fig. 2A was measured using Chemi Doc apparatus and analyzed with Quantity One software. The analysis (%) of AQP5, GM1, flotillin-2, mucin, lysozyme and amylase were expressed as the relative intensity with the data at 9 a.m.. The values shown are the means of four separate experiments±S.E. A value of $p<0.05$ was considered significant (*<0.01, **<0.05).

of salivary AQP5 concentration as shown in Fig. 2. In unstimulated conditions, the average ratio of the salivary flow rate to the AQP5 level at each time point described above was from 0.83±0.04 to 0.97±0.03 (Table 1). There is no significant difference between these figures, suggesting that the AQP5 level in resting saliva from humans of the same age, can be used as an index of salivary flow rate. The levels of lipid rafts, amylase, mucin and lysozyme in resting saliva showed similar diurnal variations to that of AQP5 level.

3.4. Age-dependent changes in AQP5 levels and salivary flow rates

It is generally thought that saliva secretion decreases markedly in the elderly. Therefore, in order to investigate the age-dependent changes in AQP5 level and the flow rate of resting saliva, the AQP5 level and volume of resting saliva from men and women ranging in age from 20 to 80 years were measured. As shown in Fig. 3, the AQP5 levels were 1.50±0.15 and 1.59±0.17 ng/ml in the resting saliva of men and women, respectively, in the 20 to 29 years age group. A gradual decline to 0.70±0.03 ng/ml for men and 0.63±0.20 ng/ml for women was observed in those aged 60 to 69 years, and a decline to 0.80±0.04 ng/ml for men and 0.55±0.26 ng/ml for women in those aged 70 to 79 years. These findings show that there were no differences in the AQP5 levels and flow rates of resting saliva from men and women ranging in age from 20 to 49, however, differences in these parameters were observed in both men and women aged from 50 to 79 years. These results clearly show that there were age-dependent decreases in resting salivary AQP5 levels and flow rates in healthy subjects. This is consistent with a previous finding that xerostomia observed in the elderly, was induced by impairment of the translocation of AQP5 to the APM of salivary gland cells [25]. These findings also show that the AQP5 level in resting saliva obtained from participants in the same age group can be used as an index of salivary flow rate.

3.5. Effect of cevimeline on salivary AQP5 levels over time

It has been reported that cevimeline induces long-lasting salivation [26] and stimulates amylase secretion by the activation of M_3 mAChRs in rat parotid glands [27–29]. The results in Fig. 4 demonstrate that, in healthy subjects aged 20 to 39 years, a single oral dose of cevimeline (30 mg/60 kg body weight) induced changes in fluid and protein flow rates. Forty minutes after cevimeline administration, increases in the fluid and protein flow rates were apparent, and maximum levels were reached at 70 min, which then gradually declined to the levels found in resting saliva at 160 min (Table 2). Immunoblot analysis showed that the changes in AQP5 levels in

