Effects of isoproterenol on aquaporin 5 levels in the parotid gland of mice in vivo

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Chen G, Yao C, Hasegawa T, Akamatsu T, Yoshimura H, Hosoi K. Effects of isoproterenol on aquaporin 5 levels in the parotid gland of mice. Am J Physiol Endocrinol Metab 306: E100–E108, 2014.—In the membrane fraction of mouse parotid gland (PG), the protein level of aquaporin 5 (AQP5), a member of the water channel family, was increased by injection (ip) of isoproterenol (IPR), a β-adrenergic agonist, at 1 h, and stayed at high levels until 6 h; this change occurred simultaneously as amylase secretion. The AQP5 level then decreased and returned toward the original level at 12–48 h. After IPR injection, the AQP5 mRNA gradually increased and reached a maximum at 24 h. The facts suggest a rapid appearance of AQP5 at plasma membrane by IPR and subsequent degradation/metabolism by activation of proteolytic systems. Pretreatment of animals with two calpain inhibitors, N-Ac-Leu-Leu-methininal (ALLM) and calpeptin, as well as a protein synthesis inhibitor, cycloheximide (CHX), significantly suppressed the IPR-induced AQP5 degradation in the PG membrane fraction; such suppression was not observed by two proteasome inhibitors, MG132 and lactacystin, or the lysosome denaturant chloroquine, although most of these inhibitors increased AQP5 protein levels in unstimulated mice. The AQP5 protein was also degraded by μ-calpain in vitro. Furthermore, we demonstrated that μ-calpain was colocalized with AQP5 in the acinar cells by immunohistochemistry, and its activity in the PG was increased at 6 h after IPR injection. These results suggest that the calpain system was responsible for IPR-induced AQP5 degradation in the parotid gland and that such a system is coupled to the secretory-restoration cycle of amylase in the PG.

AQUAPORINS (AQPs) are members of the water channel family that facilitate water movement between in and out of cells. They are expressed in various tissues and cells throughout the body and play pivotal roles in water movement. So far, 13 different AQPs (AQP 0–12) have been identified in mammals (27). Among them, AQP5 is the one identified first in the salivary gland (24), and its deficiency in mice has been shown to lead to a decrease in their survival rates at the embryonic stage (9, 13). Studies of the AQP5-mutant rat (18) and AQP5-knockout mice (13) as well as the model mouse of Sjögren’s syndrome (26) have indicated that AQP5 plays a pivotal role in maintaining the normal physiological function of the salivary gland; i.e., some of these animals produce significantly hypertonic, viscous, and smaller volumes of saliva (13, 18). The parotid gland (PG) belongs to one of the major salivary glands and expresses abundant AQP5 in the luminal membrane of its serous acinar cells (20, 31). It was reported previously that isolated and disaggregated PG acinar cells prepared from AQP5-knockout mice showed a significant decrease in transmembrane water transport (9). These results suggest that a certain quantity of fluid secreted by mouse salivary gland is due to the transcellular water transport, in which AQP5 plays a major role.

Acinar cells of the PG in mice are filled with a plenty of secretory granules containing amylase, which contributes 80% of the same enzyme present in the saliva (19). It has been well established that PG amylase is exocytosed by administration of β-adrenergic receptor agonists via an increase in intracellular cAMP following activation of adenyl cyclase (3). Several lines of evidence prompted us to investigate the possibility that the AQP5 expression/metabolism may be associated with this β-agonist-induced exocytosis because 1) AQP2, a major water channel in the kidney, is known to be regulated by a vasopressin/cAMP pathway in the renal collecting duct cells (6); and 2) 8-(4-chlorophenylthio)-cAMP and isoproterenol (IPR) increased expressions of the AQP5 mRNA and protein and induced translocation of AQP5 to the apical plasma membrane in MLE-12, a cultured lung cell line (28). Nevertheless, the molecular mechanism that regulates AQP5 levels in the IPR-stimulated PG is still unknown. On the other hand, there was an 87% reduction in the content of AQP2 protein in inner medullary cells of the collecting duct, with no significant change in the AQP2 mRNA level in the dihydrotachysterol-induced hypercalcemia/hypercalciuria (23, 25). Similarly, without affecting its mRNA level, chorda timpani denervation induces AQP5 degradation via the lysosomal system in the rat submandibular gland (2, 11). These reports imply that the degradation system also plays important roles in regulation and/or control of the AQP function.

