Degradation of submandibular gland AQP5 by parasympathetic denervation of chorda tympani and its recovery by cevimeline, an M3 muscarinic receptor agonist

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Li X, Azlina A, Karabasil MR, Purwanti N, Hasegawa T, Yao C, Akamatsu T, Hosoi K. Degradation of submandibular gland AQP5 by parasympathetic denervation of chorda tympani and its recovery by cevimeline, an M3 muscarinic receptor agonist. Am J Physiol Gastrointest Liver Physiol 295: G112–G123, 2008. First published May 1, 2008; doi:10.1152/ajpgi.00359.2007.—By chorda tympani denervation (CTD, parasympathectomy), the aquaporin-5 (AQP5), but not AQP1, protein level in the rat submandibular gland (SMG) was significantly decreased, dropping to 37% of that of the contralateral gland at 4 wk. The protein levels of AQP5 and AQP1 were not significantly affected by denervation of the cervical sympathetic trunk (sympathectomy). Administration of cevimeline hydrochloride, an M3 muscarinic receptor agonist (10 mg/kg for 7 days po), but not pilocarpine (0.3 mg/kg for 7 days po), recovered the AQP5 protein level reduced by CTD and increased the AQP1 protein level above the control one. The mRNA level of AQP5 was scarcely affected by CTD and cevimeline hydrochloride administration. Administration of chloroquine (50 mg/kg for 7 days po), a denaturant of lysosomes, increased the AQP5 protein level reduced by CTD. An extract obtained from the submandibular lysosomal fraction degraded the AQP5 protein in the total membrane fraction in vitro. These results suggest the possible regulation of the AQP5 protein level in the SMG by the parasympathetic nerves/M3 muscarinic receptor agonist and imply the involvement of lysosomal enzymes, but not a transcriptional mechanism, in this regulation.

AQP5, parasympathectomy; lysosomes; chloroquine

AQUAPORINS (AQPs) are the family of water channel proteins distributed widely in nature, being found in organisms from bacteria to animals. To date, 13 AQPs have been identified in various tissues of mammals (1, 12–14, 17, 23). These proteins exist in the cell membrane and selectively transport water or water and solutes such as glycerol and urea through the central pore of each AQP monomer in response to osmotic gradients (2, 11, 16, 20, 39, 41, 51). AQPs have been divided into two major subgroups on the basis of their permeability properties: AQP0, 1, 2, 4, 5, 6, and 8 are classically defined as water-selective channels, whereas AQP3, 7, 9, and 10 are known as aquaglyceroporins (15), which are channels with less selectivity and are permeated by water, glycerol, and other small solutes. On the basis of the differences in physiological function, each member of the AQP family appears to be distributed in particular tissues. For example, AQP1 is a constitutively active channel (40) and has been identified in multiple tissues, whereas AQP2 expression is restricted to renal collecting duct cells and cells of the endolymphatic sac of the inner ear (24), where it is regulated by vasopressin (10, 11, 36). AQP5 exists in cells of the submandibular, parotid, sublingual, and lacrimal glands, as well as in the trachea, eye, distal lung, and duodenum but not in the kidney or brain (37, 38, 41). Ma et al. (30) reported that AQP5 plays an important role in the secretion of salivary fluid; i.e., pilocarpine-stimulated salivary fluid secretion in AQP5-knockout mice was reduced by more than 60% compared with that by wild-type mice. Similar results were also found for AQP5 mutant rats (35).

The hormonal regulation of AQPs has been well studied; e.g., AQP2 trafficking in the kidney collecting tubular cells is triggered by short-term exposure to vasopressin, and expression of this aquaporin is upregulated by long-term exposure to this hormone (33, 42). On the other hand, the participation of the autonomic nervous system in the regulation of AQP expression has not been studied much. The salivary glands are innervated by both sympathetic and parasympathetic nerves (7). The submandibular (SMG) and sublingual glands are innervated by the parasympathetic nerve derived from the superior salivatory nucleus of the medulla oblongata, whereas the parasympathetic innervation of the parotid glands is from the inferior salivatory nucleus. The sympathetic nerves that innervate the major salivary glands are from the superior cervical ganglion, which lies in front of the second and third cervical vertebrae. In general, parasympathetic stimulation induces a larger amount of salivary secretion than the sympathetic one. It is, therefore, of interest to examine how the expression (and/or trafficking) of AQPs is regulated by the autonomic nervous system.

In the present study, we examined the effects of sympathectomy, parasympathectomy, and the combination of parasympathectomy and administration of cevimeline hydrochloride on the AQP5 expression in the rat SMG. We found that the level of AQP5 protein in the SMG was positively regulated by the parasympathetic nervous system and that such regulation was based on the alteration of activity of a protein degradation pathway involving a lysosomal enzyme(s).
AQP5 gene, resulting in lower membrane expression of its gene product (34), we selected the rats expressing the normal amount of AQP5, as described previously (35), and mated them in our animal facility. They were kept in our animal facility with free access to water and laboratory chow. Illumination in the animal house was controlled so that it was lighted during the period of 0600–1800. Cervical sympathectomy (CSTD) or chorda tympani denervation (CTD) were performed under anesthesia with pentobarbital sodium (50 mg/kg body wt ip) as described previously (55). The front cervical skin of a group of rats was incised, and they were regarded as a sham-operated control for CSTD rats. To some denervated rats, cevimeline hydrochloride (10 mg/kg body wt po), pilocarpine (0.3 mg/kg body wt po), or chloroquine diphosphate (50 mg/kg body wt po) was administered every day for 7 days starting from the 15th day after the operation. The nonoperated animals were given water or the above drugs for the same period of time. All reagents were dissolved in sterile distilled water at a concentration so that 0.1 ml/100 g body wt of the reagents would be given. The exact amount of the reagents was given after the body weight of each animal was measured. On the 21st day after the operation, the animals were killed and their SMGs were immediately dissected.