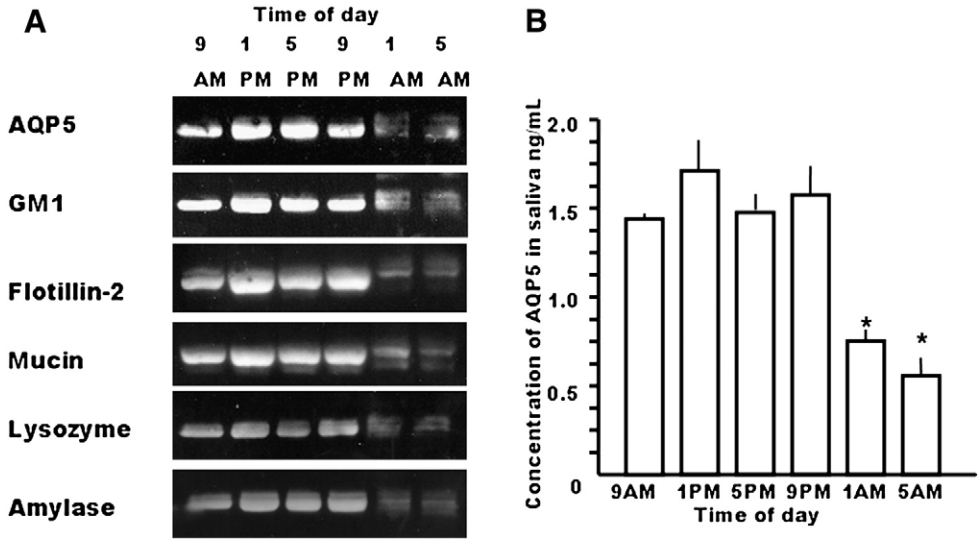

Fig. 2. Immunoblots of the diurnal variation of AQP5, lipid rafts, amylase, mucin and lysozyme in human saliva. Saliva was collected for 1 min from healthy subjects, ranging in age from 20 to 39 years, at intervals over a 24-hour period and was centrifuged at 1000 g for 10 min. (A) The resultant supernatant was used for volume and protein concentration measurements. 15 μl of the supernatant was subjected to SDS-PAGE for Western blotting with anti-human AQP5, anti-ganglioside GM1, anti-flotillin-2, anti-amylase, anti-mucin, and anti-lysozyme antibodies. (B) 50 μl of the supernatant was applied to the 96-well plate coated with the anti-human AQP5 (OPP21702). After incubation, the plate was washed well and then incubated with biotinylated-recombinant AQP5 (OPP21703). The binding of OPP21703 was determined using the horseradish peroxidase streptavidin method. A value of $p<0.05$ was considered significant (*<0.01 vs. 9 a.m.).

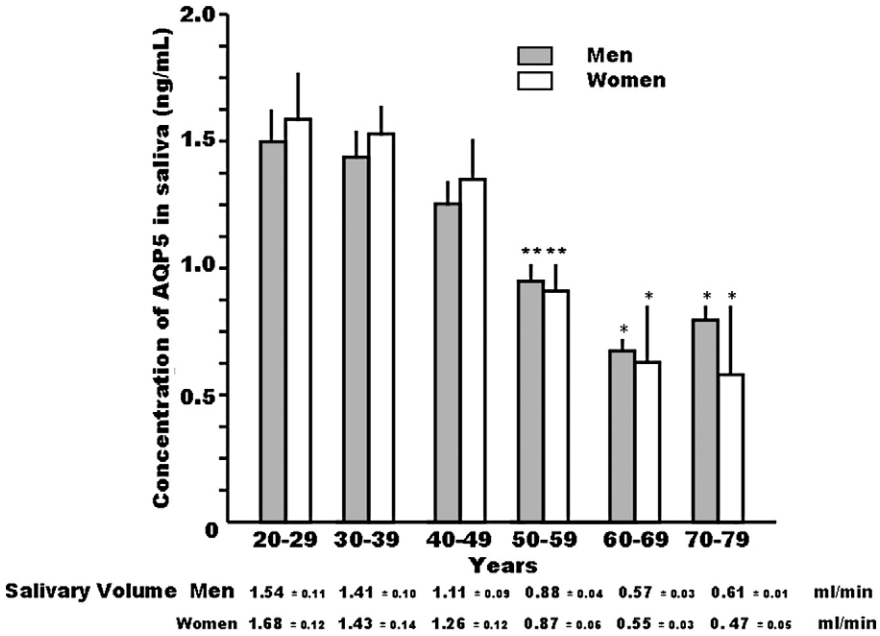

Fig. 3. Age-related changes in AQP5 levels and saliva flow rates in human resting saliva. Saliva was collected for 1 min from men and women, ranging in age from 20 to 80 years, and was centrifuged at 1000 g for 10 min. The resultant supernatant was used for volume measurements and the concentration of AQP5 using ELISA. The values and bars show the means of 3 to 6 separate experiments ± S.E. A value of $p<0.05$ was considered significant (*<0.01 vs. 20–29 years, **<0.05 vs. 20–29 years).

human saliva after cevimeline administration were parallel to the changes in lipid rafts, amylase, mucin, and lysozyme (Fig. 4). The changes in these levels were proportional to the flow rates of saliva and protein in the saliva. A strong negative correlation was observed between osmolality and the concentration of AQP5 in saliva after a single oral dose of cevimeline ($R=-0.813$, $p<0.001$) (Table 2). These findings show that, in humans of the same age, changes in salivary AQP5 levels can be used as an index of salivary flow rate and of the action of M_3 mAChR agonists on salivary glands.

