JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 46 (6): 187–197, 2013
doi:10.1267/ahc.13037

Effects of Repeated Administration of Pilocarpine and Isoproterenol on Aquaporin-5 Expression in Rat Salivary Glands

Taketo Susa[1,2], Nobuhiko Sawai[2], Takeo Aoki[2], Akiko Iizuka-Kogo[2], Hiroshi Kogo[2], Akihide Negishi[1], Satoshi Yokoo[1], Kuniaki Takata[2] and Toshiyuki Matsuzaki[2]

[1]Department of Stomatology and Maxillofacial Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma and [2]Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

Received October 30, 2013; accepted November 28, 2013; published online December 25, 2013

Aquaporins are water channel proteins which enable rapid water movement across the plasma membrane. Aquaporin-5 (AQP5) is the major aquaporin and is expressed on the apical membrane of salivary gland acinar cells. We examined the effects of repeated administration of pilocarpine, a clinically useful stimulant for salivary fluid secretion, and isoproterenol (IPR), a stimulant for salivary protein secretion, on the abundance of AQP5 protein in rat salivary glands by immunofluorescence microscopy and semi-quantitative immunoblotting. Unexpectedly AQP5 was decreased in pilocarpine-administered salivary glands, in which fluid secretion must be highly stimulated, implying that AQP5 might not be required for fluid secretion at least in pilocarpine-administered state. The abundance of AQP5, on the other hand, was found to be significantly increased in IPR-administered submandibular and parotid glands. To address the possible mechanism of the elevation of AQP5 abundance in IPR-administered animals, changes of AQP5 level in fasting animals, in which the exocytotic events are reduced, were examined. AQP5 was found to be decreased in fasting animals as expected. These results suggested that the elevation of cAMP and/or frequent exocytotic events could increase AQP5 protein. AQP5 expression seems to be easily changed by salivary stimulants, although these changes do not always reflect the ability in salivary fluid secretion.

Key words: aquaporin-5 (AQP5), pilocarpine, isoproterenol, repeated administration, salivary gland

I. Introduction

The major components of saliva are water, ions, and proteins secreted by salivary glands. Salivary glands receive variable innervations from both the sympathetic and parasympathetic nervous system [22]. Acetylcholine released from the parasympathetic nerves acts on M_3 and M_1 muscarinic cholinergic receptors which basically evokes fluid secretion [22]. Norepinephrine released from sympathetic nerves affects both fluid secretion and salivary protein secretion via granular exocytosis through α1- and β1-adorenoreceptors, respectively [22].

Membrane water channel aquaporins provide specific pathway for transcellular water transfer in water-handling organs and tissues, such as kidney and salivary glands [11, 12, 14, 15, 29]. Aquaporin-5 (AQP5), which is predominantly found in the glandular tissues, is present in the luminal surface membrane of salivary gland acinar cells and provides a transcellular water transfer pathway for salivary fluid secretion [10, 16, 23]. There have been some studies focused on AQP5 as a possible molecular target for the treatment of xerostomia [1, 18, 28]. In the present study, we focused on the effect of two drugs, pilocarpine and isoproterenol (IPR), on AQP5 expression. Pilocarpine is a commonly used and effective medicine to treat mouth

Correspondence to: Toshiyuki Matsuzaki, Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, 3–39–22, Showa-machi, Maebashi, Gunma 371–8511, Japan.
E-mail: matoshi@gunma-u.ac.jp

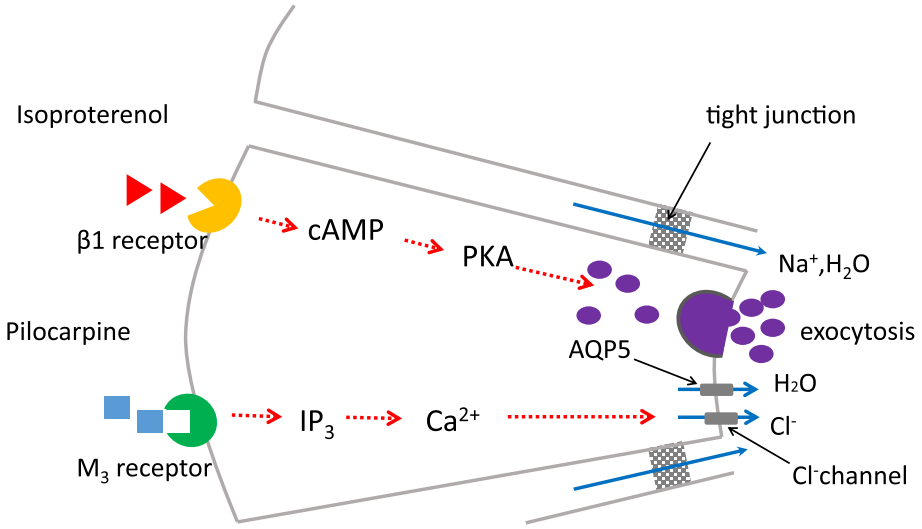

Fig. 1. Pharmacological effects of pilocarpine and isoproterenol on primary saliva secretion from salivary gland acinar cells. Pilocarpine stimulates M_3 receptors, which induces a rise in the intracellular Ca^{2+} level thereby causing chiefly the activation of luminal Cl^- channels. Cl^- release mediates Na^+ flow from the interstitium to the lumen, which consequently causes near-isosmolar water transfer through AQP5 and/or tight junctions. Isoproterenol activates β1-adrenergic receptors, causing chiefly the exocytosis of secretory granules containing salivary protein via the cAMP/PKA cascade.

dryness [5, 8]. Pilocarpine acts on the M_3 type muscarinic cholinergic receptors of acinar cells (Fig. 1). Stimulation via M_3 receptors chiefly mediates intracellular Ca^{2+} elevation which opens the apical membrane Cl^- channels in acinar cells and allows an efflux of Cl^- into the acinar lumen. This Cl^- efflux generates a lumen negative voltage gradient to drive Na^+ into the lumen via a paracellular pathway. The net result is the secretion of Na^+ and Cl^-, which causes the near-isosmolar water transfer into the lumen. There are two pathways for water transfer, transcellular through the membrane water channel protein AQP5 and paracellular through the tight junction [16]. We speculated some possible direct or indirect inducible effects of pilocarpine on AQP5 expression as well as its effect on Cl^- channel regulation. In our present study therefore, we examined the effect of repeated administration of pilocarpine on AQP5 protein abundance.

We further examined the effect of repeated administration of IPR, although it is not clinically used as a salivary stimulant. IPR mainly acts on β1-adorenoreceptor which causes cAMP elevation followed by activation of protein kinase A (PKA) leading to exocytosis of the storage protein (Fig. 1) [22]. It is well known that repeated administration of IPR produces hypertrophic/hyperplastic enlargements of rat salivary glands [26] and also induces the synthesis of some proteins in the salivary glands [2, 4, 17, 24]. We, therefore, expected some changes in AQP5 expression by repeated administration of IPR.

II. Materials and methods

Animals and experimental group

All animal experiments were conducted in compliance with the NIH Guide for the care and Use of Laboratory Animals and approved by the Animal care and Experimentation Committee, Gunma University, Showa Campus (admission no. 11-051). Male Wistar rats were obtained from Japan SLC (Shizuoka, Japan) and housed in polycarbonate cages under a 12-hr light-dark cycle with normal chow.