The tissues were processed for use in the assays to determine AQP protein and mRNA levels and for immunohistochemistry. The SMG tissue was also used to prepare acinar cells and lysosomal fraction, which is described below. For the time-course study and the study to examine the effect of denervation (Figs. 1, 2, and 5), unilateral CTD (left side) was performed, while, in the experiment where effects of agonists/inhibitors (chloroquine) were examined (Figs. 3, 4, 6, 7, and 9), bilateral CTD was performed. This is because the contralateral gland would become a good control in experiments to examine the effects of denervation. On the other hand, in experiments where effects of agonists/chloroquine were examined, bilateral CTD was carried out to avoid the possible and unnecessary effects on the experimental gland from contralateral gland when unilateral CTD was performed.

The protocol applied for the present animal experiment was approved by the Institutional Review Board of the Animal Committee of the University of Tokushima.

Isolation of acinar cells by Percoll gradient centrifugation. The SMGs from control, CTD, and cevimeline hydrochloride-administered CTD rats were cut into small pieces and digested at 37°C for 15 min in Mg2+-free HBSS containing 0.03% collagenase, 250 U/ml hyaluronidase, 0.2 mM CaCl2 (2 ml/gland) under constant shaking at strokes of 100 cycles/min. The digested tissue was pipetted 10 times with a 5-ml pipette having a 2–3-mm opening. The tissue sample was mixed with 5 ml of prechilled Ca2+- and Mg2+-free HBSS and stood for 10 min to allow sedimentation of the organoid. The supernatant was removed, and the organoid was washed as described above and stood again for 10 min for sedimentation. Two milliliters of diluted organoid (4 ml/gland) were placed on top of a prechilled isotonic 65% Percoll solution in Ca2+- and Mg2+-free HBSS (8 ml), which was prepared as described by Vincent and Nadeau (50), and centrifuged at 500 g for 30 min in a refrigerated centrifuge (HITACHI 05PR-22, Hitachi Koki, Tokyo, Japan). The two layers were separated, and the lower one containing acini was recovered, washed, and subjected to enzyme assay and Western blotting of Na+, K+-ATPase α-subunit. The purity of isolated acini, as well as that of ducts, was confirmed by phase-contrast microscopy and by Western blotting for kallikrein.

Preparation of the total membrane fractions and Western blotting. Tissue specimens were dissected as described above and homogenized in nine volumes (wt/vol) of ice-cold homogenization buffer (5 mM HEPES buffer, pH 7.5, containing 50 mM mannitol, 10 mM MgCl2, 1 mM PMSF, 1 μg/ml aprogin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1 tablet of EDTA-free protease inhibitor cocktail per 25 ml of buffer) by using a 15-ml taster-type glass mortar fitted with a Teflon pestle (Wheaton Science Products, Millville, NJ). The homogenate was filtered through a Nylon mesh (150 mesh), and the nuclear fraction was removed by centrifugation at 600 g for 30 min at 4°C. The supernatant thus obtained was centrifuged at 105,000 g for 1 h at 4°C to obtain the pellet. The pellet was washed as described above and suspended in the same homogenization buffer. The protein concentration in each sample was measured by using a Bio-Rad protein assay kit with bovine serum albumin as the standard (22).

For analysis of AQP proteins, samples were incubated at 37°C for 30 min in a sample-loading buffer (0.0625 M Tris–HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 0.001% BPB, and 5% 2-mercaptoethanol), and subjected to SDS-PAGE; i.e., protein samples (5 μg each) and standard molecular mass protein markers were electrophoretically resolved in 12% polyacrylamide slab gels by using a Mini- Protean II apparatus (Bio-Rad). After electrophoresis, the protein was transferred onto a nitrocellulose filter by means of a Bio-Rad Mini Trans Blot apparatus according to Towbin et al. (46). The blotted filter was blocked with 3% skim milk, probed with anti-AQP5 or anti-AQP1 antisera (both 3,000 diluted in blocking solution), and then incubated with 3,000 diluted peroxidase-labeled anti-rabbit IgG. Anti-AQP5 and anti-AQP1 antibodies preabsorbed with the respective COOH-terminal peptide at a final concentration of 40 μg peptide/ml of 3,000 diluted antisem solution were also reacted with the blotted filter in place of nonpreabsorbed antiserum to verify the specificity of the band. All filters were treated with ECL chemical luminescent reagents and exposed to X-ray films. The bands appeared in X-ray film, were digitalized, and quantified by NIH Image software. The bands in one experiment, separated into more than two gels, were normalized by the intensity of the bands of the same 2–3 samples run in each gel.

