Pflugers Arch - Eur J Physiol (2016) 468:519–539
DOI 10.1007/s00424-015-1749-6

INVITED REVIEW

Physiological role of aquaporin 5 in salivary glands

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Received: 31 July 2015 / Revised: 15 October 2015 / Accepted: 16 October 2015 / Published online: 5 November 2015

Abstract Regarding the 13 known mammalian aquaporins (AQPs), their functions in their expressing tissues, effects of their mutation/polymorphisms in humans, and effects of knockout of their genes are summarized in this review article. The roles of AQP5, an exocrine gland-type water channel, in the salivary gland under normal and pathophysiological conditions are reviewed in detail. First, the involvement of AQP5 in water secretion from acinar cells was demonstrated by measuring volume changes of acini/acinar cells, as well as activation energy (E_a) in transepithelial water movement by NMR spectrometry, and a functional linkage between AQP5 and TRPV4 was suggested. Next, involvement of the parasympathetic nervous system on the AQP5 levels in the acinar cells of the submandibular and that of a β-adrenergic agonist on those in the parotid gland are described. That is, chorda tympani denervation induces autophagy of the submandibular gland, causing AQP5 degradation/metabolism, whereas isoproterenol, a β-adrenergic agonist, causes first an increase then decrease in AQP5 levels in the parotid gland, which action is coupled with the secretory-restoration cycle of amylase-containing secretory granules. The PG also responded to endotoxin, a lipopolysaccharide that activates NF-κB and MAPK pathways. Elevated NF-κB and AP-1 (c-Fos/c-Jun) form a complex that can bind to the NF-κB-responsive element on the *AQP5* promoter and thus potentially downregulate *AQP5* transcription. Salivary gland pathologies and conditions involving AQP5 and possible treatments are described as well.

Keywords Salivary glands · Autonomic regulation · Autophagy · Downregulation · Activation energy (E_a) · Lipopolysaccharide

Abbreviations

ALLM	N-Ac-Leu-Leu-methininal
AP-1	Activator protein 1
AQP	Aquaporin
CCh	Carbachol
CTD	Chorda timpani denervation
CQ	Chloroquine
DPPVI	Dipeptidyl peptidase IV
E_a	Activation energy
EMSA	Electrophoretic mobility shift assay
ERK1/2	Extracellular signal-regulated kinases 1/2
GFP	Green fluorescent protein
IPR	Isoproterenol
KO	Knockout
LC3B-II	Microtubule-associated protein 1 light chain 3 isoform B-II
LPS	Lipopolysaccharide
MDCK	Madin-Darby canine kidney
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-kappa B
PG	Parotid gland
P_d	Diffusive water permeability
P_f	Osmotic water permeability
RVD	Regulatory volume decrease

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SMG	Submandibular gland
SNP	Single nucleotide polymorphism
TRPV4	Transient receptor potential cation channel, subfamily V, member 4

Introduction

One of the characteristics of cell membranes is their semipermeability. Because of this feature, water can move in and out of the cells. However, in epithelial cells of such tissues as the mammalian kidney collecting tubules, amphibian skin, and bladder, the water permeability is much higher compared with that of other tissues. Therefore, the existence of some other mechanism(s) had long been anticipated that could allow such high permeability.

In 1992, P. Agre at The Johns Hopkins University discovered the first water channel, aquaporin 1 (AQP1), which had been given the initial name of "channel-forming integral membrane protein of 28 kDa (CHIP 28)" [128]. AQP1 was shown to participate strongly in water movement across the cell membrane. In the following year (1993), AQP2, a second AQP, was cloned from kidney tissue by Sasaki's group [26]. Thereafter, research in this field developed rapidly, and in 2000, Fujishoshi's group succeeded in X-ray crystallographic analysis of the AQP1 molecule, revealing its whole structure [109]. It is now well known that AQPs exist in many living organisms from animals and plants to microorganisms [174] and that one to several molecular species of AQPs are expressed in individual tissues.

This review will first briefly overview mammalian AQPs and then focus on salivary gland AQP5 in particular relation to its physiological regulation and the water transport mechanism.

Aquaporins

AQPs are water channels constructed by serpentine-type membrane proteins. The water movement across biological membranes via AQPs is considered to be facilitated simply dependent on the osmotic gradient.

These water channels have the following characteristics [49, 55, 61]: (1) The channel protein is a six-transmembrane protein having a tandem-repeat structure. (2) It has two Asp-Pro-Ala sequences (NPA motif) in the molecule that form hemi-channel structures. These two hemi-channel structures face each other from inside and outside of the plasma membrane forming a pore through which water can pass. (3) Within the molecule, there are phosphorylation target motifs besides glycosylation target ones, which motifs participate in the regulation of the water-channel function.

After the discovery of the first AQP, a number was given to each AQP following the order of discovery, and presently 13 AQPs, AQP0–12, are known to exist in mammals.[1] These AQPs have been divided into four major subfamilies, according to the following characteristics: facilitation of permeation of glycerol besides water, altered sequence of conservative NPA box, and molecular phylogeny. The first subfamily is the water-selective AQP one, which includes AQP0, 1, 2, 4, 5, and 6. These AQPs facilitate the movement of water mainly. The second subfamily is the aquaglyceroporin one, to which AQP3 [50, 88], AQP7 [71], AQP9 [46, 157], and AQP10 [48] have been assigned. The third subfamily, named superaquaporin [51], includes AQP11 and AQP12, which are unorthodox AQPs because each has a unique NPA box with a signature cysteine residue and low homology with AQPs in the two previously mentioned groups (see the section Super aquaporins (unorthodox aquaporins) and [44, 51]). The remaining AQP, AQP8, is a water-selective AQP but has an unusual structure with a long N-terminus, short C-terminus, and high homology with γ-TIP, a plant water channel [47, 68, 94]. The phylogenic study clearly separate AQP8 from the group of water-selective AQPs in animals. Regarding mammalian AQPs, major expressing tissues, functions, physiological roles, and effects of mutation/polymorphism in human ones are summarized in Table 1.

Water-selective aquaporins

The membrane protein AQP1 was initially found in the erythrocyte, a highly water-permeable cell [128], and AQP2 [26], 3 [50], and 6 [175] were consecutively cloned from the kidney. AQP4 was cloned from the lung and brain [37, 55], whereas AQP5, from salivary gland [132]. All of them except AQP3 belong to water-selective subfamily of AQPs (Table 1). AQP4 was found to play important functions in the nervous system, whereas AQP5 functions in fluid secretion/movement in many tissues including exocrine glands and lungs. AQP6, on the other hand, localizes in vesicles inside of kidney cells and has been suggested to play a role in anion-channel function.

Aquaglyceroporins

The glycerol permeability of AQP3 was recognized when this water channel was first cloned and characterized [50]. It has an important role in the skin. A second aquaglyceroporin, AQP7, was cloned from the rat testis [45] and was shown to be permeable to glycerol, water, and urea. On the other hand, an

[1] Major intrinsic protein (MIP/MP26/MIP26) of the lens was reported before the discovery of AQP1 [127], and its function had been unknown. Since MIP26 afforded permeation of water and was highly homologous to members of the AQP family, it was later considered to be an AQP [106]. Thus the protein and gene of MIP26 are referred to as AQP0 [99, 117].

Table 1 Tissues expressing AQP species, their functions, mutation/polymorphism, and genetic disease

AQP subfamily	AQP family member	Major expressing tissues	AQP function and physiological roles	Mutation and genetic disease in *humans*	Polymorphism and resulting phenotype in *humans*
Water-selective aquaporins	AQP0	Lens tissue of the eye	AQP0 controls osmotic substances and water content of the lens tissue. Gene abnormality results in cataract development, which increases with age [27, 30].	Cataract [7, 25]	
	AQP1	Kidney (proximal convoluted tubule and descending limb of Henle's loop)	AQP1 is involved in counter current concentration. Its deletion causes defective urinary concentration [122].	Phenotypically normal [129]. Impaired urine-concentrating ability [62]	Colton blood group polymorphism [161]
		Lung (vascular endothelium in pulmonary alveolus)	AQP1 is important for aspiration of amniotic fluid at birth. Its defect shows a protective action against edema in the lungs [63].		
	AQP2	Kidney (luminal membrane of collecting duct)	Long-term action of vasopressin regulates gene expression of AQP2 via cAMP. Individuals with an abnormal gene develop diabetes insipidus. Short-term action of vasopressin induces trafficking of AQP2-bearing vesicles from the cytosol to the apical membrane [21].	Nephrogenic diabetes insipidus [159].	
		Inner ear (endolymphatic sac)	A possibility is suggested that abnormal up-regulation of vasopressin or its receptor affects trafficking of inner ear AQP2, causing endolymphatic hydrops (Meniere's disease) [96].		
	AQP4	Brain (capillary, synaptic space, end feet of astrocytes adjacent to Ranvier's node)	AQP4 is involved in formation and disappearance of edema in the brain. AQP4 in astrocytes plays important roles in brain edema due to brain ischemia, injury, inflammation or metabolic failure. Cytotoxic brain edema is relatively mild in AQP4-deficient mice, but it aggravates vasogenic edema by causing leakage of cerebrospinal fluid [124].	D184E mutation of AQP4 combined with other genetic defects or concurrently with certain environmental stimuli could confer a high susceptibility to deafness [119].	AQP-4 genetic variations and haplotypes might contribute to the risk factors for leukoaraiosis [169].
	AQP5	Salivary gland, lacrimal gland, lung, stomach, duodenal Brunner's gland	AQP5 is involved in fluid secretion from exocrine glands and lungs [60, 91, 155]. The secretion is regulated by the autonomic nervous system under normal conditions and by the NF-κB pathway under pathologic ones [4, 155, 172] (See text for detail).	Diffuse nonepidermolytic palmoplantar keratoderma [1, 10, 13].	Polymorphisms in AQP5 were associated with the rate of lung function decline in continuous smokers with chronic obstructive pulmonary disease [33]. Positive association between SNPs in AQP5 and progesterone receptor [59]. An AQP polymorphism is associated with peritumoral brain edema in menigioma patients [74].
	AQP6	Kidney (vesicles in collecting duct cells)	The anion transport activity of AQP6 is high at low pH, whereas its water permeability is low. A possibility is suggested that this water		

