**INTRODUCTION**

Aquaporins (AQPs) are the water channel proteins that have been identified in virtually all living organisms, and they are generally responsible for rapid water movement across the plasma membrane in almost all cells (1, 2). Among the 13 mammalian aquaporins identified so far, i.e., AQP0-AQP12 (2), AQP5 is reported to be expressed in the cell membrane of multiple secretory glands, including the lacrimal, salivary, and airway submucosal glands, as well as in type 1 alveolar cells (3, 4), sweat glands (5), corneal epithelium (6), and duodenal Brunner’s gland (7). The essential role of AQP5 has been shown in the study of AQP5 gene knockout mice, which demonstrated significantly increased lethality of the embryo and problems related with water homeostasis (8, 9). In addition, defective cellular trafficking was noted in the lacrimal gland and salivary gland biopsies from patients with Sjögren’s syndrome but not in patients with non-Sjögren’s dry eye and dry mouth (10, 11), although there are some opposite reports (12).

In a murine lung epithelial cell line (MLE-12), a cAMP analog, 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), was found to induce the AQP5 mRNA and protein expression (13). It is also reported that such stimulations induce translocation of AQP5 from the intracellular storage sites to the apical membrane (14-17) ; i.e., AQP5, majority of which is distributed in the cytoplasm, can be translocated to the plasma membrane in response to cpt-cAMP in MLE-12 cells (13). AQP5 was also targeted to the cell membrane after incubation with cpt-cAMP in Madin-Darby...
canine kidney cells (MDCK) cells (14). In contrast, Sidhaye, et al. reported a biphasic effect of cpt-cAMP on AQP5 translocation in MLE-12 cells; they showed that short-term exposure to this second messenger reduces membrane expression of AQP5, which is subsequently recovered by long-term exposure (18). However, these contradictory results may be mainly due to apparent difference in the basal level of AQP5 expression in the cell membrane; i.e., the membrane expression of AQP5 was far greater in the later study (18). On the contrary, Woo, et al. found that AQP5 membrane targeting may not be regulated solely by PKA phosphorylation and that it can be regulated by more than one mechanism besides that by cAMP dependent phosphorylation (19).

The present study was aimed to determine the role of PKA phosphorylation site at the ^3SSRRTS in the regulation of AQP5 trafficking. For this purpose we prepared 3 GFP-AQP5 constructs containing mutated PKA target site where one or more of Ser and Thr are replaced with Ala and Val, respectively so that phosphorylation of this residue is restricted or completely prohibited when they were expressed in the host cells. Here, we showed that AQP5 can be expressed on the plasma membrane of MDCK-II cells even phosphorylation of PKA-target sequence is restricted or prohibited, suggesting the existence of PKA-independent trafficking.

MATERIALS AND METHODS

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and cytochalasin B were obtained from Sigma-Aldrich (St.Louis, MO). Lipofectamine 2000, OptiMEM I, and Alexa Fluor 594-conjugated streptavidin were purchased from Invitrogen (Carlsbad, CA). Sulfosuccinimidyl 6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) was obtained from Pierce Biotechnology (Rockford, IL). Restriction enzymes, Xho I, came from Takara Bio. Inc (Shiga, Japan). Ligation-Convenience Kit was from Nippon Genetic Co., Ltd (Tokyo, Japan). The QuickChange Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA).

PCR cloning and preparation of the pcDNA 3.1/Hygro (+) construct containing wild-type (wt) AQP5 cDNAs