Western blotting of Na+, K+-ATPase α-subunits and kallikrein were performed as described previously (25, 54). Na+, K+-ATPase α-subunit assay was run with 10 μg of sample protein, which was treated with sampling buffer at 37°C for 30 min, resolved by 7.5% polyacrylamide electrophoresis, and probed with 300,000 diluted antisem for Na+, K+-ATPase α-subunits. For the kallikrein assay,
1 μg protein was incubated with sampling buffer at 95°C for 5 min, resolved by 12% polyacrylamide electrophoresis, and probed with 100,000× diluted anti-kalikrein antiserum. Other processes were the same as those described above.

**Isolation of the lysosomal fraction, extraction of lysosomal enzymes, and AQP5 degradation assay in vitro.** The SMG lysosomal fraction was prepared according to Tsuji et al. (48), with a slight modification; i.e., the SMG was homogenized in 5 volumes of 0.25 M sucrose containing 10 mM Tris·HCl and centrifuged at 1,200 g at 10 min. The supernatant was recovered and centrifuged at 17,000 g for 20 min. The pellet rich in lysosomes was suspended in 10 mM sodium phosphate buffer (pH 6.0), frozen and thawed once, and homogenized in 10 volumes of 10 mM sodium phosphate buffer (pH 6.0). The pellet thus treated was centrifuged at 17,000 g, and the supernatant was recovered. The recovery and purification of lysosomes by this procedure were 47% and fourfold, respectively, on the basis of the peptatin- A-sensitive cathepsin D activity.

The protein concentration of the lysosomal extract was measured, and the AQP5-degrading activity of the extract was then assayed. The degradation of AQP5 by this lysosomal extract was assayed as follows: the total membrane fraction prepared as described in the previous section was mixed at 2°C with the lysosomal extract in a reaction mixture consisting of 16 mM acetate buffer (pH 4.0) containing 33 mM NaCl (final concentration). The mixture was then incubated at 37°C for 1 h, after which the reaction was stopped by adding sampling buffer for Western blotting. Western blotting for AQP5 was then carried out as described above.

**Measurement of cathepsin D activity.** Samples for the measurement of cathepsin D activity were prepared as described by Baskin-Bey et al. (5) with slight modifications. The SMG tissue was removed from animals anesthetized with pentobarbital sodium (50 mg/kg body wt) and cut into small pieces. The tissues were placed into a Teflon glass homogenizer and homogenized at 4°C with nine volumes (wt/vol) of buffer consisting of 70 mM sucrose, 220 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and the AQP5-degrading activity of the extract was then assayed. The protein concentration of the lysosomal extract was measured, and the AQP5-degrading activity of the extract was then assayed. The degradation of AQP5 by this lysosomal extract was assayed as follows: the total membrane fraction prepared as described in the previous section was mixed at 2°C with the lysosomal extract in a reaction mixture consisting of 16 mM acetate buffer (pH 4.0) containing 33 mM NaCl (final concentration). The mixture was then incubated at 37°C for 1 h, after which the reaction was stopped by adding sampling buffer for Western blotting. Western blotting for AQP5 was then carried out as described above.

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**Measurement of proteasome activity.** Samples for the measurement of proteasome activity were prepared as described by Bardag-Gorce et al. (4). The tissue was homogenized in nine volumes (wt/vol) of buffer comprising 50 mM Tris·HCl (pH 7.5), 10% glycerol, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 2.5 μM pepstatin A at 4°C. The homogenate was centrifuged at 105,000 g for 1 h at 4°C, and the supernatant obtained was used for protein and enzyme assays. The chymotrypsin-like proteasome activity was measured as described by Tsukahara et al. (49). The reaction mixture contained 980 μM of 50 mM Tris·HCl (pH 7.5), 10 μM of 10 mM Suc-Leu-Leu-Val-Tyr-MCA as a substrate, and 10 μM of sample solution in a total volume of 1 ml. The fluorescence emission of AMC was measured by timed scanning at the wavelength of 460 nm with excitation at 380 nm at room temperature. AMC solution (100 μM) in the reaction buffer was used as a standard to compute moles of substrate hydrolyzed.

**Preparation of total RNA and RT-PCR.** Tissues for RNA preparation were quickly processed by using TRI-reagent according to the manufacturer’s protocol. The RNA concentration was measured by use of a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). By running RT-PCR described below, each RNA sample thus prepared was verified that it was not contaminated with substantial amount of genomic DNA and was also subjected to real-time PCR. RT-PCR amplification was performed by using the SuperScript One-Step RT-PCR system in a thermal cycler (TaKaRa Thermal Cycler MP, model TP 3000). To a final volume of 25 μl, the following reagents were mixed on ice: 12.5 μl of 2× reaction mixture, 5 pmol of each primer, 0.5 μl of RT/Taq mix, and 0.5 μg of template RNA. The RT reaction (cDNA synthesis) was carried out at 45°C for 30 min. The reaction mixture was then incubated at 94°C for 2 min to inactivate the enzyme and denature the RNA/cDNA hybrid. The DNA amplification by PCR was next performed for 30 cycles, each cycle consisting of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1.5 min.

