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Research Article

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Expression and Distribution of Aquaporin of Collecting Duct Are Regulated by Vasopressin V_2 Receptor in Rat Kidney

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Abstract

To examine whether expression and distribution of aquaporin of collecting duct (AQP-CD) are regulated by vasopressin V_2 receptor (V2R), we performed immunohistochemical studies with specific antibody against AQP-CD. Normal Wistar rats were divided into four groups and treated for 3 d; control, dehydration, vasopressin V_1 receptor (V1R) antagonist (OPC-21268 120 mg/kg), V2R antagonist (OPC-31260 30 mg/kg). At time of death, urine osmolality (Uosm) in the dehydration group (1884±245 mOsm/kg) was significantly higher than that in the control (938±91). In the V2R antagonist group, Uosm was significantly decreased to 249±29, whereas V1R antagonist showed no effect on Uosm. In the control and V1R antagonist groups, immunofluorescence studies showed the AQP-CD staining of both apical membrane and subapical cytoplasm of CD cells of the cortex and the inner medulla. Dehydration increased the immunostaining of both apical membrane and subapical cytoplasm of CD cells of the inner medulla, and the degree of increase was dominant in apical membrane. In the V2R antagonist group, only faint staining of apical membrane and weak labeling of cytoplasm of CD cells of the inner medulla were observed. These changes in the localization and protein amount of AQP-CD by dehydration and V2R antagonist were quantitatively confirmed by immunogold studies and immunoblot analysis of the inner medulla. The present results indicate that the distribution and amount of AQP-CD in the CD cells are regulated by vasopressin V_2 receptor. (*J. Clin. Invest.* 1994. 94:1778–1783.) Key words: vasopressin V_1 receptor • vasopressin antagonist • water channel

Introduction

It is well recognized that water permeation through the vasopressin-sensitive epithelia is mainly transcellular. An ample amount of evidence also has shown that water channel in the apical membrane of the collecting duct (CD)[1] cells plays a major role in this transcellular water movement (1). In isolated perfused tubules, vasopressin treatment induced several alterations in the morphology of the CD cells, including dilatation of lateral intercellular spaces and an increase in the number of clusters of intramembrane particles in the apical membrane (2–4). These clusters are now believed to contain the water channels, and the increase in water permeability of CD cells by vasopressin is considered to be induced by an insertion of vesicles containing the water channel.

Recently, CHIP-28 was cloned from human red blood cells (5) and it was shown that this protein is a membrane water channel (6). Immunohistochemical study showed that CHIP-28 is present in proximal tubules and descending limb of Henle but not in the collecting duct systems (7), indicating that this water channel is not a vasopressin-regulated transporter. On the other hand, aquaporin of collecting duct (AQP-CD) was cloned from rat cDNA library and immunohistochemical studies and RT-PCR along the nephron clearly showed that its expression was limited to the CD cells (8). These results raised a possibility that AQP-CD is apical water channel which is regulated by vasopressin. The aim of this study is to examine whether protein level of AQP-CD and its distribution was regulated by vasopressin V_2 receptor in the chronic in vivo condition.

Methods

Male Wistar rats weighing 200–250 g were used throughout this study. Animals were divided into four groups and treated for 3 d as follows. In dehydration group, rats were deprived of water. In vasopressin V_1 receptor (V1R) antagonist group, 120 mg/kg of OPC-21268 was given with food. In vasopressin V_2 receptor (V2R) antagonist group, 30 mg/kg of OPC-31260 was given orally. These receptor antagonists were mixed with powdered food, and daily food amount was restricted to 10 g in all groups. By this food restriction, animals could consume all of the given food, except the dehydration group. These doses of antagonists are known to almost completely inhibit the action of exogenously administered vasopressin in the rat (9, 10). The animals were housed in individual metabolic cages and 12-h urine samples were collected for the measurements of osmolality on the last day of treatment.