Total RNA was isolated from the submandibular gland (SMG) of rats by using TRI Reagent. A full-length AQP5 cDNA was synthesized by RT-PCR with 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 components were mixed on ice: 12.5 μl of 2-times concentrated reaction mixture, 5 pmol of each primer, 0.5 μl of a mixture of reverse transcriptase and Taq DNA polymerase and 1 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, followed by 1 cycle of extension at 72 °C for 5 min. The primer set used was 5′-AACCTTCCTCC-AAGGCACCATGAAAAA-3′ (sense) and 5′-CTCGAGTCACGAAATCTCTGAGGTCTG-3′ (anti-sense), which had Hind III and Xho I restriction sites (underlined) at 5′ and 3′ end, respectively (20). The AQP5 cDNA (1073 bp) obtained by RT-PCR were cloned into the pGEM-T Easy vector, from which the insert was next cut by Hind III and Xho I restriction enzymes and subcloned into a multiple cloning site of the pcDNA 3.1/Hygro (+) vector. The resultant plasmid was termed as wild-type AQP5-Hygro (AQP5(wt)-Hygro). This construct was used for preparation of AQP5 genes with mutated PKA-consensus sequence.

Preparation of AQP5-Hygro having mutated PKA-target motif at deduced amino acids 152-156 (^3SSRRTS) by site-directed mutagenesis

The AQP5-Hygro cDNAs having mutated PKA-target motif at nucleotides 454-468 (5′-CTCTTGAGCTCACGAAATCTCTGAGGTCTG-3′), which corresponds to the amino acid sequence ^3SSRRTS, were prepared by using a QuickChange site-directed mutagenesis kit. First, AQP5(wt)-Hygro plasmid described above was used as a template,
and artificial mutations were introduced. AQP5’s with mutated nucleotide sequences were next subcloned into pEGFP-C2. The following is the experimental design employed: 3 primers, PK-1, PK-3, and PK-5 were synthesized; the sequence of PK-1 was 5’-CCACCAGACTGCGACAGCCGGGCTTGTTGGGCTC-3’, which had 3 mutated nucleotides (indicated by the underline), resulting in S152ARRTA. The sequence of PK-5 was 5’-CCACCAGACTTGGCCAGCCGGGCGCTGGTTGGGCTC-3’, which had 2 mutated nucleotides, resulting in S152ARRVA. Lastly, the sequence of PK-3 was 5’-CCACCAGACTGCGAGTCCCGCCCTGGTTG-3’, which had 5 mutated nucleotides, resulting in S152ARRTVS. The primer set, PK-3 and PK-4, was the brief experimental design employed: 3 primers, PK-1 and PK-2. Similarly, AQP5(T155V)-Hygro was prepared from AQP5 construct was verified by use of an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA).

**Cell culture, transfection, cell surface biotinylation, and observation under a confocal laser scanning microscope**

MDCK-II cells, kindly provided by Dr. Mikio Furuse (Kobe University), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C in 5% CO2; they were supplemented with fresh medium every third day. For experiments, MDCK-II cells were plated at the cell density of 6×10⁴ cells/well on 6.5-mm polycarbonate membranes 48 h prior to transfection. By use of Lipofectamine 2000, the cells were transfected with GFP-AQP5( wt) or GFP-AQP5 having mutated PKA-target motif; i.e., the cells were covered with a mixture of 0.5 μg of DNA and 2 μl of Lipofectamine 2000 in a final volume of 100 μl of Opti-MEM I medium per well as described by the manufacturer’s protocol (Invitrogen). The transfection medium was removed after 5.5 h of incubation and replaced with fresh DMEM containing 10% FBS without antibiotics. After cultivation for 24 h following transfection, in which time point MDCK II cells had been cultured for 3 days in total (22), polycarbonate membranes containing cell monolayers were washed with phosphate-buffered saline (PBS) 3 times, fixed with 3% paraformaldehyde for 20 min, washed, and incubated with 50 mM NH4Cl for 15 min. For biotinylation,
cells were washed, and blocked with 1% BSA (fraction V) in PBS for 1 h at room temperature and reacted with cell-impermeable biotinylation reagent (2 mM sulfo-NHS-LC-biotin) in PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.4 at room temperature for 40 min. After having been washed, cells were incubated with 50 mM NH₄Cl in PBS to quench excess biotinylation reagent. Cells were next reacted with Alexa Fluor 594-conjugate streptavidin (1 : 200), washed, and mounted with Vectashield mounting medium (Vector Laboratories, CA, USA) on slide glasses with frames of 70-μm thickness. They were covered with a coverslip, and sealed with nail polish. Samples were stored in the dark at 4°C until they were examined under a confocal laser scanning microscope (Leica TCN NT, Heidelberg, Germany) with excitations at 488 nm (for FITC) and 568 nm (for Alexa Fluor 594).