In the present study, the primers used were prepared according to the published sequences (13, 22); i.e., 5′-CCCCCAAGGCACCAT-GAAAAA-3′ (sense) and 5′-TCACGAATCTCTAGGTCTGGTCG-3′ (antisense) for AQP5, and 5′-ATGGCCAGGAAATCAAGAAAG-3′ (sense) and 5′-TTTGGGCTTCTAATCACCACC-3′ (antisense) for AQP1. The product sizes were 1,074 and 813 bp, respectively. No PCR product other than these bands appeared in the present experiment. A 2-μl volume of the RT-PCR product was size-fractionated in 3% agarose in 40 mM Tris-acetate buffer containing 1 mM EDTA and stained with ethidium bromide (2 μg/ml) by a standard procedure. The gels were placed in ChemiImager (Alpha Innotech, San Leandro, CA), and photographs were taken by a digital camera.

**Real-time RT-PCR.** The real-time RT-PCR was performed by using a real-time thermal cycler (Takara thermal cycler dice real-time system). To a final volume of 25 μl, the following components were mixed on ice: 12.5 μl of 2× one-step SYBR RT-PCR Buffer III, 2.5 units of TaKaRa Ex Taq HS, 0.5 μl of PrimerScript RT enzyme Mix II, 5 pmol of each primer, 0.1 ng of template RNA, and RNase-free distilled H2O to make a total volume 25 μl. The RT reaction (cDNA synthesis) was carried out at 42°C for 5 min. The reaction mixture was then incubated at 95°C for 10 s to inactivate the enzyme and denature the RNA/cDNA hybrid. The DNA amplification by PCR was next performed for 40 cycles, each cycle consisting of denaturation at 95°C for 5 s, primer annealing and extension at 60°C for 30 s. Primer sets used for quantification were as follows: 5′-TG GCCACCCCTCTACATCTCGT-3′ (sense) and 5′-GATGGGCCAACCACCTCACAG-3′ (antisense) for AQP5, 5′-AGGCTTCAATTACACCTAGTTG-3′ (sense) and 5′-TTTGGGCCAAGTAGCTGGAT-3′ (antisense) for AQP1, and 5′-ACCCTAAGGCGAACCGTGA-3′ (sense) and 5′-ACAGCCTGGATGGCGTACGTACA-3′ (antisense) for β-actin. The product sizes were 149, 98, and 85 bp, respectively. The PCR product was subjected to dissociation conditions to confirm that it had a single component. The relative mRNA amount for AQP5 and AQP1 was calculated and normalized on the basis of the amount of β-actin.

**Northern blotting.** Twenty micrograms of total RNA prepared as described above were ethanol-precipitated and dissolved in 20 μl of freshly prepared sample buffer composed of 1× MOPS solution (20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA; pH 7.0), 50% formaldehyde, 6.4% formaldehyde, and 5.3% autoclaved glycerol.
RNA sample was heated at 95°C for 2 min and then rapidly cooled on ice, after which 10 μl (10 μg) of it was applied to a 1% agarose gel containing 2% formaldehyde. The RNA separated by electrophoresis was transferred onto a Nylon filter, Hybond-N+, by the standard procedure. The filter was prehybridized in hybridization buffer without probe at 42°C for at least 3 h, and then hybridized at the same temperature for more than 15 h in hybridization buffer composed of 450 mM NaCl, 50 mM sodium phosphate, and 5 mM EDTA; pH 7.4), 50% formamide, 2.5× Denhardt’s solution, 10% SDS, 0.2 mg/ml salmon sperm DNA, 10% dextran sulfate, and 50 ng/ml DIG-labeled cDNA probe specific for AQP5 (see below for preparation of the probe). The filter was washed with 2× SSPE at room temperature for 5 min, at 42°C for 20 min, and then with 1× SSPE at 42°C twice each for 20 min. It was further washed with washing buffer for 5 min and then incubated for 1 h at room temperature in 1% blocking solution supplied in the DIG luminescence detection kit. The filter was then reacted at room temperature for 1 h with 10,000× diluted alkaline phosphatase-conjugated anti-DIG-Fab in 1% blocking solution and then washed in washing buffer twice, each time for 15 min. It was equilibrated with buffer III, which was composed of 0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM MgCl2, and subsequently incubated with 100× diluted CSPD in buffer III for 5 min. The excess liquid was blotted onto Whatman 3 MM filter paper, and the filter was placed in a hybridization bag, incubated at 37°C for 15 min in an air oven, and finally exposed to Fuji RX-X ray film.

Synthesis of DIG-labeled cDNA probes. The DIG-labeled cDNA probe was prepared by PCR using DIG-labelling mix and AQP5 cDNA as a template. An aliquot of 2.5 μl PCR DIG-labeling mix (10×), 5 pmol of each primer, 2 U of Taq DNA polymerase, 50 pg of template AQP5 cDNA, and 1× PCR buffer (supplied with Ex Taq polymerase, Takara) were mixed in a total volume of 25 μl to make the reaction mixture. The sequences of sense and antisense primers, as well as the PCR thermal condition used in this labeling experiment, were exactly those as described above (see Preparation of total RNA and RT-PCR). Complementary DNA of AQP5 used for template DNA was prepared from total RNA by RT-PCR as described in the same section above, purified by electrophoresis, and recovered by use of a Qiagen spin column 50 (Qiagen, Hamburg, Germany).