3.6. Correlation between salivary AQP5, salivary amylase and salivary volume

The relationship between the concentration of AQP5 and amylase in saliva and the salivary flow rate was investigated (Fig. 5). Salivary AQP5 levels correlated with salivary volume in resting ($R=0.896$, $p<0.001$) and stimulated saliva ($R=0.939$, $p<0.001$) (Fig. 5). In resting saliva, the concentration of AQP5 ranged from 0.4 ng/ml to 2.2 ng/ml. In stimulated saliva, AQP5 concentration ranged from 1.8 ng/ml to 8.5 ng/ml. Salivary amylase levels also correlated with salivary volume ($R=0.757$, $p<0.001$) and salivary protein concentration ($R=0.872$, $p<0.001$) in stimulated saliva. Although the concentration of AQP5 and amylase in saliva and the salivary flow rate paralleled each other, the correlation between salivary AQP5 levels and the salivary flow rate was a closer fit than that between salivary amylase levels and the salivary flow rate.

3.7. Cevimeline-induced changes in confocal immunofluorescence microscopic images of AQP5 and lipid rafts in acinar and duct cells of rat parotid glands

In order to directly visualize cevimeline-induced secretion of AQP5 and lipid rafts into saliva, cevimeline (10 mg/kg) was injected into the rat tail vein. Six minutes after cevimeline administration, AQP5 and flotillin-2 immunofluorescence was associated predominantly with the APM domains in both acinar (Fig. 6, B-1 and B-2) and duct cells (Fig. 6, B-3 to B-5), and were also found in the lumen (Fig. 6, B-3 to B-5) of the ducts in these cells in the parotid glands. Conversely, in

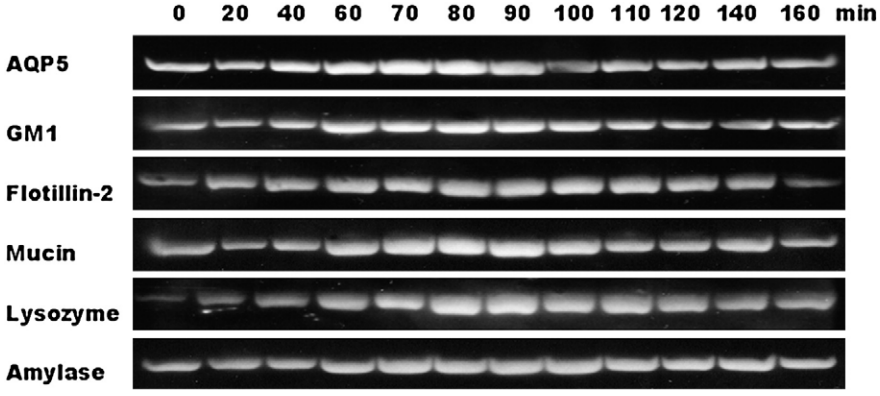

Fig. 4. Immunoblots showing the effects of cevimeline on human salivary AQP5, GM1, flotillin-2, mucin, lysozyme and amylase levels over the time course. Saliva was collected for 1 min at 20 min intervals for 160 min from healthy subjects ranging in age from 20 to 40 years after a single oral dose of cevimeline (30 mg/60 kg of body weight). After centrifugation at 1000 g for 10 min, 15 μl of the resultant supernatant was used for volume and protein concentration measurements, as well as for Western blot analysis with anti-human AQP5, anti-GM1, anti-flotillin-2, anti-amylase, anti-mucin, and anti-lysozyme antibodies.