Table 1 (continued)

AQP subfamily	AQP family member	Major expressing tissues	AQP function and physiological roles	Mutation and genetic disease in *humans*	Polymorphism and resulting phenotype in *humans*
			channel is involved in acid–base balance. AQP6 is found also in the retina, parotid gland acinar cells, inner ear, and brain synaptic vesicles [42, 173].		
Aquaglyceroporins	AQP3	Skin (cell membrane of keratinocytes in the basal layer of the epidermis)	AQP3 participates in glycerol transport. In the normal skin, its expression is elevated in response to skin stress in diseases such as atopic eczema. AQP3 helps to supply water to the skin and provides elasticity. Its deficiency results in failure of wound healing and formation of new skin [42].	Loss of GIL, a blood-group antigen [134].	Homozygous c.105G->C (conservative change without alteration of amino acid) in AQP3 was detected in 11 out of 34 Menière's Disease patients but not in 100 control chromosomes [12].
	AQP7	Fat tissue	AQP7 and 9 are involved in glycerol transport. In accordance with changes in the serum glycerol level, AQP7 mRNA in the fat tissue decreases during food intake and increases during fasting. These changes are opposite to the changes in the serum insulin concentration. AQP9 is supposed to be a glycerol channel in liver cells. Transcriptional activities of AQP7 and 9 are both negatively regulated by insulin [64, 65, 96, 158].	G264V mutation of AQP7 causes defects in glycerol transport. No increase in exercise-induced plasma glycerol [66]	
	AQP9	Liver (sinusoidal surface)			Genetic variation in the AQP9 gene is associated with femoral neck bone mineral density in postmenopausal women, and may represent one of the susceptibility genes for phenotypes related to bone mass [14].
	AQP10	Duodenum and jejunum	AQP10 transports water and glycerol [48].		
Super-aquaporins	AQP11	Testis, liver, kidney (proximal convoluted tubules)	Function is unknown. Animals with AQP11 deleted by genetic knockout develop polycystic kidney disease and die within 2 months [104].		
	AQP12	Pancreatic acinar cells	AQP12 is localized in the cytosol of pancreatic acinar cells. Based on knockout data, this channel protein has been implicated in exocrine water secretion [53, 120].		
	AQP8	Large intestine Pancreatic acinar cells liver	AQP8 is distributed in the large intestine and other digestive organs.		Single-nucleotide polymorphism in AQP8 may be associated with polycystic ovary syndrome among Chinese Han women [80].

Numbers in brackets indicate references

aquaporin with high homology to rat AQP7 was cloned from human adipose tissues [71]. Based on similar tissue distribution, primary sequence, and function, Ishibashi et al. suggested that human aquaglyceroporin cloned from adipocytes is most likely the human ortholog of rat *AQP7* [52]. AQP7 (designated as AQPap by Kishida) was shown to be downregulated by insulin and elevated by fasting [64, 65]. AQP9, a human water channel capable of permeation of water and urea, was first detected in leukocytes [46]. The homolog of *AQP9* was cloned from rat liver, and when expressed in *Xenopus* oocytes, it was shown to allow passage of a wide variety of noncharged solutes including glycerol [157]. In addition, the liver AQP9 level was shown to be regulated negatively by binding with insulin at the insulin responsive element in the promoter of *AQP9* [72]. The coordinated regulation of AQP9 and AQP7 by insulin and/or fasting condition suggested the pathophysiological importance of these two aquaglyceroporins in glycerol transport between the fat tissue and liver [72, 76]. As to *AQP10*, it was cloned from a human jejunum cDNA library [39]. AQP10 is strongly expressed in the duodenum and jejunum and supposedly functions mainly as a water transporter, although its structural homology implies that AQP10 belongs to the aquaglyceroporin subfamily [39]. Recently, AQP10 was reported to be expressed in the stratum corneum and adipocytes, in which this water channel supposedly facilitates the transport of glycerol [56, 73]. Aquaglyceroporins are important for not only the transport of glycerol between the fat tissue and liver, as mentioned above, but also for providing moisture to the skin [35, 95, 158]. Further investigation focused on AQP10 is necessary to better understand its function.

Super aquaporins (unorthodox aquaporins)

Recently, *AQP11* and *AQP12* were cloned and shown to be less homologous to the already known AQPs [44]. Although some water permeability of AQP11 was demonstrated in liposomes and cultured cells [51], its permeability to glycerol is unknown. AQP11-null mice develop polycystic kidneys following the formation of large intracellular vacuoles in the proximal tubule, but the function of AQP12 still remains to be clarified [51].

Aquaporin 8

As described earlier, AQP8 is structurally unusual compared to other members in the AQP family, being rather close to the plant water channel. Thus, this water channel has been separated from other classified AQPs in this review. With respect to its function, AQP8 is close to water-selective AQPs as it is permeable to water and does not permeate glycerol. Interestingly, it has the unique property of permeating H_2O_2 [8].

Functions other than as a water channel

In recent years, it was reported that some molecules in the AQP family function other than as water channels. For instance, AQP0 functions as an osmometer and thereby enables normal microcirculation within the lens tissue, preventing swelling of the lens fiber [16]. Simultaneously, AQP0 is involved in helping each fiber to adhere to its neighbor, resulting in narrowing of the interfibrous spaces, a feature important for maintaining lens transparency [16]. Among mammalian aquaglyceroporins (AQP3, 7, 9, and 10), at least AQP7 and AQP9 transport trivalent arsenic ions [85], and AQP3, AQP7, and AQP9 transport urea [83]. These aquaglyceroporins (AQP3, 7, 9, and 10) as well as AQP8 transport ammonia as well. It is known also that AQP6 [114, 173], AQP1 [40], and AQP8 [9] transport ions, CO_2/NO, and H_2O_2, respectively. Furthermore, some AQPs are reportedly involved in cell adhesion, cell movement, and cell division [163]. The major characteristics of mammalian AQPs are summarized in Table 1.

Mutations and polymorphisms of human aquaporin genes

A number of mutations and polymorphism/SNP of genes in the AQP family members were reported to occur in humans. Some of them cause diseases and pathologic conditions, whereas others apparently do not have any effect, or if so, only a minor one. For example, individuals with a mutation in *AQP0* [27, 30] and *AQP2* [159] develop cataract and nephrogenic diabetes insipidus, respectively; a mutation in *AQP1* apparently has no phenotypic effect, although affected patients have an impaired ability to concentrate urine [62, 129]. These cases along with others are summarized in Table 1.

Aquaporin gene knockout used to reveal new aquaporin functions

Phenotype analysis of gene-knockout (KO) mice can generally predict the pathophysiological roles of the gene product of interest in various tissues. Especially important cases would be those that show unexpected roles of the protein product. Today, all knockout mice for each AQP gene, except *AQP6* and *AQP10*, has been established (*AQP10* in mice has been shown to be a pseudo-gene [43, 103]). The phenotypes observed for each type of *AQP*-KO mouse are summarized in the literature [162] and in Table 2.