Immunohistochemistry. For AQP5 immunohistochemistry, rats were anesthetized, and a fixative (3% paraformaldehyde and 0.1% glutaraldehyde in 50 mM Na-Pi buffer, pH 7.4) was injected via the left ventricle and circulated throughout the body for 5 min. The SMG was dissected, cut into 5-mm³ cubes, and immersed in the same fixative at 4°C for 2 h. The fixed tissue was washed at 4°C overnight in PBS (pH 7.4) containing 6.8% sucrose. The specimens were then embedded in Tissue-Tec Oct compound and rapidly frozen in liquid nitrogen. Frozen sections of 5-μm thickness were cut and fixed further in ethanol at −20°C for 1 min. The sections were washed in PBS and blocked with 1.5% goat serum in PBS and immunoreacted with 1,000× diluted rabbit anti-AQP5 antiserum (primary antibody). After being washed with PBS, the sections were reacted with 200× diluted FITC-conjugated affinity purified goat anti-rabbit IgG (H+L) (second antibody) and washed with PBS. For control staining, sections were incubated with the same concentration of antibody preabsorbed with the peptide used as the immunogen (40 μg/ml). All sections were next incubated at room temperature for 15 min with PBS containing 0.1 μg/ml of propidium iodide and 20 μg/ml of RNase A and were then washed with PBS to allow the nucleus to become stained. The stained specimens were examined under a fluorescence microscope equipped with a DVM 1200 digital camera (Nikon, Tokyo, Japan), with excitation at 450–490 nm (for FITC) and 510–560 nm (for propidium iodide).

Statistics. For statistical analysis of time-course experiments, one-way ANOVA followed by Tukey’s compromise multiple comparison was applied as a post hoc test. Mann-Whitney U-test was used for other comparisons. Although the U-test is nonparametric analysis, SEs were provided for all data.

RESULTS

Effects of parasympathectomy and sympathectomy on the expression of AQP5 and AQP1. To explore the possible involvement of the autonomic nervous system in the regulation of expression of SMG AQP5 and AQP1, we examined the effects of parasympathectomy and sympathectomy on the level of these salivary gland water-channel proteins. First we examined the effects of CTD on the AQP levels as well as on the relative SMG weight (gland weight/body wt). When the relative SMG weights were measured from 1 wk to 4 wk after the operation, the weight of the denervated gland had decreased to 64.7 ± 1.37% of that of the contralateral gland at 1 wk after the operation, and this level was sustained at least up to 4 wk. The differences were statistically significant (P < 0.01, n = 5; Fig. 1A), confirming that the SMG was strongly dependent on parasympathetic nerves. The ratio of AQP5 expression in the denervated gland to that in the contralateral gland decreased slowly and linearly, becoming significantly different (P < 0.05 and P < 0.01, n = 5) at 2, 3, and 4 wk after the operation (with no significant change between values of the contralateral gland and the gland from nontreated control). At 4 wk after CTD, this ratio for the operated animal was 37 ± 9.45% of that for the nontreated animal (Fig. 1B).

We next examined 1) whether AQP1 was also affected by CTD and 2) whether sympathetic innervation was also indispensable for persistent expression of AQP5 in the SMG. As shown in Fig. 2A, the AQP1 level was hardly affected by CTD. This result does not contradict the fact that SMG AQP1 is expressed in the capillary endothelial cells (3, 28), which are not innervated by autonomic nerves. Secondly, to confirm whether or not the SMG AQP5 level was specifically regulated by parasympathetic nerves, we bisected the cervical sympathectomy (CTD) (cervical sympathetic trunk) and examined the AQP level. As shown in Fig. 2B, sympathectomy did not decrease the expression level of AQP5 protein, even at 4 wk after the operation. By CTD, however, the gland weight was decreased to ~81.0 ± 3.61% (P < 0.01, n = 5), indicating that the sympathetic nerves, although not so strongly as the parasympathetic ones, also support the SMG. The data presented in Fig. 2B compared with the result obtained by CTD, also shown in this figure, obviously indicate that the protein level of SMG AQP5 was positively regulated by parasympathetic, not sympathetic, nerves.

Effects of administration of cevimeline hydrochloride and pilocarpine on AQP protein expression. Since the expression of AQP5 protein was significantly reduced by CTD, the involvement of muscarinic receptors in upregulation of AQP5 was considered likely. Therefore, the effects of two muscarinic agonists, cevimeline hydrochloride and pilocarpine, on AQP5 in the SMG of CTD rats were examined next (Fig. 3, A and B). In this experiment and experiments thereafter, except that for Fig. 5, bilateral CTD was performed (see also MATERIALS AND METHODS). The result of Western blotting showed that bilateral CTD resulted in prominent decrease compared with unilateral CTD, implying the existence of a “cross-talk” between the two glands. The oral administration of cevimeline hydrochloride (10 mg/kg body wt), an M3 receptor agonist for 7 days significantly (P < 0.01, n = 5) elevated the AQP5 protein level reduced by CTD without affecting the gland weight. The level of AQP1, which was not changed by CTD, was significantly
(P < 0.05, n = 5) increased above the control one by the administration of cevimeline hydrochloride (Fig. 3A). For the protein levels of AQP1 in the SMG of nonoperated rats, the effects of cevimeline were minimal. On the other hand, the oral administration of pilocarpine had no effect on the SMG AQP5 protein level of the nontreated or CTD rat (Fig. 3B). We used pilocarpine at a dose (0.3 mg/kg body wt) previously reported to stimulate salivary secretion (18), which, in fact, provoked salivary secretion significantly (1,236 ± 210 mg/30 min vs. control, 112 ± 65 mg/30 min; P = 0.0002, by the Mann-Whitney U-test).