After the treatments, rats were anesthetized by pentobarbital. The kidneys of five rats of each group were preserved for immunocytochemical studies by retrograde perfusion through the abdominal aorta, initially with phosphate-buffered saline (PBS) at pH 7.4 and then with a periodate-lysine-paraformaldehyde mixture (PLP) for 5 min. After perfusion, the kidneys were removed and cut into 1–2-mm-thick slices that were fixed additionally by immersion in the PLP for 24 h at 4°C. Tissues were obtained from the outer half of the cortex and the inner one third

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1. *Abbreviations used in this paper:* AQP, aquaporin; CD, collecting duct; PLP, periodate-lysine-paraformaldehyde mixture; Uosm, urine osmolality; V1R, vasopressin V_1 receptor; V2R, vasopressin V_2 receptor.

of the inner medulla. For immunofluorescence studies, the kidney pieces were immersed in graded series of sucrose and embedded in the OCT compound in dry ice and isopentane. These tissue samples were kept at −70°C and thin sections (~ 6 μm) were obtained by cryotome. For immunogold labeling, the kidney was further cut into smaller pieces (~ 0.5 mm in diameter) and then dehydrated in a graded series of acetone and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, FRG). Urine osmolality was determined by Advanced Micro Osmometer (Advanced Instruments Inc., Needham Heights, MA).

Immunofluorescence studies. Thin sections of the kidney were stained using polyclonal antibody raised against COOH terminus of AQP-CD (8) detected by FITC-labeled swine anti–rabbit IgG as follows. The sections were incubated overnight at 4°C with the rabbit antiserum against AQP-CD (1:500) or preimmune rabbit serum diluted in PBS. The tissues sections were then washed in PBS (four times) and incubated for 2 h in FITC-labeled swine anti–rabbit IgG diluted 1:200 in PBS. The sections were washed in PBS (four times) and viewed using an epifluorescence microscope. With preimmune rabbit serum, no significant labeling was observed. Furthermore, staining observed in the CD cells disappeared after preincubation of specific antibody with the peptide immunogen (8).

Immunogold procedure. Tissues from the kidney inner medulla of five animals from each group were embedded in Lowicryl K4M as described above. Thin sections were cut with glass knives and mounted on Fromvar-coated nickel grids. Sections were first rinsed on droplets of PBS containing 0.5% BSA and 0.02% NaN_3 for 15 min. The sections were then transferred onto drops of the rabbit antiserum against AQP-CD diluted 1:500 in the same solution overnight at 4°C in a moist chamber. Sections were then rinsed two times for 15 min by floating on drops of PBS and transferred to drops of gold-labeled goat anti–rabbit IgG (Zymed Laboratories Inc., S. San Francisco, CA) diluted 1:50 in PBS containing BSA and NaN_3 for 60 min at room temperature. Sections were washed two times for 10 min in PBS and counterstained with a saturated solution of uranyl acetate for 2 min and lead citrate for 2 min. The sections were examined and photographed by a Hitachi transmission electron microscope.

To quantitate the distribution of AQP-CD in immunogold studies, we counted the number of the gold particles of inner medullary collecting duct cells. The photographs of 4 to 5 inner medullary CD cells in each kidney were taken at low magnification. The particles on the apical membrane and subapical cytoplasmic vesicles (within 0.5 μm from the apical membrane) were counted. The length of the membrane was determined with curvemeter. Cell area was measured with picture analyzing system by personal computer (MOP-AM01 Quantitative Picture Analyzing System; Eko Seiki, Tokyo, Japan). The number of gold particles was expressed as the number per μm membrane length or per μm^2 cell area. The mean value of 4 to 5 cells was taken as a value of one animal. Statistical analysis was performed with one-way analysis variance followed by Schèffe's multiple comparison method.

Electrophoresis and immunoblotting. Membrane fraction was prepared from kidneys from four rats of each group. The kidneys were perfused through the abdominal aorta with PBS, and the inner medulla was dissected. Crude homogenate of the tissue were prepared by homogenization in ice cold buffer (in mM; sucrose 320, Tris-HCl 10, EDTA 2, PMSF 0.1, pepstatin A 1 μg/ml, leupeptin 1 μg/ml) with Teflon-glass homogenizer. Crude homogenate was centrifuged at 1,000 *g* for 20 min to remove nucleus and tissue debris, and supernatant was obtained. The supernatant was further centrifuged at 105,000 *g* for 20 min, and the pellet was suspended in the same buffer (membrane fraction). This fraction is considered to contain not only plasma membranes but subapical vesicles (11). Total protein concentrations were determined by Lowry method (12).