In the present study, we investigated the effect of IPR on the expression of AQP5 during exocytosis and reaccumulation of secretory granules in the mouse PG. Here, we demonstrate that the AQP5 level in the PG membrane fraction was first increased after IPR treatment and then followed by its potential reduction. The reduction in the level of membrane AQP5 was a postnuclear phenomenon mediated by μ-calpain proteolysis.

MATERIALS AND METHODS

Materials. Calpeptin (Z-Leu-Nle-CHO) was obtained from Calbiochem (Billerica, MA). Calpain Activity Assay Kit and active human μ-calpain were obtained from BioVision (Mountain View, CA). Trypsin tablet, cycloheximide (CHX), lactacystin, N-Ac-Leu-Leu-methininal (ALLM) and mammalian two calpain inhibitors, N-Ac-Leu-Leu-methininal (ALLM) and calpeptin, as well as a protein synthesis inhibitor, cycloheximide (CHX), significantly suppressed the IPR-induced AQP5 degradation in the PG membrane fraction; such suppression was not observed by two proteasome inhibitors, MG132 and lactacystin, or the lysosome denaturant chloroquine, although most of these inhibitors increased AQP5 protein levels in unstimulated mice. The AQP5 protein was also degraded by μ-calpain in vitro. Furthermore, we demonstrated that μ-calpain was colocalized with AQP5 in the acinar cells by immunohistochemistry, and its activity in the PG was increased at 6 h after IPR injection. These results suggest that the calpain system was responsible for IPR-induced AQP5 degradation in the parotid gland and that such a system is coupled to the secretory-restoration cycle of amylase in the PG.

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nal (ALLM), Z-Leu-Leu-Leu-CHO (MG132), PMSF, and peroxidase-labeled mouse anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO). 3-Aminopropyltriethoxysilane-coated microslide glasses and microcover glasses were purchased from Matsunami Glass (Osaka, Japan). IPR, chloroquine diphosphate (CQ), DTT, and aprotinin were procured from Wako Pure Chemicals (Osaka, Japan). Complete EDTA-free protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Indianapolis, IN). The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Goat polyclonal anti-human μ-calpain antibody and goat anti-mouse amylase antibody, as well as their blocking solutions, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas rabbit anti-AQP5 antibody was prepared in our laboratory (31). Donkey anti-goat and donkey anti-rabbit IgGs conjugated with FITC were from Jackson ImmunoResearch laboratories, (West Grove, PA). Alexa Fluor 594 donkey anti-rabbit IgG (H + L) was from Molecular Probes (Invitrogen, Eugene, OR). Donkey anti-goat and goat anti-rabbit IgGs conjugated with horseradish peroxidase (HRP) and enhanced chemiluminesence (ECL) Detection Kit were from GE Healthcare (Buckinghamshire, UK). The Fujii RX X-ray film was a product of Fujifilm (Kanagawa, Japan). All other reagents were procured as described previously (31).

Animals and drug treatments. Male mice (Jcl:ICR strain) at the age of 7–8 wk were purchased from Japan SLC (Hamamatsu, Japan) and housed under standard conditions in our animal facility. They were euthanized for experiments at the age of 8–9 wk. To study the effect of IPR on the PG, IPR dissolved in the sterile saline at a concentration of 3 mg/ml was injected into mice intraperitoneally (ip) at a dosage of 0.45 mg/0.15 ml per animal (a pharmacological dose). The inhibitors lactacystin and CQ were also dissolved in the sterile saline, whereas the other inhibitors were dissolved in DMSO, followed by dilution at 1/200 in the sterile saline, immediately before injection. The dosage of each inhibitor was as follows (in mg/kg body wt): 5 CHX, 0.3 MG132, 0.6 lactacystin, 15 CQ, and 1.0 ALLM and calpeptin. Control mice were injected with same volume of the sterile saline or 0.5% DMSO. The final volume of each solution injected was 0.1 ml/mouse.

