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

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Submitted 11 June 2013; accepted in final form 21 October 2013

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 in vivo. Am J Physiol Endocrinol Metab 306: E100–E108, 2014. First published November 5, 2013; doi:10.1152/ajpendo.00317.2013.—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-methinal (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 was coupled to the secretory-restoration cycle of amylase in the PG.

Aquaporins (AQP) 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. Am J Physiol Endocrinol Metab 2014.1

AQP2, a major water transport regulator, is expressed in the collecting duct cells of the kidney, particularly in inner medullary cells (1, 2). AQP2 is known to be regulated by cyclic adenosine monophosphate (cAMP) and plays a major role in mediating water transport in the kidney (2, 3). AQP2 is also involved in regulating fluid electrolyte homeostasis. The expression of AQP2 is increased by activation of cyclic adenosine monophosphate (cAMP) following activation of adenyl cyclase (3). Several lines of evidence prompt us to investigate the possibility that the AQP5 expression/metabolism may be associated with this β-adrenergic receptor 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) AQP5-knockout mice showed a significant decrease in transmembrane water transport (9). These results suggest that a certain amount 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 β-adrenergic receptor agonists via an increase in intracellular cAMP following activation of adenyl cyclase (3). Several lines of evidence prompt us to investigate the possibility that the AQP5 expression/metabolism may be associated with this β-agostin-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) AQP5-knockout mice showed a significant decrease in transmembrane water transport (9). These results suggest that a certain amount of fluid secreted by mouse salivary gland is due to the transcellular water transport, in which AQP5 plays a major role.

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 degradation. The reduction in the level of membrane AQP5 was significant, and the IPR-induced AQP5 degradation in the PG membrane fraction was increased after IPR treatment and then followed by its potential degradation. 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-methinal (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 was coupled to the secretory-restoration cycle of amylase in the PG.

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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-methini-
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 microscope slides 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 Fuji RX X-ray film was a product of Fuji Film (Kanagawa, Japan). All other reagents were procured as described previously (31).

**Animals and drug treatments.** Male mice (Crl: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 euthanatized 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 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 membranous 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 10 min at 4°C to remove the nucleus and cell debris. The supernatant thus obtained was designated 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 electrophotorectively transferred onto a nitrocellulose filter in a Mini-protean 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 μM 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 sampling 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 μM 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 μM) 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 sampling 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 euthanatized 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 = 3:1).

**Measurement of salivary secretion.** The saliva was collected by cotton pellet procedure from mice at 0, 6, and 24 h after IPR injection, as described previously (30). Briefly, the whole saliva was collected for 5 min by aspiration into preweighed small cotton pellets, and the pellets were placed into a preweighed 1.5-ml Eppendorf tubes, which were then weighed immediately.

**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 Kulzer Histo-Technik 8100, following the manufacturer’s protocol. The tissue sections were cut at 2 μm, washed in PBS, and incubated with trypsin solution (1 tablet/ml H2O) at room temperature for 15 min to explore the antigen epitope. For immunostaining of AQP5 and µ-calpain, the PG tissue was fixed with 3% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.4) at 4°C for 2 h, washed, frozen in liquid nitrogen, and embedded in optimum cutting temperature tissue tech compound, as described previously (31). Frozen sections at 5 μm were cut, washed in PBS at room temperature for 5 min, and postfixed in ethanol at −20°C for 1 min (for AQP5 staining) or in a 1:1 mixture of ethanol and acetone 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 antisera, 1,000 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 AQP5) 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 AQP5/µ-calpain) at room temperature for 2 h, followed by washing with PBS. For control staining, primary antiserum/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 AQP5 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 AQP5 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 95% 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 dilated 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. 1A, 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 AQP5 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 AQP5 redistribution. In untreated mice, the AQP5 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 AQP5 labeling in the lateral plasma membrane was strong and sharp. At 1 h after IPR injection, the acinar lumen

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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

![Image of 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.](http://ajpendo.physiology.org/)

![Image of 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.](http://ajpendo.physiology.org/)
seen. At 24 h after IPR injection, the AQP5 labeling seen in the apical membrane returned sharp (Fig. 2E). The staining in the cytosol became very weak while that at the basal membrane was recovered. The specificity of the immunohistochemical staining was confirmed by using a preabsorption experiment; i.e., all of the positive staining disappeared completely when the sections were incubated with the same concentration of antibody solution preabsorbed with the antigen peptide (Fig. 2F), clearly indicating that the positive staining was due to a specific reaction. Some of these results are well supported by a previous report (15).