Table 2
Effect of cevimeline on human salivary volume, osmolarity, protein concentration, AQP5, GM1, flotillin-2, mucin, lysozyme and amylase over the time course

Time (min)	0	20	40	60	70	80	90	100	110	120	140	160
Salivary volume (ml/min)	0.94±0.31	1.08±0.02	1.13±0.26	1.22±0.31	2.78*±0.28	2.26*±0.81	2.06*±0.40	1.96*±0.02	1.54**±0.42	1.38±0.49	1.04±0.01	0.94±0.73
Osmolarity (mmol/kg)	147±8	118±6	104±8	97±6	82**±3	78**±5	80**±7	90**±6	99±6	106±9	116±5	138±10
Protein concentration (mg/ml)	1.36±0.02	1.28±0.02	1.67±0.19	2.19*±0.06	2.98*±0.03	2.83*±0.25	2.35*±0.02	2.27*±0.11	2.46*±0.11	1.87**±0.05	1.66±0.12	1.55±0.10
AQP5 (%)	100	102±6	127±9	150**±11	282*±9	232*±12	164**±11	146**±14	121±9	118±8	104±9	89±6
GM1 (%)	100	106±2	122±5	204*±10	205*±3	225*±5	203*±7	174**±6	135±6	121±9	107±10	105±8
Flotillin-2 (%)	100	126±8	163**±8	212*±6	258*±12	330*±5	321*±15	319*±6	260*±11	195*±9	127±5	75±10
Mucin (%)	100	113±5	162**±8	277*±6	316*±18	447*±15	430*±17	364*±16	326*±6	267*±9	157**±11	125±9
Lysozyme (%)	100	103±3	148**±8	285*±14	329*±3	428*±12	367*±9	300*±16	206*±9	201*±7	166*±9	126±5
Amylase (%)	100	102±6	102±11	154**±8	169**±9	180*±15	215*±17	189*±8	171**±12	167**±10	157**±5	140±3

Saliva was collected for 1 min at 10 min intervals for 160 min from healthy subjects after a single oral dose of cevimeline (30 mg/60 kg of body weight). After centrifugation at 1000 *g* for 10 min, the resultant supernatant was used for volume, osmolarity and protein concentration measurements. Chemiluminescence in Fig. 4 was measured using Chemi Doc apparatus and analyzed with Quantity One software. The analysis (%) of AQP5, GM1, flotillin-2, mucin, lysozyme and amylase were expressed as the relative intensity with the data at 0 min. The values shown are the means of four separate experiments±S.E. A value of $p<0.05$ was considered significant (*<0.01, **<0.05).

unstimulated condition (zero time after cevimeline), AQP5 and flotillin-2 staining was observed with a diffuse pattern in the cytoplasm and was not found in the lumen of the ducts in parotid glands (Fig. 6, A). Nuclei were stained with PI in order to compare easily the intracellular distribution of AQP5 in the unstimulated condition with that in the stimulated condition (Fig. 6, A-1, A-2, B-1, and B-2). The distribution of AQP5 was fully ablated by the anti-human antibody (OPP21702) preadsorbed with the excess