1) Pilocarpine or IPR administration

To assess the effects of pilocarpine or IPR administration, rats were divided into a pilocarpine, IPR, and control group. The animals in the pilocarpine and IPR groups received intraperitoneal administration of pilocarpine (Catalog no. P6503, Sigma-Aldrich, St Louis, MO; 0.1 mg/100 g body weight) or IPR (Catalog no. I5627, Sigma-Aldrich; 0.8 mg/100 g body weight), respectively, in the morning and again at night for 7 days. Vehicle (saline) was given intraperitoneally on the same schedule to the control group animals.

2) Fasting with/without IPR administration

To evaluate the changes in AQP5 abundance in fasting rats with/without IPR administration, the animals were divided into four groups as follows: fasting group, control group, fasting-IPR group, and fasting-vehicle group. In the fasting group, rats were fasted for 3 days whilst normal chow was given to the control group rats. In the fasting-IPR group, rats were fasted for 3 days with intraperitoneal administration of IPR (0.8 mg/100 g body weight) twice daily (morning and night). In the fasting-vehicle group, the animals were fasted for 3 days and received an intraperitoneal vehicle injection on the same morning/night schedule. Rats were deeply anesthetized by an intraperitoneal injection of ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight) and then sacrificed.

The parotid and submandibular glands were then rapidly removed for analysis.

Antibodies

The antibodies against AQP5 used in this study have been previously developed and characterized in our laboratory: AffGPTM41 [13], AffRaTM41 [13], and AffRaTM14 [10]. A mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich (Catalog no. A5316; St Louis, MO). Mouse monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Abcam (Catalog no. ab9484; Cambridge, MA). The secondary antibodies used were Rhodamine Red-X-conjugated donkey anti-rabbit IgG (Catalog no. 711-295-152; Jackson ImmunoResearch, West Grove, PA), Rhodamine Red-X-conjugated donkey anti-guinea pig IgG (Catalog no. 706-295-148, Jackson ImmunoResearch), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Catalog no. P0448; DAKO, Glostrup, Denmark), HRP-conjugated goat anti-mouse IgG (Catalog no. P0447, DAKO), and HRP-conjugated rabbit anti-guinea pig IgG (Catalog no. P0141, DAKO).

Hematoxylin-eosin staining

Parotid and submandibular gland tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and observed under an AxioPhot2 microscope (Zeiss, Overkochen, Germany) equipped with a digital camera (DP72 Olympus, Tokyo, Japan).

Immunofluorescence microscopy and laser confocal microscopy

Fresh parotid and submandibular glands isolated from rats were immediately embedded in O.C.T. compound (Sakura Fine Technical, Tokyo, Japan) and rapidly frozen in liquid nitrogen. These frozen sections were cut with a cryostat, mounted on poly-L-lysine (Sigma-Aldrich) coated glass slides, fixed with ethanol at –20°C for 30 min, and washed with PBS. For paraffin sections, tissues were immersion-fixed in 4% paraformaldehyde-0.1 M phosphate buffer solution (pH 7.4) overnight, dehydrated and embedded in paraffin. Paraffin sections were mounted on adhesive glass slides (Platinum; Matsunami, Osaka, Japan). For antigen retrieval, paraffin sections were placed in 20 mM Tris-HCl buffer (pH 9.0) and heated in a microwave oven (MI-77, AZUMAYA, Tokyo, Japan) for 30 min at 97°C [15]. Both frozen and paraffin sections were then incubated with 5% normal donkey serum-PBS to block non-specific binding of antibodies and subsequently incubated with anti-AQP5 polyclonal antibody (AffGPTM41, AffRaTM41, or AffRaTM14) overnight at 4°C. After washing with PBS, the specimens were incubated with the appropriate secondary antibody, Rhodamine Red-X-conjugated anti guinea pig or rabbit IgG, for 1 hr at room temperature. For nuclear counterstaining, 4',-6-diamidino-2-phenylindole (DAPI) was added to the secondary antibody solution. Specimens were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined with a conventional fluorescence microscope BX62 (Olympus, Tokyo, Japan) equipped with a CCD camera (Cool Snap K4, Photometrics, Tucson, AZ). Images were captured using Metamorph software (Molecular Devices, Sunnyvale, CA) and processed with Photoshop software (Adobe Systems, San Jose, CA). Laser confocal microscopy was carried out with a Fluoview FV1000 (Olympus). Captured images were processed with Photoshop software (Adobe Systems).

Immunoblotting

Parotid and submandibular glands were quickly removed from sacrificed rats, frozen in liquid nitrogen and stored at –80°C until use. To reveal the specific band for AQP5, COS7 cells were transfected with AQP5 using lipofectamine 2000 (Life Technologies, Carlsbad, CA). Rat AQP5 cDNA was a kind gift from Dr. Bruce J Baum (National Institutes of Health, Bethesda) [3]. Tissue and cell homogenates were prepared with a glass homogenizer in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Complete mini; Roche, Mannheim, Germany) on ice. Homogenates were then centrifuged at 800 g for 5 min at 4°C to remove tissue debris. Supernatants were collected and protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) with BSA as a standard. These samples were denatured in an equal volume of sample buffer (Catalog no. NP0007, Life Technologies) at 70°C for 10 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with NuPAGE 12.5% Bis-Tris Gel (Catalog no. NP0343, Life Technologies), and transferred to polyvinylidene difluoride (PVDF) membranes using a standard methods. After treating with Starting Block T20 (TBS) Blocking Buffer (Catalog no. 37543, Thermo Scientific) at room temperature for 15 min to block non-specific binding of antibodies, membranes were sequentially incubated with antibodies against AQP5 at 4°C overnight followed by HRP-coupled secondary antibodies (diluted at 1 : 10000) at room temperature for 90 min.

The specificity of AQP5-immunolabeling was verified by pre-incubating the antibodies with the peptides used as immunogens. Visualization was performed using an enhanced chemiluminescence system (ECL Prime; GE Health care, Buckinghamshire, UK). The Magic Mark XP Western Protein Standard (Catalog no. LC5602, Life Technologies) was used as molecular weight markers. After stripping the antibodies with a 1 M glycine solution (pH 2.0) containing 20% SDS, membranes were reprobed with antibodies to β-actin or GAPDH as loading controls. Finally, membranes were stained with Coomassie brilliant blue. Chemiluminescence was detected with an Image Quant LAS4000 (GE Health care) and analyzed with Image J software (National Institutes of Health).