The homogenate and membrane fractions from control and IPR-injected mice were also prepared to examine the changes in protein levels of amylase and AQP5 by Western blotting. As shown in Fig. 3A, the amylase level in homogenate decreased rapidly at 1 h by IPR injection, followed by continuous increase until 24 h. These data agree with the immunohistochemical data shown in Fig. 1. On the other hand, IPR induced a twofold increase in AQP5 levels in the membrane fraction at 1 h; AQP5 stayed at the high level until 6 h. At 12 h after IPR injection, a dramatic decrease in AQP5 levels was observed, and the level gradually returned toward its original point by 48 h (Fig. 3, A and E). The changes in AQP5 levels were inversely correlated with changes in the amylase level (see also Fig. 3E). The specificity of AQP5 and amylase antibodies was confirmed by preabsorption of the antibody with the blocking peptide (Fig. 3C).

Next, we determined whether the increase and decrease in the AQP5 level was correlated to changes of the AQP5 mRNA level. Total RNA extracted from the PG of control and IPR-injected mice was subjected to RT-PCR analysis. As shown in Fig. 3D, onset of the elevation of AQP5 mRNA expression was already detected at 1 h following the injection of IPR. However, the mRNA level reached a peak at 12–24 h after IPR injection, which was then decreased at 48 h (Fig. 3, D and E). The injection of IPR provoked the most prominent increase in the mRNA expression of AQP5 in the PG at 12–24 h. It is worthy to note that the AQP5 protein level in the membrane fraction elevated rapidly after IPR injection, whereas its mRNA level was still close to the control level and did not increase to the fullest. Also as mentioned above, the level of membrane AQP5 reached a peak at 1–3 h when amylase level in the gland became minimal.

We hypothesized the following mechanism to elucidate these phenomena (see also DISCUSSION); i.e., amylase containing secretory granules are bearing AQP5 at their membrane which, upon exocytosis, will become a part of the plasma membrane, resulting in the rapid elevation of AQP5 levels in the membrane fraction. Second, the AQP5 level in the plasma membrane returns toward the original level by proteolytic cleavage and de novo synthesis of this water channel protein. To support the first stage of this hypothesis, it is necessary that the membrane fraction we used in this experiment was not mixed with the membrane of the secretory granules (which might have been arisen during preparation of the membrane fraction) or secretory granules themselves. To test this validity, we measured the AQP5 protein level in the homogenate (Fig. 3A), and the ratio of AQP5 in the membrane fraction to that in the homogenate was calculated after having been normalized by the β-actin values (Fig. 3F). The result showed that the ratio

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**Fig. 4.** Effects of protease inhibitors on IPR-induced degradation of the AQP5 and β-actin protein levels in the PG in vivo. A: Western blotting of the PG membrane fraction obtained from mice injected with the inhibitors or vehicles. Mice were injected with IPR and the tissue were collected 1 h after injection. B: Western blotting of the PG membrane fraction obtained from mice injected with the various vehicles 1 h prior to injection with IPR. Mice were euthanized 24 h after IPR injection. C and D: Western blotting of the PG membrane fraction obtained from mice injected with the inhibitors 1 h prior to injection with IPR. Mice were euthanized 24 h after IPR injection. In A, the data were combined after having been obtained from plural gels. The variation among gels was minimum, which was verified/confirmed by comparing the intensity of the same sample applied on each gels. In C, the data obtained from a single gel were combined. Detailed experimental conditions are described in the text. NT, nontreated; DM, 0.5% DMSO; Sal, saline; MG, MG132; LC, lactacystin; CHX, cycloheximide; CQ, chloroquine diphosphate; AM, N-Ac-Leu-Leu-methininal; CP, calpeptin.

**Fig. 5.** Degradation of AQP5 by μ-calpain in vitro. A: time course of AQP5 degradation by μ-calpain. Aliquots of the 1.0-μg membrane fraction of the mouse submandibular gland (SMG) were incubated with 8 U/ml μ-calpain at 30°C for the indicated time, followed by Western blotting. B: effects of dosage of μ-calpain on AQP5 degradation. Aliquots of the 1.0-μg membrane fraction of the mouse SMG were incubated with indicated amounts of μ-calpain at 30°C for 1 h, followed by Western blotting. The band intensity of Western blot images was quantified using the NIH Image J software. NI, no incubation; AL, ALLM; CP, calpeptin. Detailed experimental conditions are described in the text.
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 treatment, and then a rapid reduction of this channel protein was observed at 12 h. The elevation of the membrane AQP5 level

**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

![Fig. 7. The hypothetical model of AQP5 dynamics coupled to the secretory-restoration cycle of amylase in acinar cells of the PG. Details about the model are described in the text. AM, apical membrane; LM, lateral membrane; BM, basal membrane.](http://ajpendo.physiology.org)
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 AQPI 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 cystosolic 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.

GRANTS

This work was supported in part by the National Natural Science Foundation of China (No. 21277078).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

6. Hasler U, Mordasini D, Bens M, Bianchi M, Cluzeaud F, Rousselot M, Vandewalle A, Feraile E, Martin PY. Long term regulation of aqua-