Aquaporin 5 in individual tissue

Tissues expressing aquaporin 5

Water secretion from exocrine glands, such as salivary, lachrymal, and sweat glands, and water movement in the lung

Table 2 Phenotype analysis of AQP gene knockout mice

AQP family members	Gene knockout and phenotype analysis	Reference
AQP0	Lens transparency is lost by knockout of *AQP0*. Transgenically expressed AQP1 in the lens fiber cells of *AQP0*-KO mouse reduces the severity of lens cataract and prevents dramatic acceleration of cataractogenesis. Lens fiber cells, however, show deformities and lack of compact cellular architecture, demonstrating that AQP0 may function in cell-to-cell adhesion.	[142, 160]
AQP1	Urine- concentrating capacity is severely impaired in *AQP1*-KO mice. Marked suppression of UT-A1 and AQP4, a urea transporter and a basolateral water channel, respectively, is observed in the inner medullary collecting duct of the kidney in *AQP1*-KO knockout mice. Osmotic water permeability of the peritoneal barrier is reduced in them as well. Growth of tumors transplanted in *AQP1*-KO mice is slower. The migration speed of endothelial cells obtained from *AQP1*-KO mice is slower also	[105, 163, 170]
AQP2	An inducible mouse model of *AQP2*-KO is accompanied with severe polyuria in adult mice. In *AQP2*-KO mice, urine osmolality decreases from approximately less than 1/4 after 4–5 days, with urine output increasing from 2 to 25 ml/day. Urine osmolality does not increase after water deprivation. AQP3 protein expression in the collecting duct is increased by about 5-fold after *AQP2* gene deletion.	[176]
AQP3	AQP3 deletion results in a more than 4-fold reduction in osmotic water permeability and more than a 2-fold reduction in glycerol permeability in the epidermis. AQP3 deletion also results in a significant reduction in glycerol content in the stratum corneum and epidermis. The growth and phenotype of AQP3 null mice is grossly normal except for polyuria. AQP3 deletion has little effect on AQP1 or AQP4 protein expression but does cause a decrease in AQP2 protein expression, particularly in the renal cortex.	[34, 90, 92]
AQP4	AQP4 deletion in mice has little or no effect on development, survival, growth or neuromuscular function but does lead to a small defect in urine- concentrating ability.	[18, 93]
AQP5	Pilocarpine-stimulated saliva production is reduced by more than 60 % in *AQP5*-KO mice. AQP5 is responsible for the majority of water transport across the apical membrane of type I alveolar epithelial cells. Compared to wild-type mice, *AQP5*-KO mice are hypersensitive to acetyl choline, showing significantly increased concentration-dependent bronchoconstriction.	[69, 89, 91]
AQP7	In *AQP7*-KO mice, the water permeability of the proximal straight tubule brush border membrane is reduced compared with that of wild-type mice. A marked elevation of the urine glycerol level is observed in *AQP7*-KO mice, suggesting that glycerol is reabsorbed by a novel pathway in the proximal straight tubules.	[145]
AQP8	The emergence of multioocyte follicles is associated with AQP8 deficiency, suggesting the involvement of AQP8 in follicle formation in the ovary. Knockout experiments suggest its involvement with cytoskeletal proteins and ammonium transport.	[97, 152]
AQP9	Permeability of urea in hepatocyte basolateral membranes isolated from *AQP9* and *UT-A1/3* (a urea transporter) double-KO mice is decreased additively compared with that from either single-KO mice, suggesting that AQP9 and unidentified UT-A urea channels constitute primary but redundant urea facilitators in murine hepatocytes.	[54]
AQP10	Pseudogene in mice	[43, 103]
AQP11	The AQP11 null mouse has a remarkable phenotype showing polycystic kidneys, which is neonatally fatal. In a liver-specific *AQP11*- KO experiment, deletion of AQP11 in the liver results in disrupted RER homeostasis and increased sensitivity to RER injury upon metabolic challenge with amino acids.	[43, 121, 135]
AQP12	In *AQP12*-KO mice, pancreatitis induced by a cholecystokinin-8 (CCK-8) analog causes more severe pathological damage to this organ than it does in wild-type mice.	[120]

alveoli are two of the most important physiological functions of these structures. The possible involvement of water-channel proteins has therefore been implied. Raina et al. cloned *AQP5* mRNA for the first time from the submandibular gland (SMG), one of the major salivary glands [132]. They showed that this AQP is expressed in such exocrine glands as the parotid (PG), sublingual, and lachrymal glands, as well as in the trachea, eyes, and lungs. Later, it was demonstrated that AQP5 is also expressed in the stomach [126], Brunner's glands in the duodenum [125, 126], pancreas [11], skin, and sweat glands [116]. Besides AQP5, several other AQPs are expressed in these tissues simultaneously, and it is recognized in general that one tissue expresses several members of the AQP family. On the other hand, most tissues having an exocrine function express AQP5. Therefore, a pivotal role is suggested for AQP5 in exocrine function.

Effects of mutation and polymorphism of *aquaporin 5*

Recently, missense mutations in the *AQP5* gene were identified in patients suffering from autosomal-dominant diffuse nonepidermolytic palmoplantar keratoderma (referred to as palmoplantar keratoderma Bothnia type), which was mapped in Swedish and UK families to chromosomal region 12q11–12q13 [1, 10]. A variant of AQP5 (c.529A>T; p.Ile177Phe) was localized in the plasma membrane in the stratum

granulosum in affected palmar epidermis, similarly as in normal subjects, thus indicating that this mutant AQP5 traffics normally [10]. A year later, another missense mutation of AQP5 (c.367A>T, p.Asn123Tyr) was identified in a large three-generation family of Chinese Han ethnicity with palmoplantar keratoderma of the Bothnia type, and study of this mutant showed that the mutant channel is leaky and more sensitive to hypotonic solution than is the wild-type one [13]. Such properties of this mutant AQP5 may account for the intensive cellular swelling resulting in the phenotype of diffuse nonepidermolytic palmoplantar keratoderma.

Single-nucleotide polymorphism (SNP) has been detected in humans. An SNP is found in the 3′ UTR region of the *AQP5* gene in a certain percentage of asthma patients, resulting in reduced production of the AQP5 protein [70]. These individuals are hypersensitive toward choline-provoked bronchoconstriction [69]. Five SNPs in *AQP5* were genotyped in European Americans with chronic obstructive pulmonary disease (n=429), and three of them showed significant association with the rate of decline in lung function [33]. Also, a positive association between SNPs in *AQP5* promoter and progesterone receptor was reported [59]. Recently, high expression of AQP5 and polymorphism in the AQP5 promoter were suggested to be associated with peritumoral brain edema in meningioma patients [74].

Aquaporin 5 in the salivary glands

Expression of aquaporin 5 and other aquaporins in the salivary glands

In the rat SMG, AQP5 is expressed in the apical/lateral and basal membranes of the acinar cells [4, 112], whereas AQP1 is detected in the cell membranes of the capillaries within salivary gland tissue [2, 4, 79]. The mRNAs for *AQP3* and *AQP4* are expressed in the fetal, but not in the adult SMG, whereas *AQP2* mRNA cannot be detected in either fetal or adult SMG [2]. The expression of AQP6 and AQP7 in the human salivary gland was examined, but the results were obscure, and such expression was not confirmed [20]. On the other hand, positive AQP8 labeling was observed in the myoepithelial cells in the salivary gland (SMG, PG, and sublingual gland), with no labeling of acinar or ductal epithelial cells [22, 166], whereas *AQP11* transcripts were present in the developing and mature duct structure of the SMG, and its expression was reduced in the adults, implying some roles for AQP11 during gland development [75].

Effects of aquaporin 5 mutation on the salivary function

Inbred rats having a point mutation at nucleotide 308 (G308A) in their *AQP5* gene and producing a mutant AQP5 protein were established [111, 112], and this mutant molecule has an aspartic acid at position 103 in place of glycine (AQP5-G103D) in the third transmembrane domain of AQP5. This mutation was initially found as an SNP among SD rats in the breeder's colony. The AQP5 protein level in the SMG and other tissues is strongly reduced in these mutant rats, though the glandular mRNA level is unchanged compared with that in wild-type rats, and the Kozack sequence (GGCACCaugA), which affects the translational efficiency, was not altered in the mutant mRNA [112]. These data support the hypothesis that the reduced AQP5 protein production in the mutant rats is caused by accelerated degradation of the mutant molecule via the protein quality control system. Actually, structures positive for AQP5 are taken up by lysosomes more in the mutant SMG than in the wild-type one [58]. The water permeability of the mutant AQP5 as tested by the *Xenopus* oocyte expression system is normal [58]. However, water secretion from the salivary gland in vivo and in situ is significantly affected [110, 112]. Namely, the initial water secretion from the mutant SMG upon cholinergic stimulation is reduced compared with that from the wild-type gland due to its extremely low expression at the acinar cell membrane. Studies using AQP5-mutant rats can provide useful information about the effects of genetic variation of the *AQP5* gene in humans. Also, *AQP5*-KO mice and *AQP5*-mutant rats are useful models for studying the physiological roles of AQP5 in the water secretion from the exocrine glands.

Effects of *aquaporin 5* knockout on salivary gland function

Mice genetically altered by knocking out the *AQP5* gene have been prepared and analyzed [91]. Though the appearance of the KO mice is normal, both their birth rate and growth rate are reduced. As compared with that of their wild-type counterpart, the salivary secretion and levels of some tight junction proteins in the acini are decreased in these *AQP5*-KO mice [60, 91], suggesting that *AQP5* KO affects salivary flow via the paracellular route as well (see the section Roles of aquaporin 5 in water secretion).