III. Results

Histology

By gross anatomical observation, IPR-administered parotid glands were found to be significantly enlarged compared with the controls as previously described [26], although no apparent changes were found in the pilocarpine-administered parotid and submandibular glands. We thus evaluated any histological changes in both the parotid and submandibular glands from the different experimental rats. Tissue sections were stained with hematoxylin and eosin and observed under a light microscope (Fig. 2). When we observed IPR-administered parotid glands, we found that 1) individual acinar cells were enlarged and 2) numerous vacuole-like structures were present in the subluminal region of each acinar cell. The IPR-administered submandibular glands were also altered, namely the cytoplasm was more eosinophilic than the controls in which

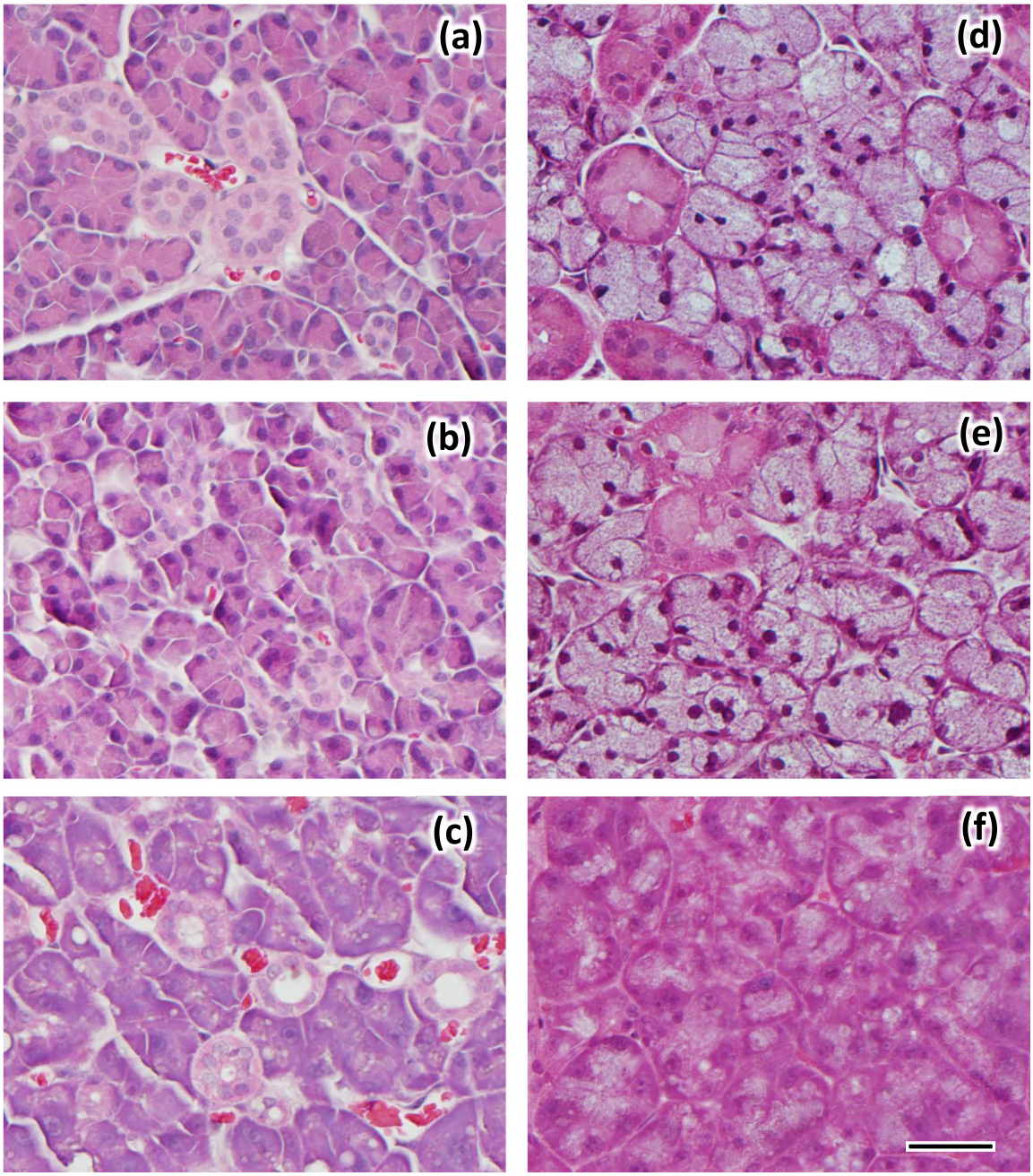

Fig. 2. Histological changes in the acinar cells of pilocarpine- or isoproterenol (IPR)-administered rat salivary glands. Rat parotid and submandibular glands are shown in the left and right columns, respectively. Control, pilocarpine-administered, IPR-administered glands are shown in the upper, middle, and lower panels, respectively. Changes are apparent in the IPR-administered parotid (**c**) and submandibular (**f**) glands. Bar=20 μm.

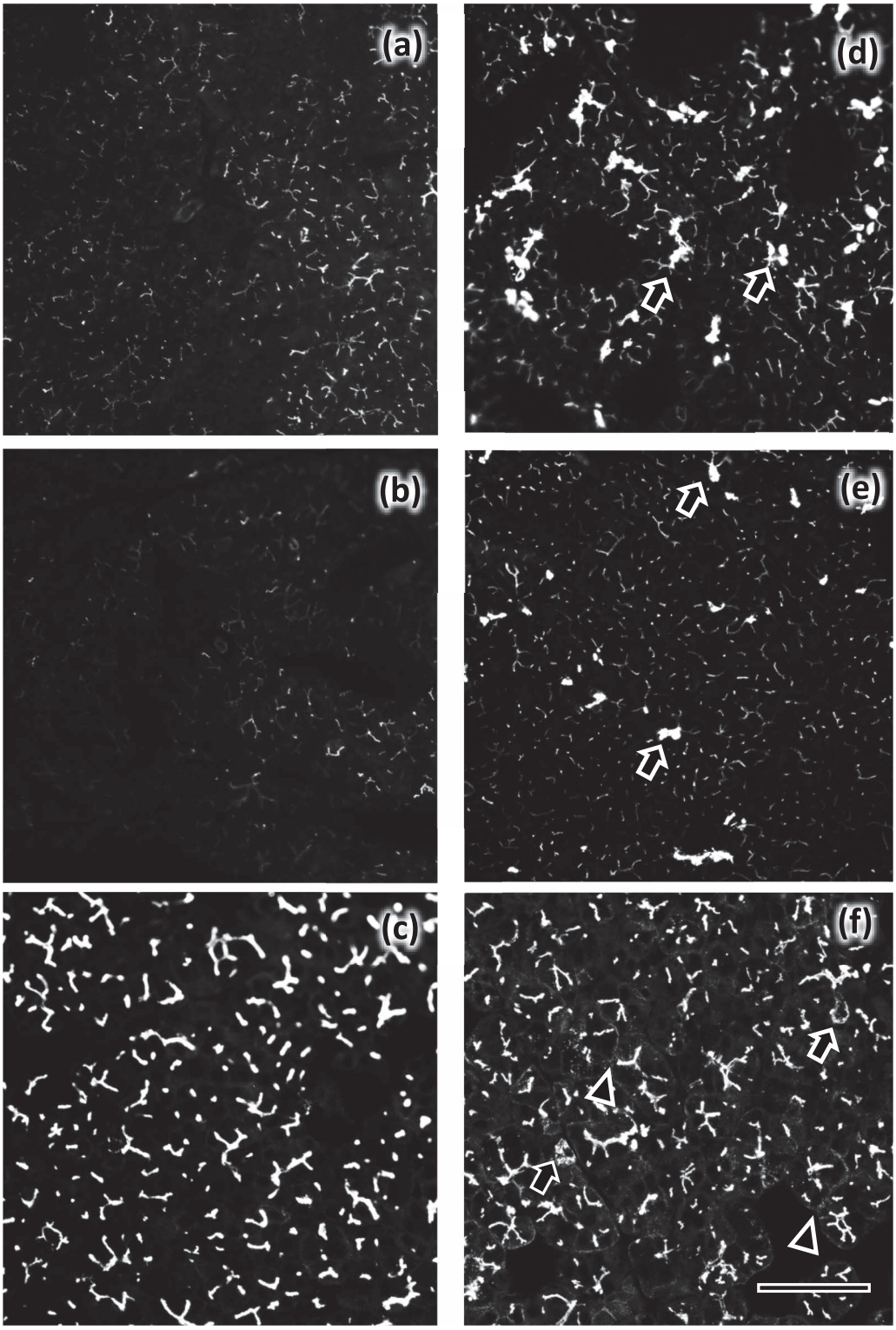

Fig. 3. Changes in the fluorescence intensity of AQP5-labeling in pilocarpine- or IPR-treated salivary glands. Parotid and submandibular glands are shown in the left and right columns, respectively. Control, pilocarpine-administered, and IPR-administered glands are shown in the upper, middle, and lower panels, respectively. Fluorescent images were captured and processed under identical conditions and representative images are shown. Arrows indicate intercalated ducts. The AQP5 labeling intensity is highly increased in IPR-administered parotid (**c**) and submandibular (**f**) glands, but decreased in pilocarpine-administered parotid (**b**) and submandibular (**e**) glands. AQP5 labeling is mainly restricted to the apical membrane. However, faint labeling was evident on the basolateral membrane of IPR-administered submandibular glands (arrowheads in **f**). Bar=100 μm.