Since apparent changes in the protein level of AQP5 were observed following CTD and cevimeline hydrochloride administration after CTD, we thought that the localization of AQP5 after these treatments should be verified. AQP5 is known to be localized predominantly in the apical (32) and basolateral membranes (35). The result of immunohistochemistry showed that such localization was not changed appreciably by CTD or by cevimeline administration after CTD (Fig. 4). However, AQP5-positive stainings of the plasma membrane and cytoplasm in the acinar cells were decreased by CTD and increased by cevimeline injection after CTD.

Effects of CTD and administration of cevimeline hydrochloride on the AQP5 and AQP1 mRNA levels in the SMG. We next examined the possibility that CTD and/or cevimeline hydrochloride might have affected the transcription of AQP mRNAs. For this purpose, AQP5 mRNA levels after CTD were first examined by Northern blotting using the DIG-labeled cDNA probe. In this experiment, a 1.6-kb band corresponding to AQP5 mRNA was specifically detected in all total RNA samples from the SMG of CTD rats as well as from those of the
The effects on the protein level of SMG AQP5 by CTD/cevimeline hydrochloride administration. Among the two protein-metabolizing systems (proteasome and lysosomal systems), possible involvement of the lysosomal system was examined. To confirm this possibility, chloroquine, which is known as a denaturant of lysosomes (44), was administered to normal and CTD rats, and the protein level of AQP5 was then examined. No significant change in AQP5 level was observed when chloroquine was administered to nonoperated rats. However, administration of chloroquine (50 mg/kg body wt po) elevated the AQP5 protein level decreased by CTD (Fig. 7), suggesting the idea that an enzyme(s) in the lysosomal system is involved in the reduction of the protein level of SMG AQP5 caused by CTD. We observed that the administration of chloroquine did not affect the gland weight in both nonoperated and CTD rats. Thus the possibility that a lysosomal extract would be able to degrade AQP5 was next confirmed by conducting an in vitro experiment (Fig. 8). As shown in Fig. 8, the lysosomal extract degraded AQP5 protein in the total membrane fraction. Although this is a preliminary experiment and requires more detailed data, this result implies the possible involvement of some lysosomal enzyme(s) in the autonomic control of the AQP5 protein level in the SMG. Since CTD, an experimental protocol employed in this study, resulted in a significant decrease in the gland weight, we assumed that this operation may have elevated the autophagy or induced similar cell physiological changes, resulting in acceleration of protein catabolism in the SMG. Thus we examined the activity of several lysosomal enzymes as well as the proteasome activity of the SMG for the purpose of knowing whether the proteolytic enzyme in lysosomes or those in the cytosol were actually changed by CTD and/or cevimeline administration. Accordingly, the activities of proteasome and some selected lysosomal proteinases, i.e., cathepsins B, D, and E, were measured. The results of these assays showed that the activities of these three cathepsins, especially cathepsin D, were increased by CTD and that this increase was suppressed by the administration of cevimeline hydrochloride (Fig. 9, A–C). On the other hand, the proteasome activity was not affected significantly by either CTD or administration of cevimeline hydrochloride (Fig. 9D). Since the SMG consists of ducts and acini as major constituents, it was indispensable to verify that the change observed in the cathepsin activity of the entire tissue also took place in the acinar cells. Thus acini were isolated from the SMG by the Percoll centrifugation method. As shown in Table 1, the purity of the acini isolated by the present procedure was verified to be 91.6–97.2% based on the assay data for kallikrein, a duct cell marker. Using such cell samples, we measured the cathepsin D activity. As shown in Table 1, the cathepsin D activity in these isolated cell samples changed similarly as in the entire tissue, suggesting a parallel relation between the changes in the entire tissues and those in the isolated acinar cells. These results suggest that CTD increased the activities of lysosomal enzyme(s) and thereby elevated protein metabolism activity in the acinar cells as well.

In addition, the amount of Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunit in the isolated acinar cells was also determined, and the effect of CTD on the level of this protein was examined. The results showed that CTD effects were not restricted to the AQP5 protein level but that the denervation also affected at least one other membrane protein of acinar cells, i.e., the Na\(^{+}\),K\(^{+}\)-

control gland (Fig. 5). The AQP5 mRNA level in the denervated SMG was almost the same as that for the contralateral gland from week 0 to week 4 after the operation.

Furthermore, the administration of cevimeline hydrochloride did not affect the mRNA levels of AQP5 nor AQP1 as determined by RT-PCR (Fig. 6A) and by real-time RT-PCR (Fig. 6B). These data suggest that the changes in the AQP5 protein level caused by CTD and/or cevimeline hydrochloride administration were regulated not transcriptionally but probably by posttranscriptional events.

Possible involvement of lysosomal system in AQP5 degradation. The results obtained above suggest that the autonomic regulation of the protein level of SMG AQP5 (and AQP1 in part) may be controlled posttranscriptionally. If this is the case, it is possible that the protein degradation system is involved in
ATPase α-subunit. Different from AQP5, however, the Na⁺,K⁺-ATPase α-subunit was not upregulated by cevimeline, thus suggesting a relatively specific effect of this agonist on AQP5 induction.