SDS-PAGE was performed using the buffers described by Laemmli (13) and 10–20% gradient acrylamide slab gel was used. 1 μg/lane of membrane protein from the inner medulla of each kidney was electrophoresed in acrylamide slab gels, which was transferred onto nitrocellulose, incubated with affinity purified anti–AQP-CD (1:500). The nitrocellulose membrane was then incubated with ^{125}I-protein A (ICN Biochemicals, Costa Mesa, CA) and autoradiography was taken. The membrane was also analyzed by image analyzing system (BAS 2000 system; Fuji Film Co., Tokyo, Japan) to quantitate the intensity of the bands.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Wako Junyaku Co. (Osaka, Japan) unless otherwise indicated. OPC-21268 and OPC-31260 were generously provided by Otsuka Pharmaceutical Co. (Osaka, Japan).

Table I. Urine Osmolality in the Four Groups

Groups	Urine osmolality
	mOsm/kg
Control	938±91
Dehydration	1884±245*
V1R antagonist	943±72
V2R antagonist	249±29*

Number of animals is six in each group. Values are means±SE. * $P < 0.01$ compared with control group.

Results

Urine osmolality in the four groups is shown in Table I. In V2R antagonist group, urine osmolality was significantly lower than that in control group, whereas V1R antagonist group did not show significant difference compared with control group. In dehydration group, urine osmolality was significantly higher than that in control group.

Representative sections of cortex and inner medulla stained with the antibody are shown in Figs. 1 and 2, respectively. In control group (Fig. 1 *A*), AQP-CD staining was intense in the apical membrane and was moderate in the cytoplasm of the cortical collecting duct cells. The staining patterns of the cortical collecting duct in the dehydration, V1R antagonist, and V2R antagonist groups were essentially similar to that of the control group, although the staining seemed slightly decreased in V2R group (Fig. 1, *B, C,* and *D*). Thus, adaptive changes were not striking in the cortex by immunofluorescence studies than the inner medulla.

In contrast to the above observations of the cortical collecting duct, we observed striking adaptive changes in the immunocytochemical staining of AQP-CD in the inner medulla. In control group, AQP-CD staining was observed in the apical membrane and cytoplasm (Fig. 2 *A*). In dehydration group, most of the cells in the inner medulla showed strong labeling on the apical membrane and in the subapical cytoplasm (Fig. 2 *B*), the change in the plasma membrane being more pronounced. In V1R antagonist group, the staining pattern of AQP-CD (Fig. 2 *C*) was similar to control group in the inner medulla. On the other hand, sections from the kidney in V2R antagonist group showed striking changes in AQP-CD staining; the inner medulla showed a loss of plasma membrane AQP-CD staining with only a faint subapical cytoplasmic labeling remaining (Fig. 2 *D*).

To quantitate these changes in the kidney, we performed the immunogold experiments with the inner medulla from the four groups, since the changes in the cortex were less evident. Representative sections are shown in Fig. 3. Labeling patterns similar to the immunofluorescence studies were observed by the immunogold study. In control kidney (Fig. 3 *A*), gold parti-