acinar cells normally show a clear cytoplasm. This may be caused by the change in the protein composition in IPR-administered salivary glands [2, 4, 17, 24]. We found no apparent changes in pilocarpine-administered parotid or submandibular glands.

Immunohistochemistry of pilocarpine- or IPR-administered salivary glands

Immunofluorescence microscopy was performed on cryostat sections of pilocarpine- or IPR-administered rat salivary glands with anti-AQP5 antibodies (Fig. 3). When the specimens were examined at lower magnification, the fluorescence labeling intensity of AQP5 was clearly increased in IPR-administered, but decreased in pilocarpine-administered, parotid and submandibular glands compared with the controls. When the tissue distribution of AQP5 was examined, no changes were apparent in either IPR- or pilocarpine-administered parotid and submandibular glands compared with the controls, i.e. AQP5 was found to be localized to the apical membrane including the intercellular secretory canaliculi of acinar cells in both glands. In the submandibular gland, the intense labeling for AQP5 was also seen in the apical surface of the intercalated duct cells and the faint labeling was on the basolateral membrane of acinar cells. These results were in accordance with previously reported findings from our laboratory [10, 13, 16]. To assess whether the high amount of AQP5 labeling in IPR-administered parotid and submandibular glands was still restricted to the surface membrane, laser confocal microscopy was carried out (Fig. 4). AQP5 was found in this analysis to be restricted to the surface membrane with no signal evident in the cytoplasm (Fig. 4).

We used three AQP5 antibodies in this study. AffRaTM41 and AffGPTM41 were raised in rabbit and guinea pig, respectively, with the same antigen peptide, whereas AffRaTM14 was raised in rabbit using a different antigen peptide. All three antibodies produced similar results. We also performed immunofluorescence micros-

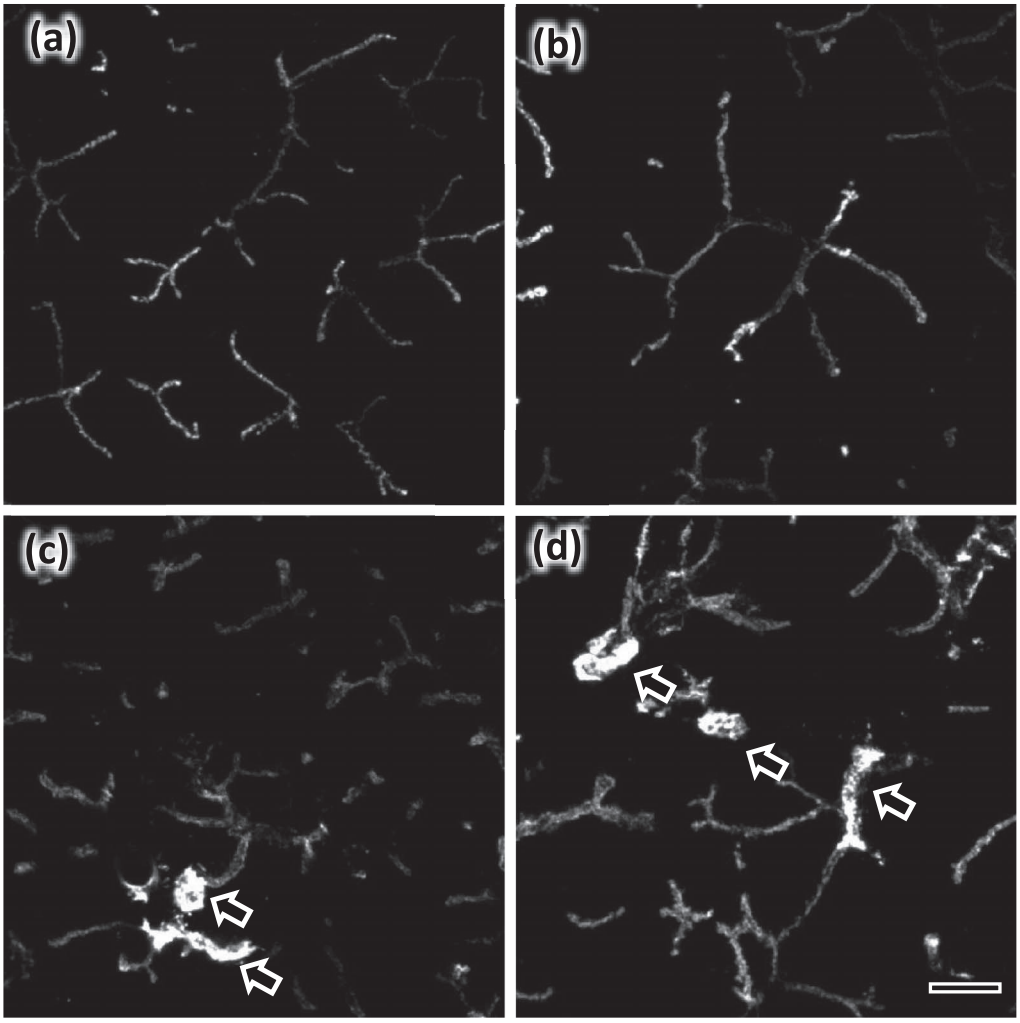

Fig. 4. Subcellular distribution of AQP5 by laser confocal microscopy. Control (**a**) and IPR-administered (**b**) parotid glands. Control (**c**) and IPR-administered (**d**) submandibular glands. Projection images of 17 consecutive confocal images are shown. AQP5 is localized to the apical membrane including the intercellular canaliculi. No intracellular labeling was apparent. Arrows indicate intercalated ducts. Bar=10 μm.