Roles of aquaporin 5 in water secretion

Water is secreted from the terminal portion of exocrine glands via paracellular and transcellular routes. This secretion takes place as a result of coupling to ion transport via the ion channel. In the salivary gland, the first trigger of this process is the release of Cl^- followed by Na^+ movement [24, 86, 175]. The Cl^- ions required for this process are co-transported into the acinar cells along with the Na^+ and K^+ ions via NKCC1, an $Na^+/K^+/2Cl^-$ co-transporter, present in the basal membrane. Evans et al. showed that saliva secretion is reduced by 60 % in

NKCC1 KO mice [23]. Their study directly demonstrated that Cl^- ions are accumulated in the cells by the action of NKCC1 and serve as a driving force for saliva secretion. As for the other two cations accumulated by NKCC1, Na^+ is transported to the outside of the cells via Na^+, K^+-ATPase and K^+, via K^+ channels. Two K^+ channels, IK1/SK4 and maxiK/Slo, have been identified, and KO of either of them does not affect saliva secretion, whereas double KO of these two K^+ channels significantly reduces it [5, 136], suggesting that the accumulated K^+ ions are transported to the outside of cells via both channels. These facts also support the idea that the Cl^- efflux from the cells is indispensable for water secretion.

Ma et al. showed that saliva secretion after the initial 5 min and 5–10 min after pilocarpine stimulation is reduced by more than 60 % in *AQP5*-KO mice [91]. The extent of the effects on saliva secretion is similar between *AQP5* KO and *NKCC1* KO animals. In AQP5-G103D mutant rats, only a trace amount of the mutant molecule is expressed in the SMG acinar cells [112], and 15 % of that of the wild type in the PG acinar cells [139]. In these rats, a similar reduction in saliva secretion is observed during the initial 12–17 min after pilocarpine stimulation in vivo [112]. These data imply consistently that AQP5 play important roles at least at the initial phase of saliva secretion. On the other hand, Menon's group determined water transport via the paracellular route by performing an experiment to measure the movement of FITC-dextran [60]. They demonstrated that water transport via this route is decreased in *AQP5*-KO mice also, although the transcellular one is much more affected by *AQP5* KO. Another observation by these authors is that the levels of claudin-7, claudin-3, and occludin in the parotid gland are decreased in *AQP5*-KO mice. Decreased expression of these tight junction proteins is thought to be correlated with decreased paracellular permeability because such a phenomenon is actually observed in Madin-Darby canine kidney (MDCK) type II cells expressing a low level of claudin-2 in vitro [82].

Since acinar cells have the cholinergic receptor, it is most possible that cholinergic agonists, via stimulation of acinar cells, trigger transcellular water transport, leading to the rapid increase in water permeability. However, extracellular tracer studies suggest that only a small fraction of water is transported through the transcellular pathway and that the majority of water moves through the paracellular pathway [107, 108]. Thus, it is important to study the initial mechanism governing the opening/closing of the paracellular pathway in the acinus of salivary glands to elucidate the entire water secretion by the exocrine glands.

Activation energy for water transport

In order to assess the involvement of AQP5 in the abovementioned water transport by the salivary gland acinar cells, a research group examined changes in the cell volume and activation energy (E_a) of diffusive water permeability (P_d) in isolated parotid acinar cells obtained from AQP5-G103D mutant rats and their wild-type counterparts (Table 3; Ref. [139]). In the unstimulated wild-type acinar cells, E_a determined by NMR spectrometry was shown to be 3.4± 0.6 kcal mol^{-1}, and no detectable change was noted after stimulation with carbachol (CCh), a cholinergic agonist. In the unstimulated mutant acinar cells, a high E_a value (5.9± 0.1 kcal mol^{-1}) was detected, and it showed a minimal decrease after CCh stimulation (5.0±0.3 kcal mol^{-1}). As mentioned earlier (see the section Effects of aquaporin 5 mutation on the salivary function), since the AQP5-G103D (mutant AQP5) expressed in *Xenopus* oocytes has a water permeability almost the same as that of the normal AQP5 [58], this high E_a value for the mutant acinar cells may not be due to the functional disorder of the mutant AQP5 but rather to the fact that a small number of AQP5 molecules are expressed in the acinar cell membrane, and therefore, the observed E_a value was affected by the high E_a for the water transport via the lipid bilayer (E_a=12–14 kcal mol^{-1}) [130, 133].

Reliability of the E_a value for mutant acini was verified by calculating its E_a assuming that the E_a for the lipid membrane per se is 12.75 kcal mol^{-1} [130, 133] and that the AQP5 fraction relative to the wild-type one is 15 % (based on Western blot analysis). The apparent E_a value expected from the simulation curve is E_a=6 kcal mol^{-1}, which value well agrees with the measured one.

The increase in E_a for water transport would result in a decrease in osmotic water permeability (P_f) of mutant acinar cells to 1/3 to 1/4 of that of wild-type rats, although the apparent P_d between the two groups of rats is the same (see Table 3). These results suggest that AQP5 is essential for reducing the activation energy for water transport in the acinar cells and therefore affects their water permeability.

These data consistently support the fact that reduced saliva secretion in *AQP5* KO mice, providing there is full deletion of AQP5 [60, 91], is at least in part due to the decrease in water transport across the plasma membrane between the two water transport routes, transcellular and paracellular pathways.

Hypothetical mechanism of water secretion

In vitro shrinkage of exocrine acinar cells upon cholinergic stimulation has generally been recognized [113, 115, 153] with exception of SMG C10 cells in culture, which show transient blebbing of cell membranes instead of cell shrinkage upon stimulation [3]. The lack of cell shrinkage in the latter study cannot be explained at present [3]. In the isolated acinus of the PG from wild-type rats, CCh (1 μM) stimulation induces transient swelling of the acinus, followed by rapid shrinkage of the acinar cells shortly thereafter. The transient swelling of acini and rapid shrinkage of acinar cells can explain the water shift through the transcellular and paracellular

Table 3 Diffusive water permeability (P_d), activation energy (E_a), and osmotic water permeability (P_f) of water transport in acinar cells of wild-type and AQP5-G103D mutant rats

	P_d (cm s^{-1}, at 25 °C)	E_a (kcal mol^{-1})		P_f (cm s^{-1})
		Under unstimulated condition	Under CCh-stimulated condition	
Wild type	1.1×10^{-3}	3.4±0.6	3.6±0.6	$5–10\times10^{-3}$
AQP5-G103D mutant	$\sim1\times10^{-3}$	5.9±0.1	5.0±0.3	$1.6–2.4\times10^{-3}$

Isolated acinar cells were suspended in KRB solution containing 10 mM gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA). NMR spectrometry was used to measure ^{1}H NMR signals and T_1 relaxation of water protons in the suspended cell solution by an inversion-recovery pulse sequence. The biexponential relaxation was detected, and the pure relaxation rate constant of the slower component (R_s) was obtained. The diffusive water permeability (P_d) of acinar cells was calculated by equations $R_s=R_n+k_n$ and $P_d=k_n\times V_n/A_n$, where R_n is the intrinsic $1/T_1$ of the intracellular water that was determined by the T_1 relaxation of the salivary gland without Gd-DTPA, and V_n/A_n is the volume/surface area ratio of the acinar cells, which was determined by light microscopy. The activation energy (E_a) of water transport was calculated from the slope of the Arrhenius plot, $\log_e(k_n)=-E_a\times(1/T)$. Osmotic water permeability (P_f) was estimated as follows: i.e., in the lipid bilayer without water channels, the P_f/P_d ratio is 1. When water flows through the narrow aqueous pore in the AQP5 molecule by single-file transport, it is expected that the P_f/P_d ratio will increase up to around 5 [137]. The P_f value in the table was calculated on the assumption that the P_f/P_d ratio for AQP5 is 5–10 (data from Satoh et al. [139])

pathways, respectively [113]. The transient swelling of the acinus is accompanied by an enlargement of the luminal canal [113]. In the initial stage of water secretion, Cl^- efflux into the lumen takes place first. A small amount of water is then shifted into the lumen via AQP5 present in the apical membrane. It is believed that the same amount of water then enters the cells through the basolateral membrane via AQP5. Therefore, the acinar cell volume is unchanged, and the luminal volume is increased. In the next step, rapid acinar cell shrinkage takes place at 8 s after CCh stimulation [139]. The volume of the acinar cells is decreased rapidly by 20 % by the CCh stimulation, and the decreased volume size stays at this low level as long as the agonist is present [139]. The agonist-induced shrinkage is explained by the isotonic release of KCl from the acinar cells [153], and both K^+ and Cl^- channels are supposed to be activated by an increase in intracellular Ca^{2+} [115]. As a result of rapid shrinkage of the acinar cells, the paracellular space between acinar cells increases. It is likely therefore that the permeability of the intercellular junctions between acinar cells would be increased. Indeed, 10 kDa dextran can pass through the paracellular tight junction in the acinus following CCh stimulation [141]. This increase in permeability allows a continuous secretion of saliva via the paracellular pathway.

Functional linkage between aquaporin 5 and cation channels

Transient receptor potential cation channel, subfamily V, member 4 (TRPV4) is an ion channel protein that belongs to a member of the vanilloid subfamily in the transient receptor potential (TRP) superfamily of ion channels [81, 151]. It functions to regulate the systemic osmotic pressure in various tissues such as the kidney, liver, heart neurosensory cells, and the central nervous system [151]. The cDNA encoding vanilloid receptor-related osmotically activated channels has been cloned from rat, mouse, human, and chicken [81].