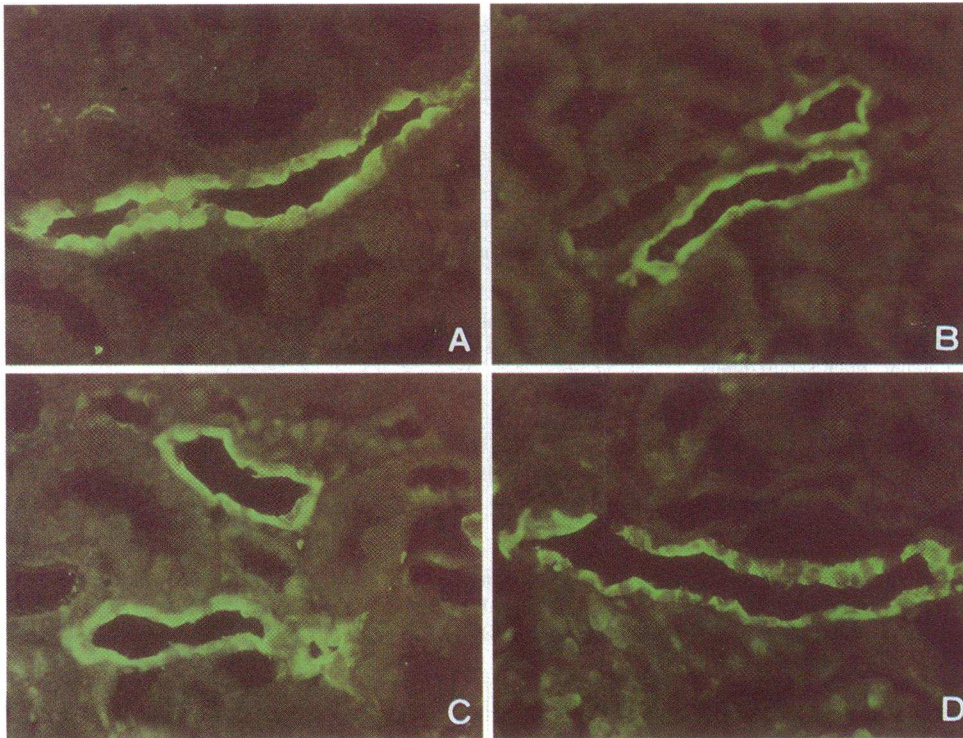

Figure 1. Semi-thin sections of rat kidney cortex incubated with antibody against the COOH terminus of AQP-CD, followed by swine anti–rabbit IgG coupled to FITC. (*A*) Control rat; (*B*) dehydrated rat; (*C*) kidney from the vasopressin V_1 receptor antagonist treated rat; (*D*) kidney from the vasopressin V_2 receptor antagonist treated rat.

cles were seen not only in the apical membrane but in the cytoplasmic vesicles. In the kidneys from dehydration group (Fig. 3 *B*), AQP-CD labelings were more enhanced in the apical membrane, whereas gold particles were also seen in the cytoplasmic vesicles. In V1R antagonist group, the pattern of AQP-CD labeling was similar to the control group (Fig. 3 *C*). The kidneys from the V2R antagonist group showed changes similar to the immunofluorescence studies (Fig. 3 *D*). We also performed these immunofluorescent and immunogold studies with affinity purified antibody in some specimens from each group, and we confirmed the same pattern of AQP-CD staining (data not shown). To quantitate the changes in AQP-CD labeling, the numbers of the gold particles in the apical membrane and subapical regions were counted in the kidneys from four groups.

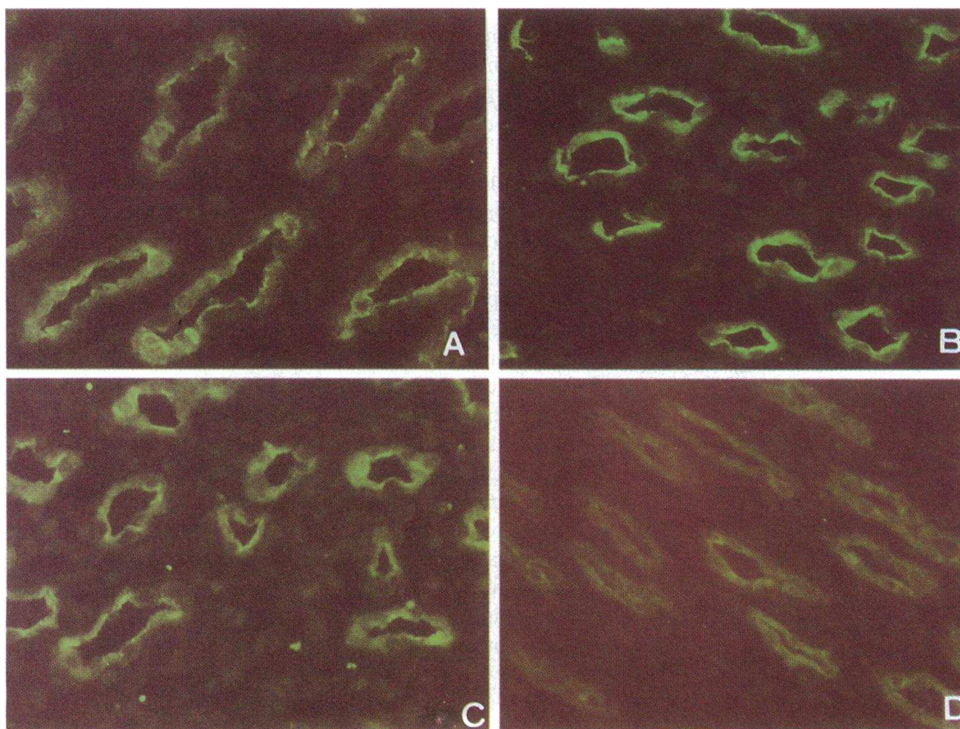

Figure 2. Semi-thin sections of rat kidney inner medulla incubated with antibody against the COOH terminus of AQP-CD, followed by swine anti–rabbit IgG coupled to FITC. (*A*) Control rat; (*B*) dehydrated rat; (*C*) kidney from the vasopressin V_1 receptor antagonist treated rat; (*D*) kidney from the vasopressin V_2 receptor antagonist treated rat.