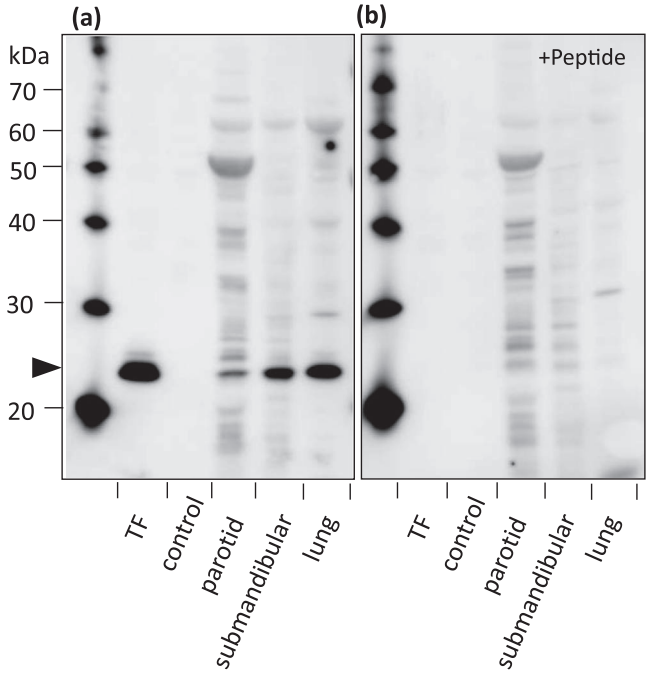

Fig. 5. Immunoblotting of AQP5 from transfected cells and tissue homogenates. (**a**) A band at approximately 24 kDa was common to all samples (arrowhead) except for the untransfected control. (**b**) When the AQP5 antibody was preabsorbed with the antigen peptide prior to immunoblotting, the band at approximately 24 kDa largely disappears. TF, AQP5-transfected COS7 cells; control, untransfected COS7 cells.

copy analysis of antigen-retrieved paraffin sections to determine whether the changes in AQP5 labeling intensity could be related to changes in immunoreactivity caused by some modifications to the antibody-binding site of AQP5 [6, 20]. However, similar results were obtained from both cryostat and antigen-retrieved paraffin sections.

Immunoblotting of pilocarpine- or IPR-administered salivary glands

To quantify changes in the AQP5 protein level in pilocarpine- or IPR-administered salivary glands, we performed semi-quantitative immunoblotting. We have previously shown that our AQP5 antibodies produced a positive band of 27 kDa in immunoblots of rat salivary gland homogenates [10]. In our present study, we used an immunoblotting system that differed from our previous studies and found that a positive band was identified at about 24 kDa in salivary gland homogenates. To reveal the AQP5-specific band in this system, AQP5-transfected COS7 cells and lung tissue homogenates in which AQP5 was expressed abundantly were simultaneously electrophoresed with parotid and submandibular gland homogenates and subjected to immunoblotting with anti-AQP5 antibodies (Fig. 5). A band at about 24 kDa was commonly detected in all of these samples by all AQP5 antibodies used and largely disappeared when the antibodies were preabsorbed with antigen peptide. Hence we considered the 24-kDa band to be the AQP5-specific product and quantified its intensity.

We tried to use β-actin and GAPDH as internal controls for AQP5 quantification but we found these proteins to be inadequate for this purpose as their levels also seemed to change in IPR- and pilocarpine-administered samples (data not shown). We therefore carefully measured the protein concentrations of our tissue homogenates and adjusted our measurements by the amount of sample electrophoresed. Our results showed that the repeated adminis-

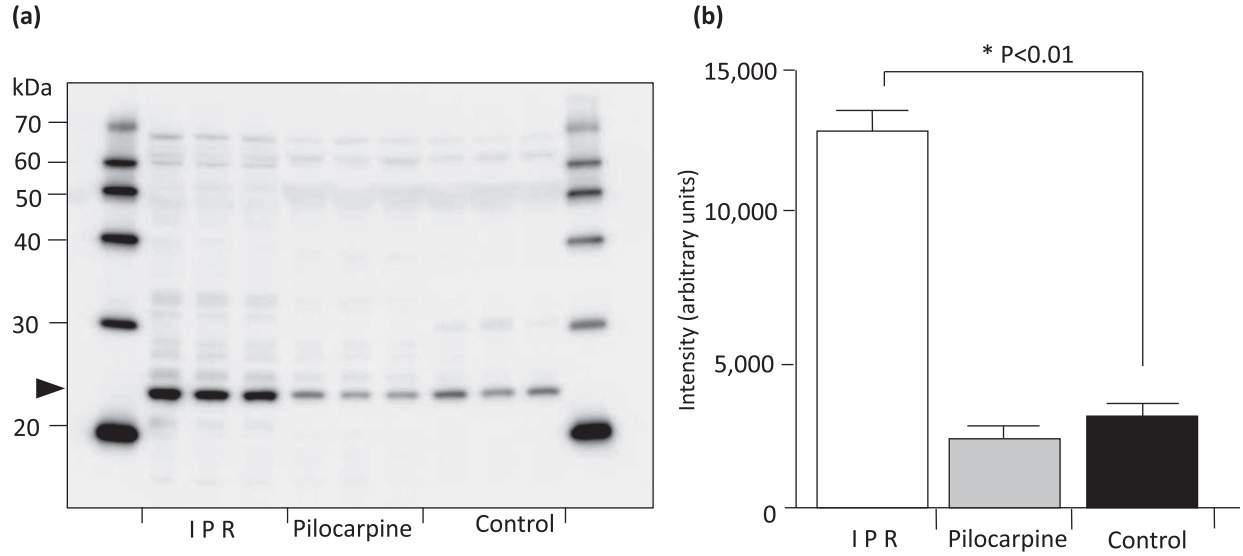

Fig. 6. Semi-quantitative immunoblotting of AQP5 in pilocarpine- or IPR-administered parotid glands. (**a**) Ten micrograms of tissue homogenate protein was prepared from parotid glands and electrophoresed. AQP5-specific bands (arrowhead) on the blot were quantified. (**b**) Band intensities are expressed as arbitrary units. Data are expressed as the mean±SEM (n=3). Statistical analysis was performed using the Student's t-test.

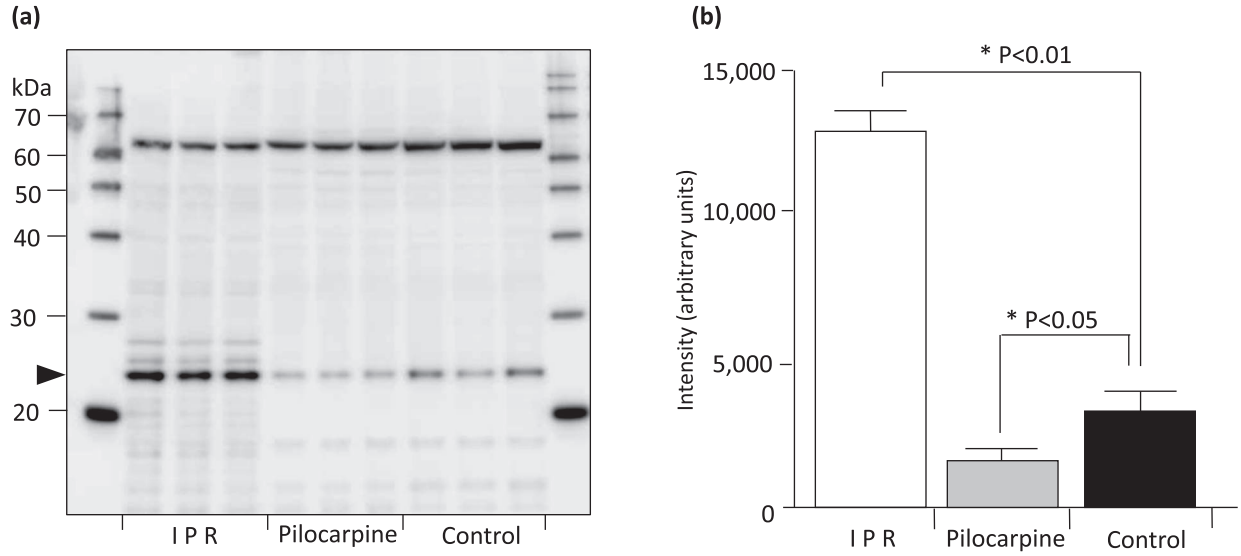

Fig. 7. Semi-quantitative immunoblotting of AQP5 in pilocarpine- or IPR-administered submandibular glands. (**a**) Ten micrograms of tissue homogenate protein was prepared from submandibular glands and electrophoresed. AQP5-specific bands (arrowhead) on the blot were then quantified. (**b**) Band intensities are expressed as arbitrary units and data are the mean±SEM (n=3). Statistical analysis was performed using the Student's t-test.