The samples were examined by scanning at horizontal directions for 32 times at different vertical planes (0.5 μm intervals). The cells expressing GFP-AQP5 were selected and their transverse images were saved. These data were examined to verify whether AQP5 was completely targeted to apical cell membrane. This can be determined by merging 2 pictures, GFP fluorescence (green) and biotin (red) as the cells expressing AQP5 at cell membrane show the yellow signal. For the sake of convenience, the cell membrane facing to the medium will be referred to as the “apical membrane,” and the one facing to the polycarbonate membrane, the “basal membrane.” In all transfection experiments, 4-6 wells were prepared and means± S.E. were calculated.

Treatment with H-89, colchicine, and cytochalasin B

For examination of the effects of inhibitors of PKA, microtubules, and microfilaments, cells cultured on polycarbonate membranes for 48 h were transfected with the chimeric gene, GFP-AQP5(wt) as described above and cultured under a growth-arrest condition (14), i.e., DMEM containing 0.5% FBS, for 18 h. The medium was then replaced with fresh medium containing 0.5% FBS and either 30 μM H-89 (14), 10 μM colchicine or 10 μM cytochalasin B (17) ; and the cells were cultured for another 6 h (14). During 6 h-culture, medium was replaced with fresh ones containing same inhibitors once at 3 h. The cells were then fixed, biotinylated, and examined under a confocal laser scanning microscope as described above.

Statistics

Data are expressed as means±SE. For statistical analysis of results, Mann-Whitney U-test was applied.

RESULTS

AQP5 expression in MDCK-II cells

We examined physiological behavior of AQP5 in MDCK-II cells, since these cells are known to provide a well-defined polarized cell model during cultivation on a polycarbonate membrane, and are widely utilized in studies of epithelial cell polarity and intracellular protein trafficking (23). Thus trafficking and/or translocation of particular proteins, including AQP5 protein (22), toward apical or basolateral membranes can be studied in this model system. MDCK-II cells were transfected with plasmids generating chimeric proteins for GFP-AQP5(wt) or GFP-AQP5 proteins with mutations in PKA consensus sequence, and cytoplasmic or apical-membrane localization of these gene products was analyzed (Figs. 1 and 3). In Fig. 1, typical MDCK-II cells expressing GFP-AQP5(wt) presenting prominent GFP signals and indicating the localization of the AQP5 chimeric protein in them are shown (Fig. 1A). We counted the number of cells that expressed GFP-AQP5(wt) and those showing localization of GFP-AQP5(wt) at apical membrane (Fig. 1B) ; membrane localization of the chimeric protein was confirmed by yellow signal which verified its co-localization with surface-labeled biotin.

At 6 h after transfection, GFP-AQP5(wt) was already expressed but it stayed in the cytosol until 12 h. At 24 h after transfection, 33% of the cells among those expressing GFP-AQP5(wt) showed localization at the apical membrane. This value increased to 73% at 48 h after transfection.

Effects of H-89, colchicine, and cytochalasin B on translocation of GFP-AQP5(wt)

We used several reagents known to stimulate or inhibit cell dynamics in order to understand and confirm the machinery involved in the translocation of GFP-AQP5(wt) in the MDCK-II cells.