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

Preparation of membrane fractions. From normal and IPR-injected mice, the submandibular gland (SMG) and PG tissues were isolated, and the membrane fraction was prepared as described previously (11, 31). Briefly, the SMG and PG tissues were homogenized in the ice-cold homogenization buffer (5 mM HEPES buffer, pH 7.5, containing 50 mM mannitol, 10 mM MgCl2, 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1 tablet of complete EDTA-free protease inhibitor cocktail/25 ml of buffer) by using a glass mortar fitted with a Teflon pestle and centrifuged at 800 g for 5 min at 4°C to remove the nucleus and cell debris. The supernatant thus obtained was termed as “homogenate.” The homogenate was divided into two parts; one part was served for the analysis of amylase, AQP5, and μ-calpain without further processing, whereas the other part was centrifuged at 105,000 g at 4°C for 1 h to obtain the pellet, which was resuspended in the homogenization buffer and used as the “membrane fraction” for the AQP5 analysis.

The protein concentration of all above samples was determined by a Bio-Rad protein assay, using bovine serum albumin as a standard.

Western blotting. The membrane fraction was mixed with 2× SDS sample buffer and denatured at 60°C for 30 min for AQP5 analysis. Similarly, the homogenate, having been mixed with the sample buffer, was denatured at 85°C for 15 min for the analysis of amylase and μ-calpain. The samples were subjected to SDS-PAGE using 12% (for AQP5, amylase, and β-actin) or 8% (for μ-calpain) polyacrylamide gel. After electrophoresis, separated proteins were electrophoretically transferred onto a nitrocellulose filter in a Mini-protein II Electrophoresis Apparatus (Bio-Rad). The blotted filter was blocked with PBS containing 3% nonfat dry milk in 0.1% Tween-20 (0.1% T-PBS) at room temperature for 2 h and then incubated at 4°C overnight with each primary antibody. The dilution of primary antisera or antibodies used was as follows: rabbit anti-AQP5, 3,000 times; goat anti-amylase, 1,000 times; mouse anti-β-actin, 50,000 times; and goat anti-μ-calpain, 500 times; all in 0.1% T-PBS containing 1% nonfat dry milk. For a control reaction, the filter was incubated with the same concentration of the antisera or antibody that had been preabsorbed with the blocking peptides (29). The filter was washed with 0.1% T-PBS and incubated with donkey anti-rabbit IgG-HRP or with donkey anti-goat IgG-HRP, both diluted 30,000 times, at room temperature for 2 h and subsequently washed with 0.1% T-PBS. The filter was then reacted with the ECL reagent, and exposed to an X-ray film during an appropriate time.

Degradation assay of AQP5 in vitro. For the assay of the activity to degrade AQP5 by calpain, the membrane fraction (1.0 μg) obtained from the mouse SMG was used as the AQP5 substrate because this tissue contains large amount of AQP5 (24). The membrane fraction was incubated with 2.5–10 U/ml of μ-calpain in 20 μl of the reaction mixture containing 30 mM Tris-HCl (pH 7.5), 200 μM CaCl2, and 1.5 mM DTT at 30°C for 1 h (22). The reaction was terminated by adding 20 μl of 2× SDS sample buffer, followed by incubation at 60°C for 30 min. AQP5 in the reaction mixture was then analyzed by Western blotting. Similarly, for the time course study, 8 U/ml μ-calpain was mixed with the membrane fraction, and the reaction mixture (20 μl) was incubated at 30°C for 0, 0.5, 1, 2, and 3 h. To examine the effect of inhibitors of μ-calpain, the enzyme (8 U/ml) was mixed with each inhibitor (ALLM and calpeptin, 10 μM), preincubated at room temperature for 30 min, and incubated with the membrane fraction at 30°C for 1 h. The reaction was terminated by adding 20 μl of 2× SDS sample buffer and subjected to Western blotting. For determination of the amount of AQP5 degraded, the band intensity was quantified by using National Institutes of Health (NIH) Image J software.