Liu et al. [84] studied a possible linkage between AQP5 and TRPV4 in the salivary gland and found that hypotonic solution elicits an increase in cell volume and Ca^{2+} entry, followed by activation of a regulatory volume decrease (RVD). Ca^{2+} entry is associated with ruthenium red-sensitive nonselective cation current, suggesting the involvement of TRPV4. Acinar cells from *TRPV4*-KO or *AQP5*-KO mice do not activate the RVD upon their exposure to hypotonic solution, confirming the requirement of both channel proteins for RVD activation as well as their functional linkage. Hypotonicity increases the association and surface expression of TRPV4 and AQP5 in the salivary gland cells [84], while it reduces AQP5 abundance in the presence of TRPV4 in mouse lung epithelial cells [143].

On the other hand, caveolin-1, the main component of the caveolae plasma membranes, is suggested to be a critical component for salivary gland function [123]. The functional linkage of AQP5 with cation channels suggested above is supported by the fact that loss of caveolin-1 impairs agonist-stimulated salivary fluid secretion, transient receptor potential canonical 1-stroma interaction molecule 1 (TRPC1-STIM1) channel assembly, and altered apical targeting of AQP5. Further study should disclose the mechanism of regulation of AQP5 function and involvement of cation channels and/or other components.

Verification of proposed mechanism of water secretion by use of acini expressing aquaporin 5-G103D

In mutant acini, when 1 μM CCh is applied, the acinus does not swell at all, and the agonist-induced shrinkage of acinar cells is delayed by 8 s [139]. If we suppose that the fluid might shift via the paracellular pathway, it would be expected that the acinus should swell transiently even in the mutant rats.

However, no acinar swelling can be detected at all in the mutant rat. Therefore, under normal conditions, a transcellular water shift is thought to take place during this time period (after CCh), and AQP5 should be involved in such transient swelling of the acini. Since the amount of AQP5 expressed is small in mutants, even if Cl^- is effluxed into the lumen, water would not be able to move quickly from the acinar cell. As a result, no transient swelling would be observed in the acinus of the mutant rat [139].

The delay in CCh-induced shrinkage of the acinar cells is also explained by the low expression of the AQP5 in the acinar cells. As discussed above, due to the higher E_a value (5.9 kcal mol^{-1}), the P_f value of the mutant rats is decreased to 1/3–1/4 of that of the wild-type rats. This low water permeability may cause a delay in the water efflux from the acinar cells and may prevent the initial rapid shrinkage of the acinar cells. In the mutant rat, not only is the CCh-induced shrinkage of the acinar cells delayed by 8 s but also the decreased cell volume recovers spontaneously [139]. It is speculated that a decreased transcellular water shift prevents the Na^+ and Cl^- osmosis, resulting in accumulation of Na^+ ions in the acinar cells, and this may cause the spontaneous recovery of acinar cell size [139].

On the other hand, as described above, the expression levels of claudin-3 and 7 (in females) or claudin-7 (in males) in *AQP5*-KO mice were found to be reduced to 50 % of the level in wild-type mice (see the section Effects of aquaporin 5 knockout on salivary gland function); and such a reduction has been described to be closely correlated with a reduction in paracellular water secretion [60]. Also in the PG of AQP5-G103D mutant rats, claudin-7 and ZO-1 levels tend to be lower (72±6 % (P=0.07) and 75±6 % (P=0.16) of wild-type PG, respectively) than those in wild-type rats. This reduction in expression of tight junction proteins may be the direct cause of the reduced salivary secretion via the paracellular route in the AQP5-G103D mutant rats.

Autonomic regulation of salivary gland aquaporin 5

The salivary gland is double-innervated by sympathetic and parasympathetic nerves. Generally, the sympathetic nerve provokes the release of a small amount of viscous and protein-rich saliva, whereas parasympathetic nerve action triggers that of a massive amount of dilute saliva. The sympathetic nerve originates from the thoracic vertebrae and innervates the three major salivary glands via the superior cervical ganglion. The nuclei of parasympathetic nerves, the centers of salivation, are localized in the superior and inferior salivatory nuclei in the medulla oblongata. The efferent nerve from the superior salivatory nucleus is a facial nerve that reaches the SMG and sublingual gland via the submandibular ganglion. On the other hand, the efferent nerve from the inferior salivatory nucleus is a glossopharyngeal nerve, which, via the otic ganglion, innervates the parotid gland. Both efferent nerves are thought to regulate the salivary gland through muscarinic acetyl choline receptors. In addition, the chorda timpani parasympathetic nerve and isoproterenol injection are reported to regulate AQP5 in the SMG and PG, respectively [2, 15, 79].

Regulation of SMG aquaporin 5 by chorda timpani nerve

Though secretion of saliva from the three major salivary glands greatly differs according to species and to basal and stimulated state of the gland [19], among them, the SMG generally participates to the greatest extent in salivary secretion.

The possible involvement of the autonomic nervous system in the regulation of AQP5 was first studied by measuring the level of this water channel in the SMG membrane fraction after denervation of the chorda timpani nerve [79]. Chorda timpani denervation (CTD) causes a continuous reduction in the AQP5 level in the membrane fraction, reaching 50–60 % of the control by 4 weeks. The gland weight is decreased, reaching a minimum (75–80 % of the control) by as early as 1 week after denervation [4, 79].

By immunohistochemistry, the decrease in AQP5 level elicited by CTD and its recovery by injection of cevimeline, an M3 muscarinic agonist, was confirmed [4]. AQP5, concomitantly with other membrane proteins such as dipeptidyl peptidase IV (DPPVI) and Na^+, K^+-ATPase α-subunit are localized in the SMG. Whereas Na^+, K^+-ATPase is localized in the duct cells, DPPVI as well as AQP5 has been shown to be localized in the cell membrane of the acinar cells, implying that the DPPVI protein would be an appropriate control when changes in the AQP5 level are determined. A reduction in the AQP5 level in the acinar membrane by CTD was confirmed, with no detectable change in the DPPVI level [4]. Pretreatment with cevimeline prevented the decrease in the level of AQP5 [4]. These immunohistochemical data are supported by the results of a Western blotting experiment; i.e., CTD reduced the AQP5 level in the membrane fraction, and cevimeline injection prevented this reduction. No change in the AQP5 level was observed when pilocarpine, another muscarinic agonist, was used.

The reduction in the SMG AQP5 level caused by CTD is not due to a reduction in its mRNA level. Such a reduction appears to be due to activation of AQP5 metabolism/degradation because the AQP5 protein level in MLE-12 cells, a cultured lung cell line, is reportedly decreased by cAMP and recovered by chloroquine, a denaturant of lysosomes [144]. These data suggest that SMG AQP5 is metabolized by the lysosomal system upon CTD. Li et al. showed this to be the case, i.e., injection with chloroquine prevented the reduction in SMG AQP5 level caused by CTD [79]. Protein degradation by the lysosomal system is known as autophagy [100]. This

process is involved in balancing protein synthesis, degradation, and regeneration to control the proliferation of normal cells, development, and homeostasis. Thus, it is probable that AQP5 metabolism/degradation by CTD is brought about by lysosomes via autophagy in the SMG. Actually, in the SMG, microtubule-associated protein 1 light chain 3 isoform B-II (LC3B-II), a marker protein for autophagosomes, is elevated immediately and transiently after CTD, peaking at 1 day [4]. Consecutively, lysosome-associated membrane protein 2 (Lamp 2), a lysosomal marker, is elevated and remains at a high level at 7–10 days after CTD [4]. It has also been confirmed by immunohistochemistry that AQP5 is present in these structures [4]. Thus, it is probable that active autophagosomes are increased in number first, followed by elevation of the number of lysosomes upon CTD and that AQP5 is taken up by these structures (see Fig. 1). These results well agree with the continuous decrease in the AQP5 level over a 4-week period. Also, the transient increase in the LC3B-II level, i.e., indicating the onset of autophagy, for a short period well accords with the fact that the gland weight decreases by 20–25 % upon CTD [4, 79]. No apoptosis has been detected by use of the TUNEL assay or by measuring the ratio of the levels of anti-apoptosis and pro-apoptosis proteins (bcl-2/Bax) [4]. The just-cited study showed also that the soluble fraction of SMG containing the lysosomal enzymes metabolizes/degrades AQP5 in vitro and that such an activity is elevated by CTD. All these facts and other reports [57, 78] imply that this degradation system plays important roles in regulation and/or control of AQP function.

In conclusion, SMG AQP5 is dependent on chorda timpani parasympathetic innervation, and denervation of this autonomic nerve results in stimulation of autophagy and activation of the lysosomal system, by which AQP5 is supposedly metabolized (Fig. 1). Further study is needed to disclose the details of this mechanism.