Thus, at first, we determined whether PKA activity was required for GFP-AQP5(wt) translocation. An experiment to examine the possible involvement of microfilaments or microtubules was also concomitantly performed. MDCK-II cells, plated
and transiently transfected as described above, were treated with either H-89 (30 μM; 13, 14), colchicine or cytochalasin B (10 μM, each) at 18 h after transfection; they were cultured further for 6 h under a growth-arrest condition as described above. In good accordance with our previous finding (17), colchicine, an inhibitor of cytoskeleton assembly, inhibited strongly the translocation of the chimeric proteins; i.e., the number of cells expressing the GFP-AQP5(wt) at their apical membrane was only 38% of that of the non-treated control cultures (Fig. 2), suggesting that translocation of AQP5-bearing vesicles, required the microtubule system. A microfilament inhibitor, cytochalasin B had no or only little effect on the trafficking of the GFP-AQP5(wt) protein at their apical membrane among GFP-positive cells at different post transfection times is presented. The number of sample wells analyzed was 4 to 6.

On the other hand, percentage of the cells that expressed GFP-AQP5(wt) at their apical membrane among GFP-AQP5(wt) positive cells increased in the presence of the PKA inhibitor H-89, as compared with that for the non-treated cells. The percentage increase in apical translocation by H-89 was 1.5 times of that for non-treated control (Fig. 2).

The above results suggest that the translocation of AQP5-bearing vesicles towards the apical membrane was increased by decreasing the phosphorylation of Ser/Thr of AQP5. From these initial observations, we hypothesized that the phosphorylation itself is probably not crucial for membrane expression of AQP5. The phosphorylation target site blocked by H-89 may be amino acid 152-156 (SRRTS), since this is a PKA-target motif found in AQP5 (3). We, therefore constructed 3 GFP-AQP5 mutants having mutated consensus PKA-target motifs to examine the role of this PKA-target motif in membrane expression of AQP5.

**Effects of mutation in PKA-target motif (SRRTS) on translocation of the AQP5 chimeric proteins**

Twenty-four hours after transfection of MDCK-II cells with GFP-AQP5 (S152A/S156A), GFP-AQP5 (T155V) or GFP-AQP5 (S152A/T155V/S156A),
the number of cells expressing each mutant chimeric proteins at cell membrane were 76, 57, and 74%, respectively of the GFP-AQP5 positive cells. These numbers were significantly greater compared to the 33% for the cells expressing GFP-AQP5(wt) at the cell membrane (Fig. 3).

**DISCUSSION**

The salivary glands are innervated by both sympathetic and parasympathetic branches of the autonomic nervous system. The parasympathetic stimulation and activation of M3 muscarinic receptors on the acinar cells produces the largest increase in the salivary flow rate (24). The presence of an exocrine-type water channel, such as AQP5, in acinar cells is essential at least for transcellular water transport as shown by the results of its knockout experiment (8, 9). Trafficking of AQP5 is thought to be involved in the regulation of salivary water secretion via this channel protein; e.g., in the parotid gland, AQP5 was reported to be trafficked from the intracellular vesicles to the apical membrane in response to stimulation by muscarinic receptors in vitro (15).

In human salivary gland cells (HSG cells) transfected with AQP5 cDNA, the AQP5 protein is trafficked to the plasma membrane following an increase in [Ca²⁺], (17). All of these reports imply that trafficking of AQP5 from intracellular vesicles to the apical membrane is provoked by secretagogue-induced [Ca²⁺], increase leading to an increase in water permeability at the apical membrane. From the aspect of molecular phylogenetic comparison, AQP5 is known to be closest to AQP2, suggesting that their physiological and biochemical properties would be close as well (3, 25). AQP5 is trafficked from intracellular vesicles toward the apical membrane in response to a muscarinic agonist (15), which response is similar to the vasopressin-induced trafficking of AQP2 to the plasma membrane (26). A key event that triggers signals for prompting AQP2 trafficking is phosphorylation of the amino acid residue located at the carboxyl terminus, Ser-256, by protein kinase A (27). For instance, van Balkom, et al. (28) constructed several AQP2 genes having a mutation at either putative casein kinase II-target motifs (Ser-148, Ser-229, Thr-244), a protein kinase C (PKC) -target motif (Ser - 231), or a PKA-target motif (Ser-256); these constructs were then expressed in MDCK cells. All of these mutant proteins, except Ser-256 mutant, trafficked from the intracellular vesicles to the apical membrane via a forskolin-sensitive mechanism, similarly as wild-type AQP2 did, suggesting that phosphorylation of Ser-256 is essential in this cellular event (28).