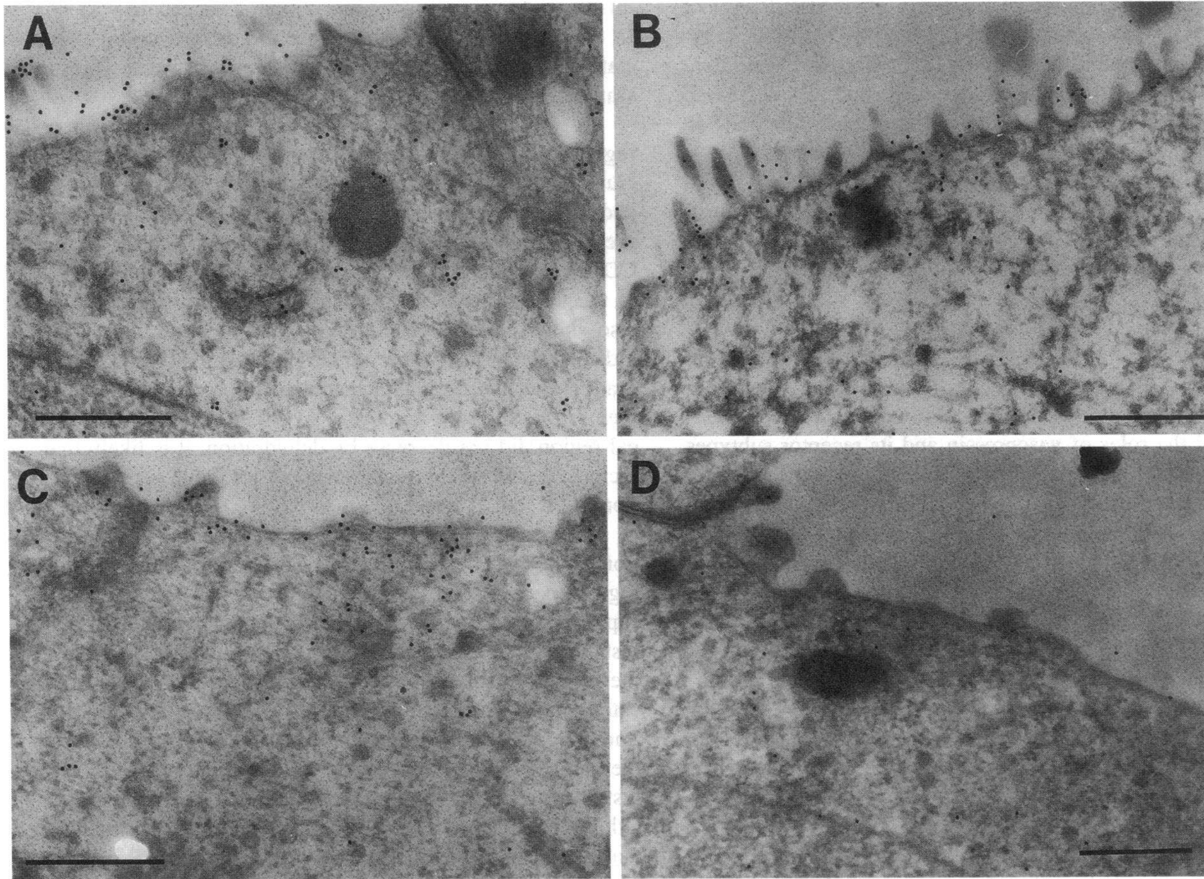

Figure 3. Electron micrograph of the apical part of a cell from the terminal inner medullary collecting ducts. Bar = 0.5 μm. 3A; control rat, 3B; dehydrated rat, 3C; kidney from the vasopressin V_1 receptor antagonist treated rat, 3D: kidney from the vasopressin V_2 receptor antagonist treated rat.