Function of PKA-target motifs in the aquaporin 5 molecule

Since AQP5 has cAMP-dependent protein kinase target motifs at the fourth loop and C-terminal tail (Ser156 and Thr259, respectively), AQP5 function may be controlled by secretagogues [36, 132]. Woo et al. reported that cAMP-dependent phosphorylation of AQP5 at its Ser156 may not be involved in the AQP5 membrane expression and trafficking in human bronchial epithelial cells [167]. Instead, they described the possibility that AQP5 phosphorylation at this site may affect cell proliferation through the Ras signaling pathway [168]. In polarized MDCK cells, AQP5 is localized at the apical membrane [164, 165]. Constructs for chimeric proteins, AQP-GFP and GFP-AQP5, having normal Thr259 or the T259A mutation, were used to transfect MDCK cells to study the function of Thr259 in AQP5 trafficking [67]. The results showed that regardless of whether or not there was a mutation at position 259, AQP-GFP is constitutively expressed at the cell membrane. By contrast, GFP-AQP5 molecules introduced into MDCK cells remain in the cytoplasm and traffic to the plasma membrane upon dbcAMP stimulation, regardless of the presence or absence of the mutation, and such movement is H-89 sensitive. These data are very similar to those seen for AQP2 [31] and suggest that phosphorylation at Thr259 is not necessary for AQP5 trafficking. In human salivary gland (HSG) cells transfected with *AQP5* or an *AQP5-T259A* mutant construct, these molecules are constitutively localized at the

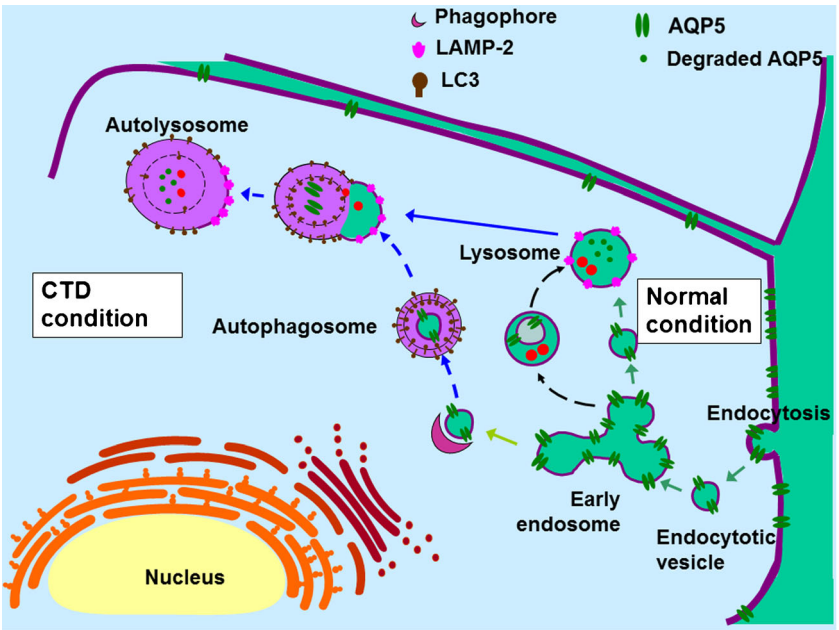

Fig. 1 Pathway for AQP5 metabolism/degradation in the acinar cells of the SMG in normal (untreated) rats and CTD rats (hypothesis). In acinar cells of the normal rat SMG, endocytotic vesicles incorporated inside the cell from AQP5-bearing apical membrane by endocytosis move toward lysosomes via early endosomes. In CTD rats, in addition to these pathways, phagospheres take in early endosomes to form autophagosomes, which will then fuse with lysosomes to become autolysosomes. AQP5 is incorporated into these structures and metabolized

plasma membrane under both resting and forskolin-stimulated conditions [36]. In the SMG and PG, Thr259, a novel phosphorylation site of AQP5, is potentially phosphorylated through cAMP signaling, but not through Ca^{2+} signaling, in vivo [36]. These data again suggest that phosphorylation of Thr259 of AQP5 is not important for AQP5 trafficking in salivary gland cells in vitro. The precise physiological role of Thr259-phosphorylated AQP5 is still unknown at present.

IPR-induced dynamic changes in PG aquaporin 5

AQP2, a major water channel in the renal collecting duct, is known to be regulated by the vasopressin/cAMP signaling pathway [38]. On the other hand, 8-(4-chlorophenylthio)-cAMP or the β-adrenergic receptor agonist isoproterenol (IPR) increases *AQP5* mRNA and protein levels in MLE-12 cells, a cultured lung cell line, and induces the translocation of AQP5 (trafficking) [171]. Also, in the PG, one of the major salivary glands, amylase, an important mammalian digestive enzyme, is secreted by the β-agonist, in which secretion process in the cAMP signaling is functioning.

These reports imply that the cAMP signaling pathway is involved in the regulation of AQP5 in the salivary gland and that β-agonists would possibly be involved in AQP5 regulation in the PG. Chen et al. [15] studied the effects of IPR on the level of AQP5 in the PG and analyzed its relation to the secretion-reaccumulation cycle of amylase by IPR as well as to the dynamic change in secretory granules. The PG secretory granules containing a large amount of amylase protein secrete their constituents by exocytosis. During this process, the secretory granules first dock to the plasma membrane and then fuse to it. It was found that IPR first upregulates the expression of AQP5 protein in the PG membrane fraction, which expression is followed by decomposition of this water channel protein via the calpain pathway [15]. Namely, the IPR treatment has three obvious effects on AQP5 expression in the PG membrane fraction. Firstly, the AQP5 protein level rapidly rises at 1 h after IPR treatment. Secondly, the level then decreases at 6 h after IPR treatment, reaching its minimum at 12 h. Thirdly, the protein level of AQP5 begins to increase again at 12 to 24 h after the treatment. The *AQP5* mRNA level, having gradually risen from 1 h after IPR treatment, reaches its peak in accordance with the pattern of increase in its protein level.

The elevation of the AQP5 protein level after the IPR injection takes place with the same timing (at 1 h) as the exocytotic amylase secretion and continues till 6 h after the injection. This elevation is thought to be the result of exocytotic transfer of AQP5 from the granule membrane to the plasma membrane. Actually, the membrane of secretory granules containing amylase is reported to express the AQP5 protein [98]. Thus, it was assumed that the amount of AQP5 in the plasma membrane is first increased by the IPR injection and then decreased by activation of the protease system (see the section IPR-induced downregulation of PG aquaporin 5).

The level of AQP5 protein in the PG membrane fraction is reduced by 85 % of the peak level (which is 200 % of the control level taken as 100 %) at 12 h after IPR treatment (to 30 % of the control level). This result is in good accord with the report that proteolytic decomposition of AQP2 protein is induced by dihydrotachysterol in the rat renal inner medullary collecting duct, although its mRNA level is unaltered [131, 138]. It is possible that IPR reduces the AQP5 protein level by affecting the posttranslational process, but not the transcriptional one.

On the other hand, the reduction in the AQP5 protein level after IPR treatment is suppressed by calpain inhibitors, but not by proteasome inhibitors nor by a lysosomal denaturant, suggesting that AQP5 is a substrate for calpain and that this water channel protein is metabolized by calpain proteolysis (see the section IPR-induced downregulation of PG aquaporin 5).

In summary, Chen et al. proposed the following mechanism (ref. [15] and Fig. 2): in accordance with the amylase secretion 1–3 h after IPR treatment, the granule membrane of secretory granules (containing amylase) becomes a part of plasma membrane by docking, resulting in elevation of AQP5 in the plasma membrane. Thereafter, from around 6 h after the IPR injection, the AQP5 protein becomes decomposed or metabolized. This reduction is due to proteolytic degradation by μ-calpain. Lastly, from around 12–24 h after IPR treatment, new AQP5 biosynthesis becomes obvious, and then the AQP5 level gradually increases toward its original one.

Downregulation of aquaporin 5 in the salivary glands

LPS-induced downregulation of PG aquaporin 5

Endotoxin or lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria, and it activates many types of cells in patients suffering from septic shock. LPS induces inflammatory proteins such as cytokines and defensins through TLR4, an important molecule in the innate immune system. The LPS/TLR4 signal transduction system has been well described [154]. Briefly, binding of ligands to the extracellular domains of TLR4 drives complex signaling systems and eventually activates NF-κB, through which numbers of inflammation-inducing proteins are elicited. In addition to the NF-κB pathway, the MAPK pathway is also activated during LPS signaling and is considered to be involved in the induction of proliferation, apoptosis, cytokine biosynthesis, and cytoskeletal reorganization. In the downstream of MAKK, there are the growth factor/chemical inducer-activated ERK1/2 pathway and the cytokine-inducible JNK and p38 pathways.