tration of IPR significantly increased the AQP5 protein level in both the parotid ($P<0.01$; Fig. 6) and submandibular ($P<0.01$; Fig. 7) glands. On the other hand, repeated administration of pilocarpine significantly reduced the AQP5 protein levels in the submandibular glands ($P<0.05$; Fig. 7). In the parotid glands, the AQP5 protein levels showed the same tendency to decrease upon pilocarpine treatment, although this was not a statistically significant change (Fig. 6).

Immunohistochemistry and immunoblotting of salivary glands from fasted rats

We speculated that repeated exocytotic events by repeated IPR administration could cause the increase of AQP5 expression. Therefore, to suppress exocytotic events in the salivary acini of the rats, the animals were fasted for 3 days and immunohistochemistry and immunoblotting was performed using similar procedures to those described above. Immunohistochemistry showed that AQP5 was decreased and almost undetectable in the fasted rat parotid glands, but was maintained or increased when IPR was administered during fasting (Fig. 8). Semi-quantitative immunoblotting confirmed a reduction of AQP5 in the parotid glands of fasted rats (Fig. 9).

IV. Discussion

We found in our current analyses that the abundance of AQP5 in the salivary gland is not increased but decreased by repeated administration of pilocarpine, one of the most effective xerostomia treatment drugs. Surprisingly, AQP5 was found to be increased by repeated administration of IPR, although this agent is not used for xerostomia treatment. We discuss these findings below.

Effect of repeated administration of pilocarpine on AQP5 abundance

We speculated that the AQP5 expression could be increased by a repeated administration of pilocarpine. Unexpectedly, however, we found that AQP5 was in fact decreased by pilocarpine, and that pilocarpine showed no enhancing effects on AQP5 expression. These results might indicate that the paracellular pathway in the acini would be predominant for water transfer in the pilocarpine-stimulated state, so that the necessity of AQP5 for transcellular pathway might be low. It was actually shown using both ultrastructural and tracer-permeability analyses that tight junctions in acinar cells appear to be leaky, suggestive of a possible paracellular pathway of water movement [27]. The beneficial effects of pilocarpine on xerostomia may therefore not be related to AQP5 expression. Our results, of course, do not deny the functional role of AQP5 in the salivary fluid secretion. It is also true that AQP5 knockout mice showed defective saliva secretion to some extent [7].

What is the mechanism underlying the decrease of AQP5 protein in pilocarpine-administered salivary gland? Pan *et al.* [21] identified AQP5 in human resting saliva and they found that the amount of AQP5 in saliva is increased in cevimeline, a M_3 agonist, -stimulated state. Ogawa *et al.* [19] found that exosomes in human whole saliva contain AQP5, although the mechanism underlying the release of exosomes into saliva and biological functions of them are still unclear. The decrease in luminal surface AQP5 in pilocarpine-administered rats in our current study might be the outcome of AQP5 release into saliva via possible some secreted microvesicles by direct budding of luminal membrane together with AQP5 and/or via exosomes containing AQP5 [30].

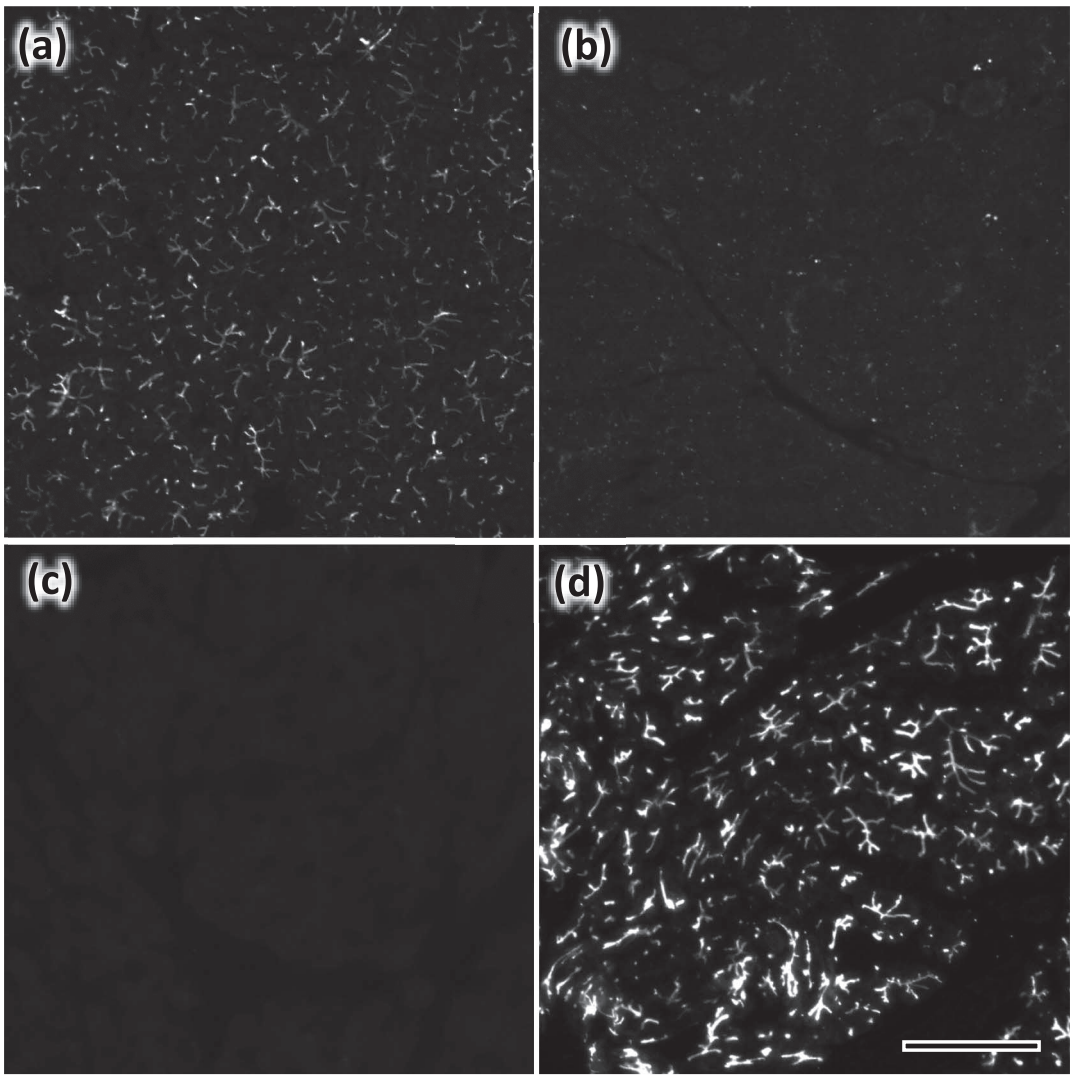

Fig. 8. Changes in the fluorescence intensity of AQP5 signals in rat parotid glands from fasted animals with or without IPR administration. Rats were either given normal chow as a control (**a**), fasted for 3 days (**b**), fasted with vehicle (saline) injection as a control (**c**), or fasted with IPR-administration (**d**). Following immunofluorescence microscopy, fluorescent images were captured and processed under identical conditions. Representative images from each group are shown. Bar=100 μm.