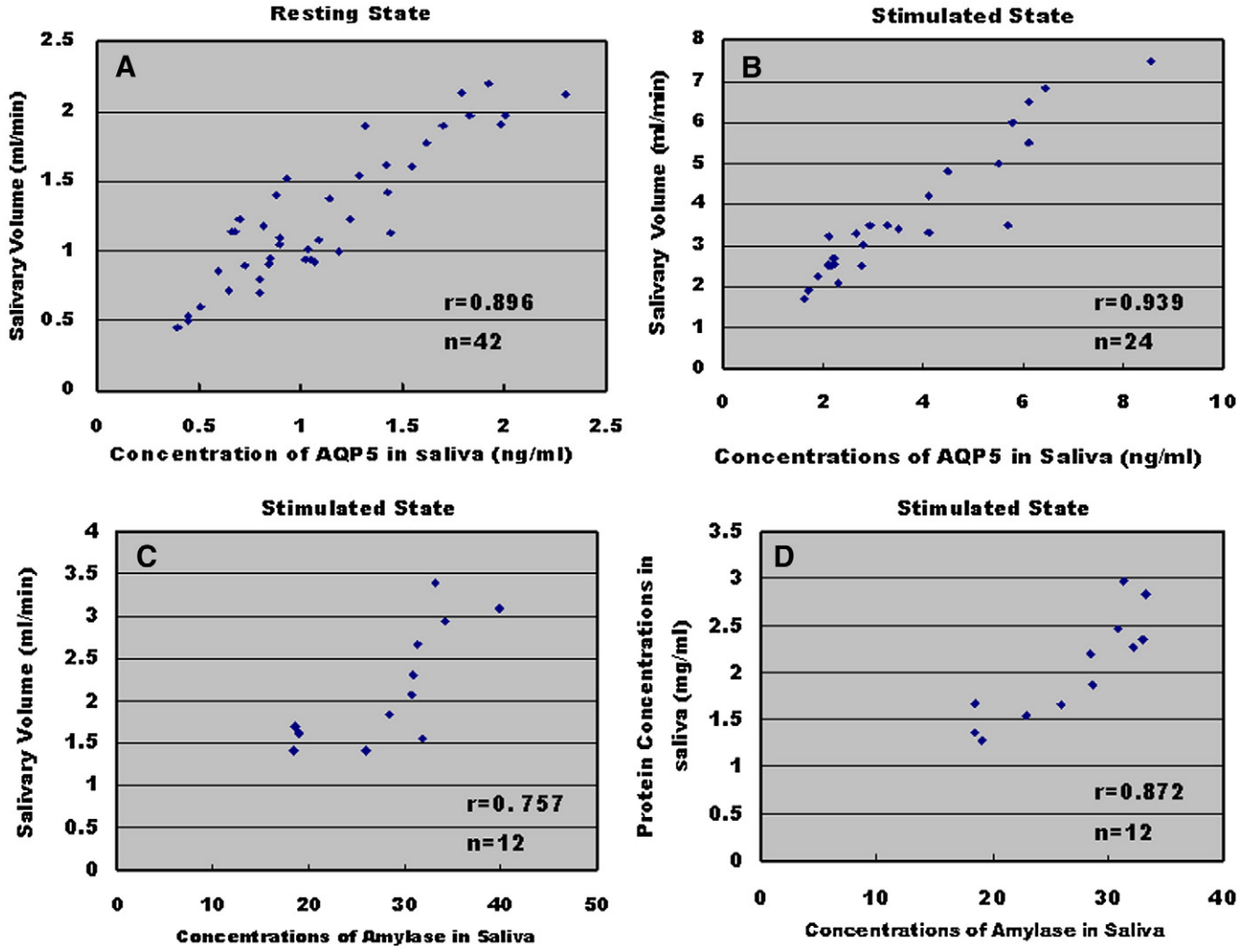

Fig. 5. Scattergrams comparing AQP5 and amylase levels to salivary volume or protein levels in human saliva. Correlations between the concentrations of salivary AQP5 and salivary volume are shown in human resting saliva (A) and stimulated saliva (B). The correlation between salivary amylase levels and salivary volume (C) and the correlation between salivary amylase levels and salivary protein levels (D) are shown in human stimulated saliva.