Preparation of total RNA and RT-PCR. Mice were euthanized at 0, 1, 3, 6, 12, 24, 48, and 72 h after IPR injection, and the PG tissue was dissected. Total RNA was isolated from the tissue using TRI Reagent, following manufacturer’s protocol. RT-PCR experiments for AQP5 and β-actin were carried out as described previously (31). All RT-PCR products were resolved by electrophoresis in 3% agarose gel (NuSieve/SEAKEM® GTAgE) in 1× TAE buffer (18.4 mM Tris-acetate, 4.5 mM sodium acetate, and 1 mM EDTA). The band intensity was quantified by NIH Image J software.

Immunohistochemistry. For amylase immunostaining, the PG tissue was fixed with the fixative containing 3% paraformaldehyde and 0.1% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.4) at 4°C for 2 h, as described previously (30). The fixed tissue was then embedded in Paraplast plus plus (Sherwood Medical, St. Louis, MO) and sectioned at 10 μm. Sections were transferred to 50% ethanol at −20°C for 1 min (for AQP5 staining) or in a 1:1 mixture of ethanol and ace tone for 30 min (for AQP5/μ-calpain double staining).

All sections were washed in PBS, blocked with 5% normal goat serum (for AQP5) or with 5% normal donkey serum (for amylase and AQP5/μ-calpain) in PBS, and immunoreacted with the diluted primary antibody at 4°C overnight. The dilution of primary antisera or antibodies was as follows: rabbit anti-AQP5, 50,000 times; goat anti-amylase antibody, 500 times; and goat anti-μ-calpain anti-
body, 100 times. All primary antiserum and antibodies were diluted in PBS containing 1% blocking serum. After having been washed with PBS three times, the sections were reacted with either 200 times-diluted goat anti-rabbit IgG FITC (for AQPS) or 200 times-diluted donkey anti-goat IgG (H + L) FITC (for amylase) or a mixture of donkey anti-goat IgG (H + L) FITC and Alexa Fluor 594 donkey anti-rabbit IgG (H + L) (200× and 500× dilutions, respectively; for AQPS/μ-calpain) at room temperature for 2 h, followed by washing with PBS. For control staining, primary antisera/antibodies preabsorbed with each antigen peptide were used. For the nuclear staining, the sections for amylase staining were incubated at room temperature for 15 min with PBS containing 0.1 μg/ml of propidium iodide and 20 μg/ml of RNase A, whereas the sections for AQPS and μ-calpain staining were incubated with 50 ng/ml DAPI at room temperature for 2 min. These stained sections were examined under a fluorescence microscope equipped with a DXM 1200 digital camera (Nikon, Tokyo, Japan).

Calpain activity assay. The μ-calpain activity in the PG at 0, 1, 3, 6, 12, and 24 h after injection of IPR was assessed according to the manufacturer’s protocol. The PG tissue was homogenized in the ice-cold “extraction buffer” supplied by the manufacturer and centrifuged at 10,000 g at 4°C for 1 min. The supernatants were collected, and the protein concentration was determined. Aliquots of 100 μg of the supernatant proteins were applied for determination of the calpain activity with the use of Ac-LLY-AFC, a fluorescent calpain substrate. Recombinant human μ-calpain was used as the positive control and the value measured in the presence of Z-LLY-FMK, a calpain inhibitor, as the negative control. The activity was measured in a microplate reader (Thermo Fisher, Nippon Thermo) with 400-nm excitation and 505-nm emission.

Statistical analysis. Results are presented as means ± SE. Data were statistically analyzed by Student’s t-test to generate two-tailed P values.