GFP has been used for studying the behavior and localization of particular proteins in the living system. For example, GFP-AQP2 chimera (GFP fused to the amino-terminus of AQP2) expressed in cultured porcine kidney epithelial cells (LLC-PK; cells) was found to traffic in a regulated pathway from the intracellular vesicles toward the basolateral plasma membrane in response to vasopressin or forskolin stimulation (21). Similar to 2 different constructs of GFP with AQP2, GFP-AQP5 was localized primarily in the intracellular vesicles, while AQP5-GFP was predominantly localized on plasma membranes under non-stimulated condition (14).
Although present and previous inhibitor experiments suggested that blocking the PKA-dependent phosphorylation increased the trafficking of AQP molecule toward the apical membrane, this does not necessarily mean that the inhibitor blocked phosphorylation of the PKA-target motif of AQP5 molecules; it might have blocked proteins to which AQP5 may be associated. The present in vitro mutagenesis study for the first time explored that blocking the PKA-target motif of AQP5 molecule increases the trafficking of this molecule toward the apical membrane.

Our study was conducted to examine the effects of the PKA phosphorylation site of the AQP5 molecule on its intracellular translocation or trafficking. The consensus sequence of the PKA-target motif is located in cytoplasmic loop D of AQP5 at amino acid residues 152-156 (Ser-Arg-Arg-Thr-Ser; 3). Since there are not many reports which describe the function of this motif in the trafficking of AQP5, we investigated the cell physiological properties of rat AQP5 expressed in MDCK-II cells used as a model system. We prepared GFP-AQP5 constructs in which PKA consensus sequence motif was mutated to Ala and/or Val to generate the un-phosphorylated state and these constructs were used to transfect MDCK-II cells.

The amino-terminal fusion chimera, GFP-AQP5 (wt), was shown to be translocated to the apical membrane from the intracellular compartments in polarized MDCK-II cells during cultivation for more than 24 h after transfection. In cells transfected with GFP-AQP5(wt) or mutant GFP-AQP5s, all chimeric proteins were expressed on the plasma membrane; i.e., even PKA-dependent phosphorylation was blocked, GFP-AQP5 was trafficked toward apical membrane. By the experiment using GFP-AQP5 with replacement of various amino acids at 152SRRTS (S152A/T155V/S156A), we found that blocking the phosphorylation of AQP5 at this PKA-target motif increased its translocation to the apical membrane. Also, the cells expressing the wild-type molecule at their apical membrane increased in number by treatment with H-89 comparing to non-stimulated conditions. This membrane trafficking of GFP-AQP5 (wt) required the involvement of microtubules.

In the present study, we have demonstrated here that GFP-AQP5 chimeric molecules having mutation at their PKA consensus sequence can be expressed at the apical membrane. In contrast with phosphorylation-dependent translocation of AQP2 (28), our data suggest that AQP5 can be trafficked toward the cell membrane irrespective of phosphorylation of PKA-target motif. Thus the present study imply that a mechanism(s) independent of phosphorylation of PKA-target motif (152SRRTS) is involved in translocation of AQP5 in MDCK-II cells.

ACKNOWLEDGEMENTS

This work was a part of a dissertation for the Doctorate of Philosophy Degree submitted to the Graduate School of Oral Sciences, the University of Tokushima. We are grateful to Professor Mikio Furuse, Faculty of Medicine, Kobe University, for providing MDCK-II cell line. We thank Dr. Masayuki Shono for his technical assistance in operating the laser confocal microscope.

GRANTS

This work was supported in part by Scientific Research (B) (18390493) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. M.R. Karabasil, A. Azlina, and N. Purwanti were supported by a scholarship from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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