**DISCUSSION**

In the present study, we examined the role of the autonomic nervous system in the expression of water channels AQP5 and AQP1 in the rat SMG by assessing the effects of parasympathectomy, sympathectomy, and the administration of muscarinic agonists cevimeline hydrochloride and pilocarpine. The level of the AQP5 protein in the membrane fraction of the SMG was decreased by unilateral denervation of the chorda tympani. This decrease was accompanied by a reduction in the gland weight although the time course of the AQP5 decrease was a little different from that seen for the decrease in the gland weight. The AQP5 protein level did not decrease after CSTD, and sham operation in this experiment, suggesting the selective regulation of AQP5 by parasympathetic nerves in the SMG, and that operation-accompanying inflammation did not affect the AQP5 expression, which is different from lung tissue (44).

Effects of CTD and/or cevimeline treatment on Na⁺, K⁺-ATPase α-subunit and dipeptidyl peptidase IV (DPPIV), a general marker of plasma membrane, were also examined as control experiments. Similar to their effects on AQP5, the Na⁺, K⁺-ATPase α-subunit level was decreased by CTD and increased by cevimeline treatment in nonoperated control and CTD rats. However, we found that no difference was observed in the DPPIV level when effects of CTD and/or cevimeline treatment were examined (data not shown). Thus the changes in the level of Na⁺, K⁺-ATPase α-subunit, as well as AQP5, appear to be specific effects caused by CTD and/or cevimeline treatment.

The secretion of submandibular saliva was measured in rats that received CTD and cevimeline administration. The secretion during the initial 5 min after pilocarpine administration was hardly affected by CTD and increased by cevimeline treatment in nonoperated control and CTD rats. However, we found that no difference was observed in the DPPIV level when effects of CTD and/or cevimeline treatment were examined (data not shown). Thus the changes in the level of Na⁺, K⁺-ATPase α-subunit, as well as AQP5, appear to be specific effects caused by CTD and/or cevimeline treatment.

The neural mechanism regulating AQP channels has been reported for the kidney (19) and muscle (26) although denervation is reported to upregulate the AQP5 protein level in...
sweat gland (29). Thus the neural control of AQP does not appear to be special for the salivary gland.

The rate of decrease in the AQP5 protein level following CTD was very slow. The level decreased linearly, and its T1/2 (half-life time) was 25 days. Since the AQP5 mRNA level was not changed by CTD, it is probable that the activity of the protein degradation system was elevated by CTD, provided that the rate of protein synthesis was not increased. Such changes would have led to a new equilibrium of synthesis and degradation of AQP5. This possibility was implied also by immunohistochemical observation, in which the number of AQP5-bearing vesicles was changed by CTD/cevimeline injection.

Generally, the affinity of an agonist to the receptor determines the physiological response and selectivity of the agonist among the receptor subtypes. Thus both cevimeline and pilocarpine provoke the saliva secretion although its intensity is different (18). The effects of cevimeline on the nondenervated rats were minimal, whereas the administration of cevimeline hydrochloride after CTD increased the AQP5 levels reduced by the operation. This difference may be explained by a possibility that CTD may have attenuated the sensitivity of the muscarinic receptor, since chronic excess agonist can lead to a downregulation of receptors, whereas a deficit of transmitter can lead to increased numbers of receptors and supper sensitivity of the system (6). In our present study, the second muscarinic agonist, pilocarpine, did not have such an effect as cevimeline. We speculate that the potency of CTD-induced supper sensitivity may be different for each receptor subtype. All these elucidations are based on the assumption that effects of cevimeline on the AQP5 level were due to stimulation of M3 muscarinic receptors (18). This suggestion still remains tentative and requires more experiments using other M3 muscarinic antagonists or the receptor knockout mice, experiments that are now being considered.

Leith et al. (27) showed that the AQP1 protein level increases after application of proteasome inhibitors in BALB/c fibroblasts spontaneously expressing AQP1, suggesting
involvement of proteasomes in regulation of the AQP1 protein level. In the present study, on the contrary, the AQP1 protein level in the denervated gland did not change at all compared with that in the contralateral gland, and it increased above the control by administration of cevimeline hydrochloride. In the SMG, AQP1 is known to be localized in capillaries (3, 28), which are not innervated. Despite the lack of apparent cholinergic innervation, the presence of muscarinic receptors, primarily of the M3 subtype, in the endothelial cells of most of blood vessel is described (8), receptors that play a pivotal role for relaxation. This is probably the reason that denervation did not affect the SMG AQP1 protein level. However, it is not clear by what mechanism cevimeline hydrochloride induced AQP1 in the SMG. More experiments are required using M3/M1 muscarinic receptor knockout mice to determine whether these receptors are involved in this induction.