RESULTS

Effects of IPR on expressions of amylase and AQP5 in the PG in vivo. In the present study, we first tried to determine whether the level of AQPS expression is changed during the secretory-restoration cycle of secretory proteins in the exocrine gland. As an experimental model for protein secretion and restoration, we used the system of IPR-induced amylase secretion of the PG (3); IPR, a β-adrenergic agonist, has been used for many years and is known to cause secretion of 96% of the cellular content of amylase from the PG by its ip injection (1). At first, the immunohistochemical staining was employed to confirm the changes in the level/localization of amylase in response to IPR (Fig. 1). In the control group, amylase was detected in almost all secretory granules, which were distributed in the cytosol of entire acinar cells (Fig. 1A). One hour after injection of IPR, the staining intensity of amylase in the PG became very weak, suggesting its secretion from PG (Fig. 1B); the amylase staining subsequently increased in 3 h, indicating the onset of amylase reaccumulation by this time (Fig. 1C). At 6 h after IPR injection, acinar cells were diluted and became filled with a number of secretory granules strongly positive for amylase (Fig. 1D), and more prominent changes were observed at 24 h (Fig. 1E). The specificity of the amylase staining was confirmed by preabsorption experiment, using the blocking peptide (Fig. 1F). These patterns of changes in the amylase content after IPR injection are similar to that described previously (1). Western blotting data also indicated that the amylase protein amount in the PG homogenate was decreased dramatically by ~90% at 1 h and retrieved from 3 h (Fig. 3A, top); the amount returned mostly to the original level at 6 h. Taken together, these data suggest that IPR strongly induced secretion of the granule content in the PG and that the amylase amount in PG was restored completely by 24 h.

We next pursued changes in AQPS levels in the PG upon IPR injection, trying to explore the possibility of whether such changes are linked to the amylase secretion-restoration cycle. Thus, immunohistochemistry was employed to determine the IPR-mediated AQPS redistribution. In untreated mice, the AQPS protein was localized in the apical, lateral, and basal aspect of the plasma membrane of acinar cells (Fig. 2A). There was also a moderate staining in the cytosolic area of the same cells. The AQPS labeling in the lateral plasma membrane was strong and sharp. At 1 h after IPR injection, the acinar lumen

Fig. 1. Immunohistochemical localization of amylase in the mouse parotid gland (PG) after isoproterenol (IPR) injection. A–E: amylase was immunostained with anti-amylase polyclonal antibody at 1 (B), 3 (C), 6 (D), and 24 h (E) after IPR injection, and its localization was compared with that of nontreated mouse (A). F: control staining in which anti-amylase antibody was preabsorbed with its blocking peptide. The red and green stainings indicate the localization of nuclei and amylase, respectively.
was dilated, and irregularity became much prominent (Fig. 2B). The AQP5 labeling in the basal membrane and cytosol was clearly decreased. The labeling in cytosol and basal membrane was little increased at 3 h after IPR injection (Fig. 2C). At 6 h after IPR injection, the AQP5 labeling in the cytosolic area was more intense, and some dots or vesicle-like structures positive for AQP5 became marked in the cytosol of acinar cells (Fig. 2D); intercellular canaliculi were now hardly

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**Fig. 2.** Immunohistochemical localization of aquaporin 5 (AQP5) in the mouse PG after IPR injection. A–E: AQP5 was immunostained with anti-AQP5 antiserum at 1 (B), 3 (C), 6 (D), and 24 h (E) after IPR injection, and its localization was compared with that of nontreated mouse (A). F: control staining in which anti-AQP5 antiserum was preabsorbed with its blocking peptide. The blue and green stainings indicate the localization of nuclei and AQP5, respectively.