Effect of repeated administration of IPR on AQP5 abundance

IPR is a β1-adrenoreceptor stimulant that causes protein secretion via granular exocytosis triggered by cAMP pathway activation (Fig. 1). IPR is commonly used in experiments on parotid gland acinar cells as a secretagogue, although it is not used clinically for the treatment of salivary gland dysfunction. Surprisingly, we found in our current analyses that the AQP5 protein level was highly elevated by repeated administration of IPR, most notably in the parotid gland. The question of why IPR increases the AQP5 level is an important consideration and we anticipated two possible mechanisms. One is that an elevation in the intracellular cAMP level would directly increase AQP5 transcription. Yeh *et al.* [33] have previously reported that IPR induces the phosphorylation of the cAMP response element binding protein (CREB) and augments its binding to DNA in the rat parotid gland. It has also been reported that AQP5 expression is induced by the cAMP-PKA/CREB pathway in rat cultured nasal epithelial cells [31]. Yang *et al.* [32] showed that elevation of intracellular cAMP caused the increase in AQP5 mRNA and protein level in murine lung epithelial cell line and mouse lung tissue slices. Taken together with these earlier findings, our present data suggest that AQP5 expression in the salivary glands may be induced via cAMP-PKA/CREB pathway activation following repeated IPR administration. A second possible mechanism is that frequent exocytotic events themselves, which are also caused via cAMP-PKA pathway activation, indirectly increase AQP5 expression [25]. To examine this point, changes in AQP5 abundance were assessed when exocytosis was suppressed in our subject rats by fasting the animal for 3 days. As expected, AQP5 protein was found

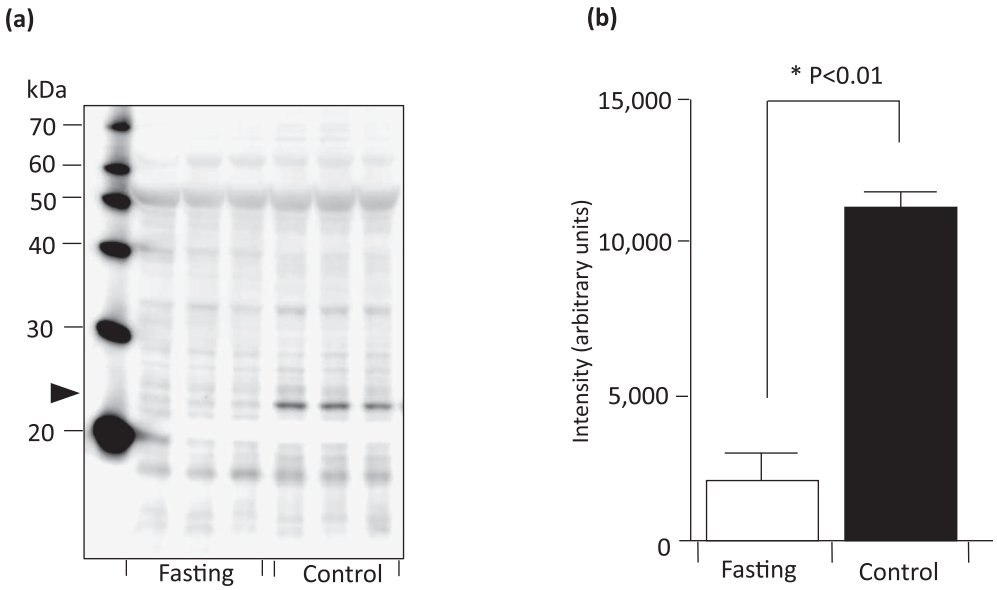

Fig. 9. Semi-quantitative AQP5 immunoblotting of rat parotid glands from fasted animals. (**a**) Ten micrograms of tissue homogenate protein was prepared from parotid glands and electrophoresed. AQP5-specific bands (arrowhead) were quantified. (**b**) Band intensities are expressed as arbitrary units. Data are the mean±SEM (n=3). Statistical analysis was performed using the Student's t-test.

to be decreased under fasting conditions. Interestingly, however, AQP5 protein was not decreased, and if anything was actually increased, by repeated IPR administration in rats, even if the animals were fasted. These results suggest that the AQP5 expression may be influenced by the frequency of exocytotic events.

The physiological importance of increased AQP5 expression following repeated IPR administration is unclear at present and may be relevant to our understanding of the real function of AQP5 in the salivary glands, since neither the elevation of cAMP nor the exocytosis of secretory granules are directly related to water secretion. AQP5 may have some important roles in addition to or other than salivary fluid secretion at the apical membrane. Matsuki *et al.* [9] have reported the localization of AQP5 in the secretory granule membrane and discussed the possible functional role of AQP5 in osmoregulation in the secretory granules. To ascertain whether some AQP5 molecules become localized to the secretory granules when AQP5 abundance is increased by repeated IPR administration, we performed laser confocal microscopy. However, no apparent intracellular AQP5 signals were detectable in this analysis. Further studies are required to reveal the real function of AQP5 in the salivary glands.

In summary, we found that the abundance of AQP5 in the salivary gland is decreased by repeated administration of pilocarpine, one of the most effective xerostomia treatment drugs, implying that the beneficial effect of pilocarpine for fluid secretion may not be related to AQP5. On the other hand, IPR, a stimulant for granule protein secretion, was found to increase AQP5 protein following repeated administration. These findings evoked us to consider the physiological roles of AQP5, which are not just for salivary fluid secretion, in the salivary glands.

V. Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 25463068. We thank Dr. Bruce J Baum for providing rat AQP5 cDNA. We also thank Yukiko Tajika-Takahashi and Mutsumi Shimoda for their assistance.

VI. References

1. Asari, T., Maruyama, K. and Kusama, H. (2009) Salivation triggered by pilocarpine involves aquaporin-5 in normal rats but not in irradiated rats. *Clin. Exp. Pharmacol. Physiol.* 36; 531–538.
2. Barka, T., Yagil, C., van der Noen, H. and Naito, Y. (1986) Induction of the synthesis of a specific protein in rat submandibular gland by isoproterenol. *Lab. Invest.* 54; 165–171.
3. Delporte, C., O'Connell, B. C., He, X., Ambudkar, I. S., Agre, P. and Baum, B. J. (1996) Adenovirus-mediated expression of aquaporin-5 in epithelial cells. *J. Biol. Chem.* 271; 22070–22075.
4. Denny, P. A., Hong, S. H., Klauser, D. K. and Denny, P. C. (1992) Increased mucin levels in submandibular saliva from mice following repeated isoproterenol treatment. *Arch. Oral Biol.* 37; 73–75.
5. Fox, P. C., van der Ven, P. F., Baum, B. J. and Mandel, I. D. (1986) Pilocarpine for the treatment of xerostomia associated with salivary gland dysfunction. *Oral Surg. Oral Med. Oral Pathol.* 61; 243–248.
6. Ishida, N., Hirai, S. and Mita, S. (1997) Immunolocalization of aquaporin homologs in mouse lacrimal glands. *Biochem. Biophys. Res. Commun.* 238; 891–895.
7. Ma, T., Song, Y., Gillespie, A., Carlson, E. J., Epstein, C. J. and Verkman, A. S. (1999) Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. *J. Biol. Chem.* 274; 20071–20074.