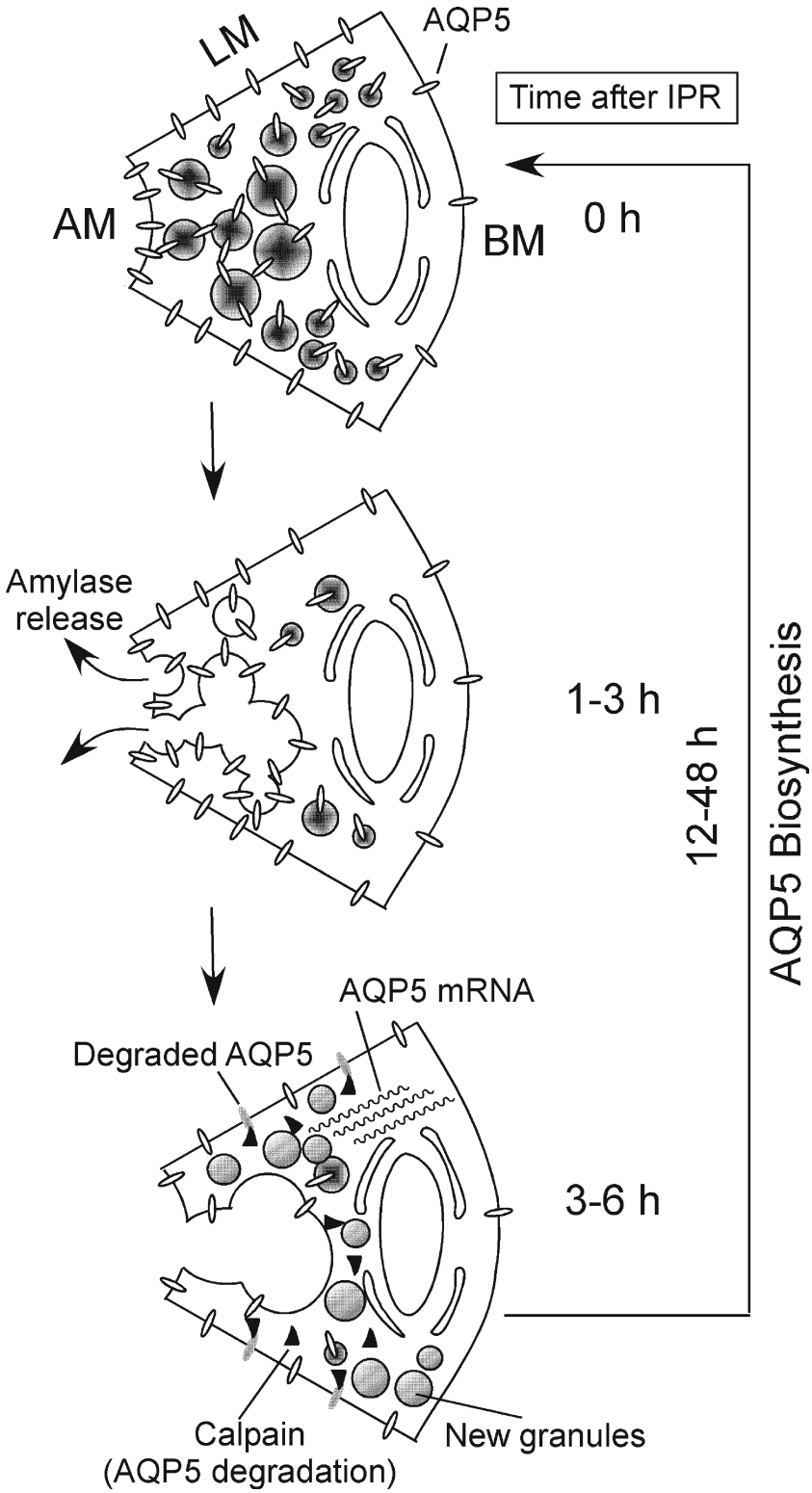

Fig. 2 Hypothetical model of AQP5 dynamics coupled to the secretory-restoration cycle of amylase in acinar cells of the parotid gland. In accordance with amylase secretion, the number of AQP5 molecules in the plasma membrane is increased at 1–3 h after IPR as the granule membrane has become a part of the plasma membrane. AQP5 in the membrane is then degraded/metabolized from around 6 h after IPR, when the amylase level in the gland has been recovering. This reduction in AQP5 is due to proteolysis by μ-calpain. New AQP5 biosynthesis gradually increases at 12–48 h after IPR to return its level to the original state. AM, apical membrane; LM, lateral membrane; BM, basal membrane (from Chen et al. [15])

The transcription of salivary gland AQP5 and AQP1 genes is inhibited by LPS, and the mechanism underlying this inhibition was studied by using an in vitro organ culture system and inhibitors of signaling pathways [172]. Inhibitors of NF-κB and MAPK pathways did not suppress transcriptional inhibition of AQP1 by LPS. One of the reasons for this result would be that there is no NF-κB-responsive element in the AQP1 promoter. However, the transcriptional inhibition of AQP5 by LPS is completely suppressed by PDTC or MG132, an I-κB kinase inhibitor or proteasome inhibitor,

respectively. In the presence of either of these inhibitors, it is conceivable that I-κB would accumulate and that the formation of free NF-κB would be suppressed. Furthermore, MAPK inhibitors, AG126 and SP600125 (inhibitors of ERK1/2 and JNK, respectively), also suppress the transcriptional inhibition of *AQP5* by LPS. Namely, it has become clear that both NF-κB and MAPK pathways are involved in the suppression of transcriptional inhibition of *AQP5* by LPS.

Another fact revealed from this experiment is that when the formation of any one of the three molecules p-c-Jun, p-c-Fos, (phosphorylated c-Jun and phosphorylated c-Fos formed by JNK and ERK1/2, respectively) or NF-κB is inhibited, then transcriptional inhibition of *AQP5* by LPS is completely suppressed, meaning that transcriptional inhibition of *AQP5* requires all three of these molecules. For confirmation of this interpretation, immunoprecipitation experiments using antibodies against the above transcription factors were performed, assuming that p-c-Jun, p-c-Fos, and NF-κB become associated to make a complex that suppresses the transcription of the AQP5 gene. PG extracts prepared 3 h after the LPS injection were reacted with Sepharose gels bearing antibodies specific for NF-κB (p65), p-c-Jun, or p-c-Fos (Fig. 3). Proteins absorbed to the gels were eluted and subjected to Western blotting and probed with anti p-c-Jun antibody. p-c-Jun was detected in the eluates from all three gels. In eluates from the anti-p-c-Jun and anti-p-c-Fos gels reacted with extracts of the PG from animals before LPS stimulation, only a small amount of p-c-Jun was detected. However, p-c-Jun was not detected at all in the eluate from the anti-NF-κB (p65) gel reacted with extracts of the PG from animals not stimulated with LPS. These results suggest that very little or no NF-κB (p65) was present in the unstimulated PG but that certain levels of p-c-Jun and p-c-Fos were present and that a complex of the three transcription factors had been formed by LPS stimulation.

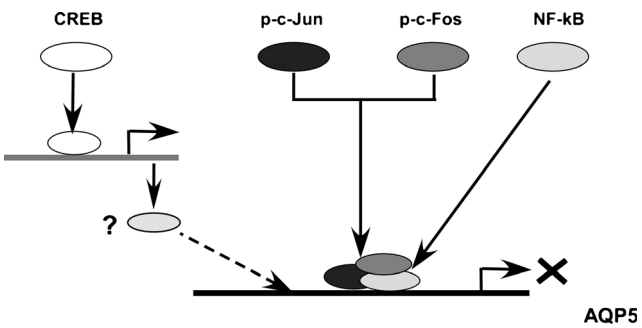

Fig. 3 Association of p-c-Jun with NF-κB subunit p65 and p-c-Fos in consequence of the LPS signaling (hypothesis). LPS injection has been shown to elevate the levels of transcription factors NF-κB, p-c-Jun, and p-c-Fos in the mouse PG in vivo [172]. The results of immunoprecipitation experiments using Sepharose gel bearing immobilized anti-NF-κB antibody, anti-p-c-Jun antibody, and anti-p-c-Fos antibody, and probed with anti-p-c-Jun, have shown that elevated p-c-Jun can bind anti-NF-κB antibody gel and anti-p-c-Fos antibody gel, suggesting that all three transcription factors form a complex. The complex of p-c-Jun, NF-κB, and p-c-Fos supposedly binds to the NF-κB-responsive element present in the promoter of *AQP5*. Though CREB is also elevated by LPS, its function in the transcriptional regulation of the AQP5 gene is not yet clear

Two NF-κB-responsive elements and two AP-1 binding sequences are found in the promoter of the *AQP5* gene. The results of an electrophoretic mobility shift assay (EMSA) performed with 2 NF-κB probes confirmed that the binding activity toward sequences for these 2 NF-κB-responsive elements is elevated in the extract obtained from the LPS-injected mice. However, the binding activity toward the AP-1 binding sequences is not elevated in extracts prepared after the LPS injection. It is possible that most of the p-c-Jun and p-c-Fos elevated by LPS forms a complex with NF-κB, which complex then binds preferentially to the NF-κB-responsive element, not to the AP-1 binding sequences. Also, phosphorylation of ERK1/2 (p-ERK1/2 is known to activate c-Fos by phosphorylation) and c-Jun are activated by LPS, and this activation is inhibited by their respective inhibitors, AG126 and SP600125. Furthermore, nuclear translocation of p65 and p50, which are subunits of NF-κB, is activated by LPS, whereas such activation is inhibited by PDTC and MG132, confirming that the pathways to activate the above transcription factors are functioning in the PG.