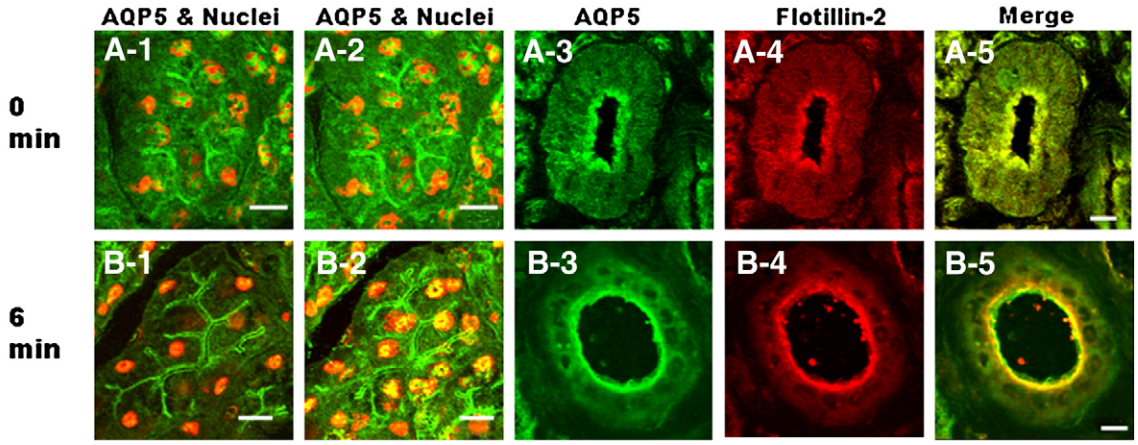

Fig. 6. Changes in confocal immunofluorescence microscopic images of tissue slices with AQP5 in acinar and duct cells of rat parotid glands treated with cevimeline. The rat parotid glands removed at 0 (A) and 6 (B) min after intravenous injection of cevimeline (10 mg/kg) were rapidly fixed by immersion and then sectioned. The sections were immunostained with anti-human AQP5 antibody (OPP21702) to detect AQP5 in acinar (-1 and -2) and duct (-3, -4, and -5) cells using Alexa Fluor 488 (green). Nuclei were counterstained with PI (red) (-1 and -2). Sixteen consecutive section images obtained with a laser confocal microscope were projected to generate a single image (-2). The sections were also immunostained with anti-flotillin-2 antibody using Alexa Fluor 568 (red) (-3,-4, and -5). Bars, 10 μm.

immunizing antigen (OPP21703) (data not shown). These findings show that cevimeline induced the translocation of AQP5 with lipid rafts to the APM regions of acinar and duct cells of rat parotid glands and then secreted them into the saliva.

4. Discussion

Resting saliva is secreted by the salivary glands into the oral cavity and has an important role in preventing xerostomic conditions. In this study, we prepared a polyclonal anti-human AQP5 antibody (Fig. 1), and using this antibody we detected intact AQP5 with lipid rafts and salivary proteins not only in human saliva stimulated by cevimeline, an M_3 mAChR agonist, (Fig. 4), but also in resting saliva (Figs. 2 and 3). These findings show that, in humans, the release of AQP5 into resting and stimulated saliva is controlled by M_3 mAChR-related mechanisms in the salivary glands. This was supported by the confocal immunofluorescence microscopic images which showed that cevimeline induced translocation of AQP5 with lipid rafts from the cytoplasm to APM in both acinar and duct cells of rat parotid glands and released them into saliva (Fig. 6). The changes in AQP5 levels in resting saliva showed a diurnal variation with one peak. High AQP5 concentrations were observed during waking hours and low concentrations were observed during sleeping hours (Fig. 2). However, in healthy human subjects, there was no circadian variation in the changes in the ratio of the flow rate of resting saliva to the amount of AQP5 in saliva (Table 1). This shows that the responsiveness of human salivary glands to cholinergic stimulation does not change during a 24-hour period, and that the amount of AQP5 in resting saliva can be used as an index of salivary flow rate.

It was previously reported that the amount of amylase secreted into the resting saliva of rats showed a diurnal variation, which coincided with changes in the number of maximal binding sites (B_{max} values) for [^{3}H] dihydroalprenolol in rat parotid membranes [30]. In addition, the clock gene is known to be expressed in salivary glands [31]. The diurnal variation of the AQP5 level in the resting saliva of healthy subjects may be regulated by diurnal variation of M_3 mAChR and α_1-ADR activities.

Elderly people often complain of xerostomia. However, the mechanisms underlying xerostomia have not been clarified. As shown in Fig. 3, AQP5 levels and salivary flow rate decrease markedly with age. It was previously reported that an ACh-induced increase in the amount of AQP5 in the APM of parotid glands in young adult rats was not observed in senescent rats, and that AQP5 responsiveness to cholinergic stimulation in the parotid gland cells of senescent rats decreased significantly [25]. In rats, cevimeline induces long-lasting salivation by persistent oscillatory increases in [Ca^{2+}]i via M_3 mAChRs in isolated parotid acinar cells, and increases the amount of AQP5 in the APM [8]. sGC/cGMP signal transduction has a crucial role in Ca^{2+} homeostasis in the cevimeline-stimulated increase in AQP5 levels in the APM of rat parotid glands [7]. It has also been shown that there was no difference in the total amount of AQP5 in parotid glands between young adult and senescent rats [25]. However, under stimulated conditions with cevimeline or ACh, there was a marked increase in fluorescence for AQP5 in the APM of parotid cells in young adult rats, but not in senescent rats [25]. A correlation between the AQP5 level in saliva and the salivary flow rate before and after cevimeline administration was clearly observed (Fig. 4). The AQP5 level and the resting salivary flow rate decreased markedly in participants older than 50 years (Fig. 3). These findings indicate that an age-related impairment in the responsiveness of AQP5 in rat parotid gland cells to muscarinic stimulation might account for the concomitant changes in protein kinase G activity in the cells, and may induce age-related xerostomia.