Since apical membrane foldings were increased in dehydration group, we normalized the particle number by membrane length. As shown in the Table II, the number of labeling in the apical membrane showed 2.4-fold increase in the dehydration group compared with control group, whereas that in V2R antagonist group decreased to 26% of that in control. The changes in subapical region showed the same tendency, but the magnitude of changes were smaller than that of the apical membrane; in dehydration group, the particle number was increased to 1.4-fold, and that in V2R antagonist group was decreased to 45% of the control. These results suggest that AQP-CD amount in both apical and subapical vesicles are regulated by vasopressin V2 receptor in association with the change in its, distributional pattern.

In Fig. 4, the representative pattern of the immunoblotting is shown. In each lane, two bands, a smaller molecule narrow band (29 kD) and a higher molecule broad band (35–45 kD), were observed in consistence with the previous paper (14). Low molecule band is corresponding to AQP-CD without gly-

Table II. Quantification of AQP-CD Antigenic Sites in the Terminal Collecting Duct Cells

Groups	Gold particles: Apical membrane	Gold particles: Subapical region
	per μm membrane	*per μm²*
Control	6.9±2.0	8.5±2.3
Dehydration	16.7±4.1*	12.0±2.3
V1R antagonist	6.4±2.1	9.5±3.6
V2R antagonist	1.8±0.5*§	3.8±1.6§

Values are the means±SD. * $P < 0.05$ compared with control group. § $P < 0.01$ compared with dehydration group. Number of animals is five in each group.

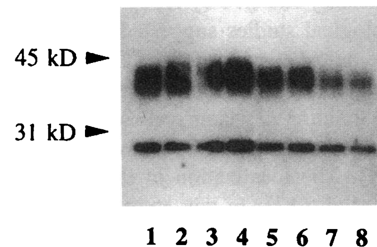

Figure 4. Immunoblot of proteins from crude homogenate and membrane fractions of the inner medulla. 1 μg protein of membrane fraction were loaded in each lane. (Lanes *1* and *2*) Control group; (lanes *3* and *4*) dehydration group; (lanes *5* and *6*) vasopressin V_1 receptor antagonist group; (lanes *7* and *8*) vasopressin V_2 receptor antagonist group. Number of lane is designated to animal number. The lower bands in each lane are corresponding to AQP-CD. Broad band above AQP-CD is glycosilated form of AQP-CD.

cosilation and higher molecule band is a glycosilated form of AQP-CD. By densitometer, the 29-kD AQP-CD band in dehydration was increased to 164% of the control group. The intensity of AQP-CD band in V2R antagonist group was decreased to 64% of that in control group. Since this membrane fraction contains plasma membranes and other intracellular membranes, this result in immunoblotting study indicates that total amount of AQP-CD was altered by dehydration and V2R antagonist. In each of the four study groups, the staining pattern of the immunoblot analysis was almost identical among the four sample animals.

Discussion

In the present study, we performed immunohistochemical studies to reveal the roles of vasopressin and its receptor subtypes on the regulation of aquaporin of collecting duct in the rat kidney. In water-deprived animals, which are supposed to have high levels of blood vasopressin, it was shown that AQP-CD was increased in both apical membrane and subapical region, while the increase in apical membrane was predominant. With the treatment by vasopressin V2 receptor antagonist, OPC-31260, AQP-CD staining was less intense in both the apical membrane and cytoplasmic vesicles than in the control rat kidneys. On the other hand, treatment with vasopressin V_1 receptor antagonist, OPC-21268, did not induce any apparent changes in the staining pattern of AQP-CD. Furthermore, the immunoblotting studies confirmed that the amount of AQP-CD protein was also regulated by vasopressin V_2 receptor.

It is well known that CD cells play a central role in the regulation of water reabsorption by vasopressin. In histological studies of isolated perfused collecting ducts, it was shown that vasopressin induced a reversible increase in thickness of the cellular layer, prominence of lateral cell membranes, and formation of intracellular vacuoles (2–4). Ultrafine structural changes by vasopressin were observed in toad bladders; it was shown that vasopressin and cyclic AMP induced membrane particle clustering in the apical plasma membrane of granular cells in the toad bladder with or without osmotic gradient (15), suggesting that these changes were induced by vasopressin itself but not by water flow. In mammalian collecting ducts, in vivo treatment of Brattleboro rats by vasopressin also induced the clustered particles in the luminal membrane (2), although some aspects of these changes in the collecting duct were different from the toad bladders (3). Functional study with the in vitro microperfused inner medullary collecting duct revealed that vasopressin treatment resulted in the increase of luminal membrane water permeability, although basolateral membrane water permeability was not significantly changed by vasopressin (16). These functional and histological studies suggested that water channel was present in the apical membrane of CD cells and toad bladders and that the localization of that channel might play a central role in the regulation of water reabsorption.