8. Maruyama, K. and Koshihara, N. (2006) Pharmacological and clinical profile of Pilocarpine hydrochloride (SALAGEN) Tab.5mg,. *Nippon Yakurigaku Zasshi* 127; 399–407 (In Japanese).
9. Matsuki, M., Hashimoto, S., Shimono, M., Murakami, M., Fujita-Yoshigaki, J., Furuyama, S. and Sugiya, H. (2005) Involvement of aquaporin-5 water channel in osmoregulation in parotid secretory granules. *J. Membrane Biol.* 203; 119–126.
10. Matsuzaki, T., Suzuki, T., Koyama, H., Tanaka, S. and Takata, K. (1999) Aquaporin-5 (AQP5), a water channel protein, in the rat salivary and lacrimal glands: immunolocalization and effect of secretory stimulation. *Cell Tissue Res.* 295; 513–521.
11. Matsuzaki, T., Tajika, Y., Tserentsoodol, N., Suzuki, T., Aoki, T., Hagiwara, H. and Takata, K. (2002) Aquaporins: a water channel family. *Anat. Sci. Int.* 77; 85–93.
12. Matsuzaki, T., Ablimit, A., Tajika, Y., Suzuki, T., Aoki, T., Hagiwara, H. and Takata, K. (2005) Water channel aquaporin 1 (AQP1) is present in the perineurium and perichondrium. *Acta Histochem. Cytochem.* 38; 37–42.
13. Matsuzaki, T., Ablimit, A., Suzuki, T., Aoki, T., Hagiwara, H. and Takata, K. (2006) Changes of aquaporin 5-distribution during release and reaccumulation of secretory granules in IPR-administered mouse parotid gland. *J. Electron Microsc.* 55; 183–189.
14. Matsuzaki, T., Hata, H., Ozawa, H. and Takata, K. (2009) Immunohistochemical localization of the aquaporins AQP1, AQP3, AQP4, and AQP5 in the mouse respiratory system. *Acta Histochem. Cytochem.* 42; 159–169.
15. Matsuzaki, T., Inahata, Y., Sawai, N., Yang, C. Y., Kobayashi, M., Takata, K. and Ozawa, H. (2011) Immunohistochemical localization of the water channels AQP4 and AQP5 in the rat pituitary gland. *Acta Histochem. Cytochem.* 44; 259–266.
16. Matsuzaki, T., Susa, T., Shimizu, K., Sawai, N., Suzuki, T., Aoki, T., Yokoo, S. and Takata, K. (2012) Function of the membrane water channel aquaporin-5 in the salivary gland. *Acta Histochem. Cytochem.* 45; 251–259.
17. Menaker, L., Sheetz, J. H., Cobb, C. M. and Navia, J. M. (1974) Gel electrophoresis of whole saliva and associated histologic changes in submandibular glands of isoproterenol-treated rats. *Lab. Invest.* 30; 341–349.
18. Motegi, K., Azuma, M., Tamatani, T., Ashida, Y. and Sato, M. (2005) Expression of aquaporin-5 in and fluid secretion from immortalized human salivary gland ductal cells by treatment with 5-aza-20-deoxycytidine: a possibility for improvement of xerostomia in patients with Sjögren's syndrome. *Lab. Invest.* 85; 342–353.
19. Ogawa, Y., Miura, Y., Harazono, A., Kanai-Azuma, M., Akimoto, Y., Kawakami, H., Yamaguchi, T., Toda, T., Endo, T., Tsubuki, M. and Yanoshita, R. (2011) Proteomic analysis of two types of exosomes in human whole saliva. *Biol. Pharm. Bull.* 34; 13–23.
20. Ohashi, Y., Tsuzaka, K., Takeuchi, T., Sasaki, Y. and Tsubota, K. (2008) Altered distribution of aquaporin 5 and its C-terminal binding protein in the lacrimal glands of a mouse model for Sjögren's syndrome. *Curr. Eye Res.* 33; 621–629.
21. Pan, Y., Iwata, F., Wang, D., Muraguchi, M., Ooga, K., Ohmoto, Y., Takai, M., Cho, G., Kang, J., Shono, M., Li, X., Okamura, K., Mori, T. and Ishikawa, Y. (2009) Identification of aquaporin-5 and lipid rafts in human resting saliva and their release into cevimeline-stimulated saliva. *Biochem. Biophys. Acta* 1790; 49–56.
22. Proctor, G. B. and Carpenter, G. H. (2007) Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci.* 133; 3–18.
23. Raina, S., Preston, G. M., Guggiono, W. B. and Agre, P. (1995) Molecular cloning and characterization of an aquaporin cDNA from saliva, lacrimal, and respiratory tissues. *J. Biol. Chem.* 270; 1908–1912.
24. Sasaki, T., Litwack, G. and Baserga, R. (1969) Protein synthesis in the early prereplicative phase of isoproterenol-stimulated synthesis of deoxyribonucleic acid. *J. Biol. Chem.* 244; 4831–4837.
25. Satoh, K., Matsuki-Fukushima, M., Qi, B., Guo, M. Y., Narita, T., Fujita-Yoshigaki, J. and Sugiya, H. (2009) Phosphorylation of myristoylated alanine-rich C kinase substrate is involved in the cAMP-dependent amylase release in parotid acinar cells. *Am. J. Physiol. Gastrointest Liver Physiol.* 296; G1382–G1390.
26. Selye, H., Veilleux, R. and Cantin, M. (1961) Excessive stimulation of salivary gland growth by isoproterenol. *Science* 133; 44–45.
27. Simson, J. A. and Bank, H. L. (1984) Freeze-fracture and lead ion tracer evidence for a paracellular fluid secretory pathway in rat parotid glands. *Anat. Rec.* 208; 69–80.
28. Takakura, K., Takaki, S., Takeda, I., Hanaue, N., Kizu, Y., Tonogi, M. and Yamane, G. (2007) Effect of cevimeline on radiation-induced salivary gland dysfunction and AQP5 in submandibular gland in mice. *Bull. Tokyo Dent. Coll.* 48; 47–56.
29. Takata, K., Matsuzaki, T. and Tajika, Y. (2004) Aquaporins: water channel proteins of the cell membrane. *Prog. Histochem. Cytochem.* 39; 1–83.
30. Thery, C., Ostrowski, M. and Segura, E. (2009) Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* 9; 581–593.
31. Wang, W. and Zheng, M. (2011) Role of cAMP-PKA/CREB pathway in regulation of AQP 5 production in rat nasal epithelium. *Rhinology* 49; 464–469.
32. Yang, F., Kawedia, J. D. and Menon, A. G. (2003) Cyclic AMP regulates aquaporin 5 expression at both transcriptional and post-transcriptional levels through a protein kinase A pathway. *J. Biol. Chem.* 278; 32173–32180.
33. Yeh, C. K., Chandrasekar, B., Lin, A. L., Dang, H., Kamat, A., Zhu, B. and Katz, M. S. (2012) Cellular signals underlying β-adrenergic receptor mediated salivary gland enlargement. *Differentiation* 83; 68–76.