Lipid rafts are known as cholesterol- and glycolipid-enriched microdomains [12] and have been implicated in membrane sorting and trafficking [12,13,18], as well as receptor signaling [19,20]. Lipid rafts are also rich in integral proteins such as flotillins, caveolins, and stomatins [13]. Flotillin-1 [32] and flotillin-2 [33] have been implicated in vesicular cycling between the plasma membrane and intracellular compartments in an unconventional, clathrin- and caveolin-independent pathway. Cevimeline induced sorting of AQP5 and flotillin-2 from the cytoplasm to the APM and released AQP5 and flotillin-2 to saliva (Fig. 6). As previously reported, cevimeline induced the translocation of AQP5 with lipid rafts from the cytoplasm to the APM by Ca^{2+} signaling and the dissociation of AQP5 from lipid rafts to non-rafts in the APM [5]. The increase in AQP5 levels in the APM of rat parotid glands enhanced the permeability of the membrane to water in response to osmolarity and the secretion of saliva. After stimulation was stopped, AQP5 was released in saliva to retrieve the resting APM. Salivary glands are innervated by the autonomic nervous system. Even in the resting condition, spontaneous secretion is present as a normal phenomenon using AQP5 located in the APM. AQP5 and lipid rafts are also released to saliva even in the resting condition.

In this study, we developed an ELISA system to measure the concentration of salivary AQP5. Using this system in healthy humans, the quantity of AQP5 ranged from 0.4 ng/ml to 2.2 ng/ml in resting saliva and from 1.8 ng/ml to 8.5 ng/ml in cevimeline-stimulated saliva. In both types of saliva, there was a good correlation between salivary

AQP5 levels and the salivary flow rate (Fig. 5). When measuring saliva volume, humans accumulate saliva in the oral cavity. To prevent artificial modification, the measurement of salivary AQP5 concentrations is a useful tool in determining physiological conditions related to salivary volume.

On activation of M_3 mAChRs with cevimeline, AQP5 was translocated to the APM and then released into the saliva. In the confocal microscopy experiments (Fig. 6), AQP5 and the lipid rafts were detected in the lumen of the parotid ducts (Fig. 6, B-3 to B-5). Primary saliva is formed in acinar cells and this primary saliva is modified in duct cells [21]. The physiological function of AQP5 in duct cells is not clear. It was reported that the activation of M_3 mAChRs induced fluid secretion in rat parotid intralobular ducts [34], suggesting that parotid ducts had a water secretion function. In parotid ducts, AQP5 may play a role in secretion or absorption depending on salivary osmolarity. After completing this function, some AQP5 may be released into the lumen to rearrange the membrane function.

It was reported that AQP2 located in the kidneys was translocated to the APM in response to vasopressin, that an increase in the amount of AQP2 enhanced the permeability of the APM to water, and that vasopressin stimulated endocytosis of the APM containing AQP2 [35]. This retrieval of membrane may be involved in readjustment of the permeability of vasopressin-responsive tissue to water. Human saliva is gaining interest in proteomic and biomarker discovery studies [36]. As shown in this study, AQP5 and lipid rafts were identified in the resting and stimulated saliva of normal subjects (Figs. 2–4). These findings indicate that the release of AQP5 and lipid rafts into saliva may also be involved in readjustment of the permeability of the APM in salivary glands to water. Further studies are needed to clarify the physiologic role of the secretion of AQP5 and lipid rafts into saliva. Finally, we determined using polyclonal anti-human AQP5 antibody that cevimeline stimulated the secretion of AQP5 with lipid rafts into human saliva, and that the changes in salivary AQP5 level could be used as an index of salivary flow rates and of the action of M_3 mAChR agonists on salivary glands.

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