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**Fig. 3.** Effects of IPR on the amylase protein level and on AQP5 protein/mRNA levels in the mouse PG. A and B: Western blotting data showing the time course of changes in amylase, AQP5, and β-actin protein levels in the PG after injection of IPR in vivo. A: PG homogenate. B: PG membrane fraction. Detail experimental conditions are described in the text. C: Western blotting showing the specificity of antibodies used in the experiment. D: RT-PCR data showing the time course of changes in AQP5 mRNA levels in the PG after injection of IPR in vivo. Expression of β-actin is also shown as an internal standard. E: amylase data in A and AQP5 data shown in B and D are quantified by using the National Institutes of Health (NIH) Image J software and presented graphically. F: the ratio of AQP5 in the membrane fraction (B) to that in the homogenate (A) was calculated after having been normalized by the β-actin values. Ab, antibody; p-Ab, peptide-preabsorbed antibody; MF/H, membrane fraction/homogenate.
The injection of IPR provoked the most prominent increase in AQP5 mRNA expression, which was then decreased at 48 h (Fig. 3, onset of the elevation of AQP5 mRNA expression was already detected at 1 h following the injection of IPR. How-
was increased at 1 h until 6 h after IPR injection, followed by a decrease. These data suggest that the membrane of secretory granules and/or secretory granules themselves have scarcely been contaminated to our membrane fraction.

To verify the second stage of this hypothesis we tested whether degradation/metabolism of AQP5 is activated in the PG after IPR injection.

Effects of protease inhibitors on the PG in vivo. Since the AQP5 protein changed irrelevantly with its transcriptional expression after IPR injection in the mouse PG, it was likely that protein degradation/metabolism was involved in regulation of the AQP5 protein level by IPR. Generally, two major protein degradation pathways are known in mammalian cells: 1) the 26S proteasome system (4), which uses cysteine proteases, including cytoplasmic calcium-dependent calpains (17) and 2) lysosomal acidic cathepsins (16). Thus, we examined the effects of the inhibitors of these proteases on IPR-induced reduction of the AQP5 protein level in the PG. As shown in Fig. 4A, in untreated mice (with no IPR injection), the AQP5 protein level in the PG membrane fraction was increased by treatment with MG132 (a proteasome inhibitor) for 25 h. Similar results were obtained by treatment with CQ (a lysosomal inhibitor) and ALLM and calpeptin (calpain inhibitors). On the contrary, in IPR-treated mice, only CHX (an inhibitor

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Fig. 6. Effects of IPR on localization of AQP5/μ-calpain, μ-calpain activity, and pilocarpine-provoked saliva secretion. A: immunohistochemical detection of AQP5 and μ-calpain in the PG of nontreated mice. Image a: red fluorescence of Alexa Fluor 594 showing AQP5 localization; image b: green fluorescence of FITC showing μ-calpain localization; image c: blue fluorescence of DAPI showing cell nuclei; image d, merged picture of images a–c; image e, enlarged picture of the boxed area of image d; image f: control staining in which anti-μ-calpain antibody was preabsorbed with its blocking peptide. B: Western blotting showing specificity of the anti-μ-calpain antibody. C: μ-calpain activity of the PG after IPR stimulation. The calpain activity in the PG was measured as described in the text. The calpain activity was indicated as relative fluorescent units (RFU)/mg protein. D: effects of IPR on the pilocarpine-provoked saliva secretion. The saliva was collected for 5 min following pilocarpine injection at 0, 1, and 24 h after IPR treatment. Means ± SE for 4 animals are shown. **P < 0.01 and *P < 0.05, significantly different from nontreated group by Student’s t-test.
of translation elongation) as well as ALLM and calpeptin suppressed the decrease of AQP5 protein level by IPR in the PG membrane fraction (Fig. 4, C and D); MG132 and lactacystin did not suppress IPR effects to decrease the AQP5 protein level. These data suggest that the degradation pathways that use both 26S proteasome and cysteine proteases participated in the regulation of AQP5 protein levels in the PG in normal mice, whereas the calpain pathway in the PG became activated when animals were stimulated with IPR, leading to AQP5 degradation.

**Proteolysis of AQP5 by μ-calpain.** To confirm in vivo results that calpain degraded AQP5, we incubated the AQP5-containing membrane fraction from the SMG with calpain in vitro and examined the ability of this enzyme to process AQP5 by Western blotting. The SMG membrane fraction was used in this experiment since it contained the minimum amount of AQP5-proteolytic activity (see bands in Fig. 5B). As shown in Fig. 5, A and B, the amount of AQP5 was decreased by μ-calpain in dose- and time-dependent manner, suggesting that AQP5 protein was actually degraded by this enzyme; i.e., the AQP5 protein level decreased to ~40–50% of the original level during 1- to 3-h incubation with 8–10 U/ml of μ-calpain (Fig. 5, A and B). Furthermore, the degradation of AQP5 protein was inhibited by calpain inhibitors ALLM and calpeptin (Fig. 5B), indicating that calpain-mediated proteolysis is responsible for AQP5 degradation.