Schüle et al. reported that transcription of the osteocalcin gene in ROS17/2.8 cells (rat osteoblastic cell line) transfected with c-*fos* and c-*jun* genes for Jun/Fos expression is inhibited under both vitamin-stimulated and nonstimulated conditions. These authors termed such a phenomenon as "cross coupling" [139]. By performing a CAT reporter assay, Stein et al. [146] examined the LTR promoter activity of HIV-1, which contains NF-κB-responsive sequences, and found that co-transfection of HeLa cells with this reporter gene and c-*fos* and c-*jun* genes results in a potentiation of the transcriptional activity of LTR (introduction of a mutation in the NF-κB responsive sequence disables the transcriptional activation). Thus, NF-κB and AP-1 family transcription factors p-c-Fos/p-c-Jun were proved to be physically associated to increase DNA binding capacity as well as biological activity, resulting in synergism of these two classes of transcription factors.

This section has given the PG as an example showing that NF-κB and AP-1 form a complex as a result of LPS signaling in the salivary glands and that such a complex actually potentiates transcriptional regulation (transcriptional inhibition) in this tissue [172]. In this example, the MAPK pathway is thought to generate p-c-Fos/p-c-Jun (AP-1), which then binds to NF-κB that has simultaneously been activated and thus potentiating its function. On the other hand, saliva secretion is supposedly reduced by LPS since the *AQP5* transcription is strongly suppressed by the endotoxin action. A similar phenomenon is observed in the lungs where the transcription of *AQP5* mRNA is suppressed by TNF-α via the NF-κB pathway, causing lung edema, but the MAPK pathway is not involved in this case [155]. Thus, potentiation of NF-κB function by AP-1 or p-c-Fos/p-c-Jun has an important

physiological meaning for the proinflammatory action of LPS. The physiological function of negative regulation of AQP5 by LPS needs to be determined further.

IPR-induced downregulation of PG aquaporin 5

Purified μ-calpain cleaves AQP0 protein at 4 positions in vitro, and these cleavage sites correspond to 4 of the 21 positions that are cleaved with age in vivo [87]. Further, calpain can degrade AQP2 expressed in the inner medullary collecting duct of the kidney [131]. As described above, AQP5 is also considered to be a substrate for calpain in the IPR-treated PG. Actually, purified calpain decomposes AQP5 in the membrane fraction of the SMG [15]. It has been suggested that μ-calpain is involved in the IPR-mediated regulation of the AQP5 level since calpain is a calcium (Ca^{2+})-dependent cysteine protease and the level of cytosolic Ca^{2+} ions is elevated by IPR [41].

The reduction in the AQP5 protein level by IPR is suppressed by two calpain inhibitors, ALLM and calpeptin, but not by MG132 or lactacystin (both proteasome inhibitors) nor by CQ (a lysosomal denaturant; ref. 15). These data confirmed that AQP5 is a substrate for calpain and that IPR stimulation induces calpain proteolysis to metabolize/decompose AQP5 in the PG. Under normal condition (untreated or without IPR), on the other hand, treatment of mice with MG132, CQ, ALLM, or calpeptin in vivo increases the AQP5 protein level in the PG [15]. This fact implies that ubiquitin-proteasome and lysosomal proteases, the two major proteolytic systems, are functioning in the PG under the normal condition. These results are supported by the report that AQP2 is degraded by proteasomal and lysosomal pathways soon after its synthesis [38].

Aquaporin 5 and salivary gland malfunction under pathological conditions

Impaired saliva secretion takes place under several pathological conditions such as those in patients suffering from Sjögren's syndrome, those who received irradiation therapy for head and neck cancers, or in patients with sialoadenitis which is caused by various types of pathogenesis.

In patients with Sjögren's syndrome, abnormal distribution of AQP5 in acinar cells of the salivary gland and lachrymal gland was reported, based on an experiment in which affinity-purified anti-human AQP5 was utilized to localize the antigen by immunohistochemical staining [148, 156]. These studies showed that localization of AQP5 in the apical membrane of acinar cells is minimum while that in the basal membrane is positive, and defective AQP5 trafficking was suggested as the cause of the abnormal distribution of AQP5, which would have resulted in impaired salivary secretion. However, contrary reports have appeared, indicating that AQP5 in Sjögren's patient remain in the apical membrane and that there is no difference in subcellular localization between Sjögren's patients and normal subjects ([6] and letter to editor in [29]). These authors used anti-rat AQP5 antibodies and confirmed antibody specificity by preabsorption with the human peptide. Such discrepancies may have been caused by the use of different antibodies in the staining techniques (letter to editor and authors' reply in [149]). Regarding this issue, however, no conclusion has yet been reached because AQP5 staining at apical membrane is observed in Sjögren's syndrome patients even when antibody specific for human AQP5 is used [28].

On the other hand, production of autoantibodies against the cell-surface muscarinic cholinergic receptor (M3) is observed in the sera from Sjögren's syndrome patients. Mouse SMG cells treated with anti-M3 antibody fail to translocate AQP5 to the plasma membrane upon muscarinic agonist stimulation in vitro [118]. Further, the sera from Sjögren's syndrome patients, but not from those of normal subjects, block the carbachol-induced AQP5 trafficking in HSG cells transfected with GFP-tagged human AQP5 [77]. These studies suggest that defective AQP5 trafficking toward the apical membrane in Sjögren's syndrome patients may be due to the production of autoantibodies against M3 receptors.

Sjögren's patients have been given infliximab (anti-TNF α) therapy under the assumption that tumor necrosis factor-α (TNF-α) would be involved as an immunopathogen in the development of this autoimmune disease. The results showed that this antibody therapy increases the unstimulated salivary flow and trafficking of AQP5 to the apical membrane in the acinar cells of the labial gland [147, 150]. It is noteworthy that TNF-α downregulates AQP5 expression in mouse lung epithelial cells via the NF-κB pathway [155] and that activation of this pathway in the mouse parotid gland downregulate AQP5 expression also [172] (see the section LPS-induced downregulation of PG aquaporin 5). These facts support the possibility that medication with anti-TNF-α/infliximab or even inhibitors of the NF-κB pathway is a candidate therapy for a successful treatment to recover from the patients' symptoms.

Care for head and neck cancer requires surgical removal of the tumor, followed by X-ray irradiation. The negative side effects of radiotherapy will be extended to adjacent normal tissues, which include increased apoptosis and marked decreases in the expression levels of AQP5 and TGF-β in the salivary gland [17]. The basic mechanism underlying such effects and possible treatments to protect the glands from the radiotherapy are being studied [32, 101, 102]. Morgan-Bathke et al. [102] used *Atg5$^{f/f}$;Aqp5-Cre* mice, which harbor a conditional KO of *Atg5*, in their salivary acinar cells and are deficient in autophagy. These autophagy-deficient mice have increased radiosensitivity, indicating them to be useful

controls to investigate the effects of radiation and/or radiation *plus* medication on the physiological function of the salivary gland. These researchers found that pretreatment with IGF-1 increases autophagosome formation in normal mice, but not in their Atg5 KO counterparts. The same group showed also that the rapalogue CCI-779 significantly improves the physiological function of the salivary gland diminished by radiation, as measured by determination of the saliva flow rate [101]. Han et al. [32] reported that pretreatment with phenylephrine, an α1 adrenergic agonist, completely protect against the reduction in AQP5 protein levels by irradiation in the rat SMG, although the mechanism is unclear.

Conclusions

AQP5 is involved in the water secretion from the salivary gland acinar cells in both paracellular and transcellular routes, which involvement is supported by the fact that water permeability is actually decreased in *AQP5*-KO mice and in rats expressing diminished levels of AQP5. Evidence is accumulating that indicates that TRPV4, which regulates the systemic osmotic pressure, is functionally linked to AQP5 in the salivary gland. Physiologically, the AQP5 level is controlled via parasympathetic nerves in the SMG and sympathetic agonists in the PG, and autophagy and lysosome-mediated degradation, as well as calpain proteolysis, are involved in regulating it. Under the pathophysiological condition of an LPS challenge to the PG, NF-κB/MAPK pathways are activated, leading to the activation of the transcriptional factors NF-κB and AP-1 (c-Fos/c-Jun), which form a complex that binds to the NF-κB-responsive element to downregulate potentially the transcription of *AQP5* mRNA. Further study is required to establish the actual molecular mechanisms by which AQP5 acts in water secretion from the salivary glands and other exocrine glands. Pathophysiological studies on AQP5 will aid in elucidating the biological significance of this channel protein in the exocrine glands.

Acknowledgments Parts of this review are based on studies conducted in the Department of Molecular Oral Physiology, Institute of Biomedical Sciences, Tokushima University (formerly, Institute of Health Biosciences, The University of Tokushima), Japan. The author appreciates deeply the laboratory staff and graduate students who participated in the project, as well as the Ministry of Education, Culture, Sports, Science, and Technology, Japan, for having supported the project for many years. The author would like to acknowledge Professor Y. Seo, Dokkyo Medical University School of Medicine; Professor K. Inenaga, Kyushu Dental University; Dr. M. Murakami, National Institute for Physiological Sciences; and Dr. T. Nakahari, Osaka Medical College, for collaboration in some of the studies cited and for thoughtful discussion regarding the water transport mechanism. The author also expresses his gratitude to Dr. Larry Frye for reviewing this article and gives thanks to all other people who contributed to the project.

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