A recently cloned water channel, AQP-CD, was exclusively present in the CD cells (8). The localization of this channel was confirmed by Nielsen et al. (17). In that study, AQP-CD was mostly present in the apical membrane of the CD cells, and depriving rats of water for 1 to 2 d markedly increased collecting-duct water-channel protein expression determined by immunoblotting and qualitative immunolabeling studies compared with water-loaded rats. Our Western blot results in dehydration group are consistent with their findings, although the difference between control and dehydration groups was smaller in our studies. We speculate that the difference in hydration state of control rats made this discrepancy, since they used water-loaded rats as control.

In the present study, we tried to confirm that vasopressin regulates AQP-CD distribution and expression quantitatively. Number of immunolabeling was increased by water deprivation and the distributional changes in AQP-CD by endogenous vasopressin levels were quantitatively confirmed (Table II). AQP-CD labeling in dehydration group was significantly increased in the apical membrane, and the labeling in subapical region also showed a small but insignificant increase. These results indicate that total number of AQP-CD was increased by dehydration and that the major increase was occurred in the apical membrane. Since the number of AQP-CD labeling in the subapical region did not decrease by dehydration, it is likely that the increase in the apical membrane was not induced by redistribution of AQP-CD alone.

Our results clearly showed that the expression and distribution of AQP-CD is regulated via vasopressin V_2 receptor, using highly specific V_1 receptor antagonist, OPC-21268, and V_2 receptor antagonist, OPC-31260. The specificity of these antagonists were well characterized (9, 10, and unpublished data) and the results in urine osmolality in our present studies also indicated that OPC-21268 had little effects on the V_2 receptor. By immunogold studies, number of gold particles in both apical membrane and subapical region was decreased by V2R antagonist group as compared to the control and dehydration groups, although difference in the subapical region was smaller than that in apical membrane between control and V2R antagonist. The decrease in protein amount of AQP-CD with V2R antagonist was also confirmed by immunoblotting.

Localization of vasopressin receptor subtypes has been revealed by functional and in situ hybridization studies. Vasopressin V_1 receptor is mainly present in the vascular beds (18) and regulates the contraction of vascular smooth cells, whereas vasopressin V_2 receptor localizes exclusively in the collecting duct systems (19) and plays an important role in the regulation of water reabsorption. Recent report suggested, however, that vasopressin V_1 receptor might also exist in the CD cells (20). The roles of V_1 receptor in CD cells are not fully understood yet, although it is suggested that activation of V_1 receptor may inhibit the water permeability in response to V_2 receptor (1). Our present results by immunolabeling and immunoblotting studies do not support the possibility that V_1 receptor plays an any important role in the distribution of AQP-CD in the terminal collecting ducts.

It has been demonstrated by Lankford et al. (21) that the osmotic water permeability in the rat terminal inner medullary collecting duct is regulated by in vivo factor related to water intake; the isolated perfused inner medullary collecting duct from water-deprived rats showed high osmotic water permeability, which lasted up to 90 min after the start of the in vitro microperfusion without vasopressin or cAMP. On the contrary, the tubules from the water-loaded rats revealed low water permeability in the in vitro microperfusion. Furthermore, these authors showed that the intramembrane particle clusters in the terminal CD cells were markedly increased in dehydration rats, and that this change was dominant in the terminal part of the inner medullary collecting duct compared with its initial part. The mechanism of this adaptive change has not been clarified, and our present study gives a part of explanation for this phe-

nomenon. Vasopressin V2 receptor-mediated regulation of apical membrane AQP-CD was conserved after the microperfusion; increased apical AQP-CD by dehydration should result in high water permeability and diminished apical AQP-CD by V2R antagonist or water load should induce low permeability. We speculate that regulation by vasopressin in the terminal segment of the inner meddulary collecting duct is more dynamic than the other part of the collecting duct. In chronic in vivo state, we conclude that vasopressin V2 receptor regulates water permeability via the changes in apical AQP-CD amount.

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