**Expression of μ-calpain in the PG and its activity changes by IPR.** Since AQP5 was degraded by μ-calpain in vitro and the degradation could be suppressed by two calpain inhibitors, ALLM and calpeptin, we next investigated localization of the μ-calpain protein in the PG by using a specific polyclonal antibody. By a double-staining experiment for μ-calpain and AQP5, this proteolytic enzyme was localized in or near the plasma membrane at the apical side in acinar cells (Fig. 6A). Specificity of the anti-μ-calpain antibody was confirmed by preabsorption with the blocking peptide (Fig. 6, A and B).

To further examine calpain activation in response to IPR, the PG homogenate was prepared from mice injected with IPR, and calpain activity was measured using Ac-LLY-AFC, a synthetic fluorescent calpain substrate. As shown in Fig. 6C, the endogenous calpain activity began to increase at 1 h, and the level reached the maximum at 6 h after IPR injection, which was in accord with the time at which the AQP5 level was decreased. This result, in conjunction with the results of calpain inhibitor experiments (Fig. 4D), indicates the possibility that calpain proteolysis was responsible for IPR-induced AQP5 reduction or degradation in the PG plasma membrane.

Since IPR induced a prominent decrease in the AQP5 protein level, the saliva secretion was measured to confirm relevance of AQP5 reduction to the physiological function. The pilocarpine-stimulated whole saliva was collected for an initial 5 min at 0, 1, and 24 h after IPR injection (Fig. 6D). At 1 h after IPR treatment, strong salivation was observed as reported previously (32). On the other hand, a significant reduction in salivation compared with control (0 h) was observed in mice treated with IPR for 24 h. These data agreed with the previous report (21) and support the present increase and decrease in the AQP5 level in the membrane after IPR injection (Figs. 2 and 3).

**DISCUSSION**

The PG is an exocrine salivary gland that stores a large amount of proteins such as amylase in the secretory granules; their constituents are secreted into saliva by exocytosis. Exocytosis is induced primarily by the activation of β-adrenergic receptors, which leads to the intracellular accumulation of cAMP (3). During exocytosis, the secretory granules are first docked then fused to the plasma membrane. The objective of the present study was to explore the molecular mechanism that could regulate the level of AQP5 in the PG of IPR-injected mice. Here, we found that IPR, a β-adrenergic receptor agonist, induced upregulation of AQP5 in the PG membrane fraction, followed by its degradation via the calpain pathway.

By Western blot and RT-PCR analyses of the PG samples obtained from mice injected with IPR, we found that IPR treatment had two distinct effects on AQP5 expression; first, it caused a gradual increase in AQP5 mRNA levels, peaking at 24 h. Second, it significantly decreased AQP5 protein levels in the membrane fraction 12 h after injection. Therefore, our subsequent studies were directed to identify the molecular mechanisms underlying these effects.

In the present study, an increase of the AQP5 protein level in the membrane fraction was detected from 1 to 6 h after IPR injection, and then a rapid reduction of this channel protein was observed at 12 h. The elevation of the membrane AQP5 level...
1 h after IPR injection concurs with the timing of amylase secretion. This increase was continued until 6 h after IPR stimulation and is suggested to be due to exocytotic transfer of AQP5 on secretory granules to the plasma membrane. It is probable that the granule membrane contains AQP5, which by fusion to the plasma membrane upon IPR stimulation became a part of the apical/lateral membrane, resulting in an increase in the AQP5 protein in the membrane. In fact, it was reported that the membrane of secretory granules isolated from the rat parotid gland bears AQP5 (14). Therefore, the increase in the area of the plasma membrane would have resulted in dilatation of the luminal membrane positive for AQP5.