**Table 1. Effects of CTD and administration of cevimeline hydrochloride on the cathepsin D activity and Na\(^+\),K\(^+-\) ATPase α-subunit levels of isolated SMG acinar cells**

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>Cathepsin D Activity, mU/mg protein, %a</th>
<th>Na(^+),K(^+-)ATPase α-Subunit, %</th>
<th>Relative Purity of Acinar Cell Samples, %c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>3.44±0.36 (100±10.47)b</td>
<td>100±4.48</td>
<td>97.06±1.95</td>
</tr>
<tr>
<td>CTD</td>
<td>4.51±0.17 (131.10±4.94)*</td>
<td>65.7±1.34†</td>
<td>97.20±0.42</td>
</tr>
<tr>
<td>CTD + cevimeline hydrochloride</td>
<td>2.69±0.30 (78.20±8.72)§</td>
<td>57.6±3.28§</td>
<td>91.56±2.5</td>
</tr>
</tbody>
</table>

*aThe value after subtraction of the cathepsin E activity from the cathepsin D plus E activities. bThe values in parentheses indicate the percentage of that of the nontreated group. cThe kallikrein protein in the dissociated cell sample before Percoll separation and in acinar cell samples after separation was determined by Western blotting. The amount of kallikrein protein present in the samples before Percoll separation was regarded as 100%. The percentage of duct cells present in the isolated acinar cell samples was calculated, and the purity of the acinar cells was determined. Data are expressed as means ± SE of 4 independent determinations. *P < 0.05, †P < 0.01, significantly different from the respective nontreated group. ‡P < 0.01, significantly different from the CTD group; §no significant difference from the chorda tympani denervation (CTD) group. SMG, submandibular gland.
Assessment by Northern blotting, RT-PCR, and real-time RT-PCR experiments showed that the AQP5 mRNA level in the SMG was not altered by CTD and/or administration of cevimeline hydrochloride. It is reported that AQP5 mRNA transcription is suppressed by TNF-α and LPS (47, 52) and induced by hypoxia and hyperosmolality (57). In a short-term experiment using a murine lung epithelial cell line (MLE-12), chlorophenylthio-cAMP was shown to induce AQP5 mRNA expression at 6 h (53). There is no report, however, that muscarinic receptors and/or their signaling molecules are responsible for the regulation of AQP5 mRNA transcription. Our data also show that this is not the case by long-term treatment.

Although the level of AQP5 mRNA did not change after autonomic denervation and/or muscarinic therapy, the AQP5 protein level was decreased by CTD and increased by cevimeline hydrochloride after CTD. These data obviously indicate the possibility that either 1) AQP5 protein synthesis is suppressed, 2) its protein is released into saliva, or 3) AQP5 protein degradation is accelerated by CTD. In the present study we verified the latter possibility.

As to protein catabolism, the existence of two pathways is generally known, i.e., lysosomal proteinases and the ubiquitin-proteasome system. In the latter system, various protein molecules are metabolized, such as those involved in cell-cycle regulation, signaling molecules, transcription factors, and Oncogene products. The quality of proteins in the endoplasmic reticulum and cytosol is also known to be controlled by the ubiquitin-proteasome system. This system removes abnormal proteins such as mutant molecules or proteins that have failed in normal folding/assembly. The lysosomal enzymes, the other hand, degrade extrinsic and intrinsic proteins, and these events are termed heterophagy and autophagy, respectively (31). The ligands and receptors bound to ligands are decomposed by heterophagy, whereas various cellular proteins are degraded by autophagy. Autophagy is known to be greatly accelerated under physiological conditions where animals cannot utilize the food protein, e.g., during fasting/starvation.

In the present study, the SMG weight was decreased by CTD, implying an increase in autophagy, or similar physiological changes took place in acinar cells due to this operation. This idea is supported by the fact that the activity of some lysosomal proteinases in homogenates (containing lysate of lysosomes) was increased by CTD and returned to their normal level by treatment with cevimeline. Using acinar cells isolated from the SMG, we examined the effects of CTD and administration of cevimeline on the Na⁺/K⁺-ATPase α-subunit level. The results showed that the level of Na⁺/K⁺-ATPase α-subunits was decreased by CTD but was not increased by concomitant cevimeline administration. Since AQP5 and AQP1 protein levels were increased by cevimeline, it is evident that the cevimeline effects were specific for AQP5 and AQP1. On the other hand, the decrease in AQP5 protein and Na⁺/K⁺-ATPase α-subunits caused by CTD appeared to be specific since a similar decrease also was not found for the level of DPPIV. This result again implies an increase in autophagy or similar physiological changes caused by CTD. To establish this hypothesis, however, the CTD-induced changes of other housekeeping proteins originating form plasma membrane, cytosol, or subcellular structure need to be determined.

Sidhaye et al. (43) reported that the AQP5 protein level reduced by cAMP is increased to its original level by chloroquine, a denaturant of lysosome, suggesting the possibility that AQP5 is degraded by a lysosomal enzyme(s). There are also other reports suggesting that AQP2 and AQP8 are degraded by lysosomal enzymes; e.g., AQP2 expressed in MDCK cells is degraded by lysosomal enzymes after being ubiquitinated (21), and estrogen-caused reduction in hepatic AQP8 level is provoked by lysosomal enzymes (9). These reports support our present data suggesting that some lysosomal enzyme(s) is involved in the regulation of the AQP5 protein level in the SMG. In fact, our preliminary experiment showed that the extract obtained from the lysosomal fraction of the SMG degraded the AQP5 protein in the total membrane fraction in vitro. The data obtained in the present study suggest that the salivary gland AQP5 level is regulated by a lysosomal enzyme(s), and, therefore, the experiment to specify the proteolytic enzyme(s) responsible for this AQP5 catabolism is now being conducted.

In conclusion, the regulation of the AQP5 protein level in the SMG by CTD and cevimeline is suggested to be controlled by a lysosomal enzyme(s). In the long-term therapy, transcriptional regulation of AQP5 mRNA is not likely involved in this neural regulation.

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