We next focused on the phenomenon of IPR-induced AQP5 degradation in the membrane fraction. Reduction in the AQP5 protein in the PG membrane fraction was ~85% (30% of the control level) at 12 h after IPR treatment. Significant reduction of the AQP5 protein level in the PG was confirmed by immunohistochemistry as well (Fig. 2E). These results are supported by the reports that dihydrotachysterol induces AQP2 protein degradation despite its mRNA expression being unaltered in the inner medullary collecting duct of rats (23, 25). It is possible that IPR induced AQP5 protein reduction not through transcriptional mechanisms but rather via the post-translational mechanism.

Current advances in the molecular characterization of the two major intracellular proteolytic systems, the lysosomal and the ubiquitin-proteasome systems (16, 4), provide for the possibility that IPR triggered the degradation of AQP5 via either one of these systems. It is well known that the ubiquitin-proteasome system uses 26S proteasome (4) and cysteine proteases, including cytoplasmic calcium-dependent calpains (17). Calpains are one of the major intracellular proteolytic enzymes in mammalian cells and have generally been assumed to be regulated independently from the lysosomal system, serving distinct functions (5, 33). Here, we found that the two major systems, ubiquitin-proteasome and lysosomal proteases, played important roles in degradation of AQP5 in the mouse PG. In normal mice, the AQP5 protein level was increased after treatment with the inhibitors, MG132, CQ, ALLM, and calpeptin in vivo (Fig. 4), suggesting that the two systems are functioning under normal conditions. These results are supported by the previous reports that both proteasomal and lysosomal pathways participated in AQP2 protein degradation occurring soon after its synthesis (6). Furthermore, it has been reported that AQP1 is a target for ubiquitination when hypertonic stress is applied (10). More recently, Kamsteeg et al. (8) have shown that the short-chain ubiquitination pathway is involved in the hormone-regulated endocytosis of AQP2 in renal cells. On the other hand, after IPR treatment, a decrease in the AQP5 protein level was partially or strongly suppressed by CHX and the two calpain inhibitors ALLM and calpeptin but not by MG132, lactacystin, or CQ. Suppression of AQP5 degradation by CHX would have been caused probably as a result of this inhibitor having inhibited the synthesis of proteolytic enzyme, although such an idea needs to be verified. Our data suggest the possibility that AQP5 is a substrate for calpain and is metabolized by proteolysis.

Involvement of calpain in IPR-induced reduction of AQP5 was verified further; i.e., the tissue calpain activity after IPR injection was shown to be increased significantly at 6 h along with concomitant increase in its protein level, although its mRNA level was not induced appreciably (data not shown). By immunohistochemistry, μ-calpain was detected in the PG and was colocalized with AQP5 in or near the apical plasma membrane of the acinar cells. Since AQP5 is thought to be a substrate for calpain in the PG of IPR-treated mice, we examined whether calpain is a protease responsible for the AQP5 degradation in vitro. We found that incubation of the SMG membrane fraction (containing AQP5) with purified μ-calpain degraded AQP5. There are some reports suggesting that AQP0 and AQP2 are degraded by calpain; e.g., purified μ-calpain cleaves human AQP0 in the lens tissue at its four positions in vitro, all of which correspond to one of the 21 sites known to occur in the lens AQP0 during aging in vivo (12). Also, AQP2 expressed in the inner medullary collecting duct is reported to be degraded by calpain (23). Since calpain is a calcium (Ca2+)-dependent cysteine protease and cytosolic free Ca2+ is known to be induced by IPR (7), these reports support our present data that μ-calpain is involved in the regulation of the AQP5 protein level by IPR in the PG.

In summary, the following mechanism is proposed (Fig. 7). In accord with the amylase secretion, the number of AQP5 molecules in the plasma membrane is increased 1–3 h after IPR treatment, 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 AQP5 reduction is due to proteolysis by μ-calpain. The new AQP5 biosynthesis is increased gradually 12–48 h after IPR to return toward the original state.

**REFERENCES**


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