Original Article

Differential Effects of Hyperosmolality on Na-K-ATPase and Vasopressin-Dependent cAMP Generation in the Medullary Thick Ascending Limb and Outer Medullary Collecting Duct

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Hyperosmolality in the renal medullary interstitium is generated by the renal countercurrent multiplication system, in which the medullary thick ascending limb (MAL) and the outer medullary collecting duct (OMCD) primarily participate. Since arginine vasopressin (AVP) regulates Na-K-ATPase activity directly *via* protein kinase A and indirectly *via* hyperosmolality, we investigated the acute and chronic effects of hyperosmolality on Na-K-ATPase and AVP-dependent cAMP generation in the MAL and OMCD. Microdissected MAL and OMCD from control and dehydrated rats were used for the measurement of Na-K-ATPase activity, mRNA expression of α -1, β -1, and β -2 subunits of Na-K-ATPase, and AVP-dependent cAMP generation. Na-K-ATPase activity in the MAL from dehydrated rats, as measured in isotonic medium, was higher than that of control rats. Moreover, incubation of samples in hypertonic medium (490 mOsm/kg H₂O) further increased Na-K-ATPase activity. Dehydration increased α -1, β -1, and β -2 mRNA expression in the MAL without changing that in the OMCD. Western blot analysis revealed that in the outer medulla, the expression of β -1, but not that of α -1 or β -2, was stimulated by dehydration. Incubation of MAL or OMCD in hypertonic medium increased AVP-dependent cAMP generation. Higher levels of AVP-dependent cAMP were generated in the MAL from dehydrated rats than that of controls, although incubation in hypertonic medium did not lead to additional increases in AVP-dependent cAMP accumulation. In contrast, AVP-dependent cAMP generation in the OMCD was stimulated by dehydration, and was further stimulated by incubation in hypertonic medium. These findings demonstrate that Na-K-ATPase is upregulated short- and long-term hyperosmolality in the MAL, but not in OMCD. (*Hypertens Res* 2005; 28: 671–679)

Key Words: hyperosmolality, Na-K-ATPase, cAMP, medullary thick ascending limb, outer medullary collecting duct

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Introduction

Sodium reabsorption in the medullary thick ascending limb (MAL) plays a key role in water absorption through water channels in the collecting ducts. The MAL participates in 10–15% of the reabsorption of sodium, whereas the collecting ducts only participate in 2–3% (1, 2). The energy for the active transport of sodium is driven by sodium-potassium adenosine triphosphatase (Na-K-ATPase) (3–5). Many hormones and autacoids participate in the regulation of Na-K-ATPase activity (3, 4). Since the localization of hormone receptors differs along the nephron, the regulation of Na-K-ATPase varies in different nephron segments (4). The presence of the following isoforms of Na-K-ATPase is known: four isoforms of the α -subunit, three isoforms of the β -subunit, and two isoforms of γ -subunit (6–13). Although results regarding the distribution of isoforms in the kidney conflict with each other, the α -1, β -1, β -2, γ -1, and γ -2 isoforms appear to be expressed in the kidney (14–19).

Dehydration influences sodium and water transport in the MAL and outer medullary collecting duct (OMCD) *via* hyperosmolality in the renal medulla and increased plasma levels of arginine vasopressin (AVP) (20, 21). Increased plasma levels of AVP also stimulate hyperosmolality in the renal medulla. The second messenger of AVP is cyclic adenosine monophosphate (cAMP), and AVP-sensitive cAMP generation is known to take place both in the MAL and in the OMCD (22). AVP stimulates sodium reabsorption through the Na-K-2Cl cotransporter (NKCC2) in the MAL, which stimulates water reabsorption in the collecting ducts (23). We previously reported that hyperosmolality decreases Na-K-ATPase activity in the inner medullary collecting duct (IMCD), where the interstitial osmolality is highest in the body (17). However, it remains unknown whether Na-K-ATPase activity in the MAL and OMCD is stimulated by dehydration or hyperosmolality. It is also not known which subunit of Na-K-ATPase plays a key role in the adaptation to dehydration. AVP participates in the short- and long-term regulation of sodium and water reabsorption, in part by the regulation of Na-K-ATPase activity. AVP regulates Na-K-ATPase activity in the distal nephron directly *via* the activation of protein kinase A, and indirectly by generating hyperosmolality in the renal medulla (3, 4).

It appears that Na-K-ATPase activity is upregulated by hyperosmolality in the MAL, and that AVP action is stimulated by acute hyperosmolality in the MAL and/or OMCD. To examine the mechanisms of adaptation to acute and chronic hyperosmolality in the renal outer medulla, the effects of hyperosmolality on Na-K-ATPase activity, the expression of Na-K-ATPase subunits, and vasopressin activity in the MAL and OMCD were investigated using polymerase chain reaction coupled with reverse transcription (RT-PCR), Western blot analysis, and the measurement of AVP-dependent cAMP generation.

Methods

Materials

Vasopressin was purchased from Sigma (St. Louis, USA). Calphostin C was obtained from Kyowa Hakko (Tokyo, Japan). The cDNA synthesis kit was obtained from Life Sciences (St. Petersburg, USA), and the polymerase chain reaction (PCR) master kit was purchased from Roche (Mannheim, Germany). The cAMP biotrac enzyme immunoassay system was obtained from Amersham (Buckinghamshire, UK).

Animals

Four-week-old pathogen-free male Sprague-Dawley rats weighing 50–100 g were used. Control rats were given free access to water. Dehydrated rats were placed under conditions of water restriction for 2 days. Urine and serum osmolality was measured using a micro-osmometer (Fiske, Norwood, USA). The protocol of the present study was checked and approved by the Experimental Animal Committee of the Kumamoto University School of Medicine (No. 13-077, 14-019, 15-026, and 16-063). The rats were kept in the Center for Animal Resources and Development at the Kumamoto University School of Medicine.

Microdissection of MAL and OMCD

Microdissection of the MAL and OMCD was performed as described previously (17, 24). In brief, the rats were anesthetized and the left kidney was perfused with 10 ml of ice-cold dissection solution containing 1 mg/ml collagenase and 1 mg/ml bovine serum albumin (BSA). The following segments were microdissected: glomerulus (Glm), proximal convoluted and straight tubules (PCT and PST, respectively), medullary and cortical thick ascending limbs (MAL and CAL, respectively), and cortical, outer medullary, and inner medullary collecting ducts (CCD, OMCD, and IMCD, respectively). In some experiments, short- and long-looped MALs (sMAL and lMAL, respectively) were dissected by microdissection. sMALs were identified by a bend in the initial portion of the ascending limb, and lMALs were identified by the attachment of long and straight thin limbs, as previously reported (25). The dissection solution was composed of the following (in mmol/l): 130 NaCl, 5 KCl, 1 NaH_2PO_4 , 1 MgSO_4 , 1 Ca lactate, 2 Na acetate, 5.5 glucose, 5 L-alanine, 2 L-leucine, and 10 *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (HEPES); the pH was adjusted to 7.4 by the addition of NaOH. The microdissection of the MAL and OMCD samples for RT-PCR was performed in a dissection solution containing 10 mmol/l vanadyl ribonucleotide complex (Life Technologies, Inc., Gaithersburg, USA). After microdissection, a 0.5- to 5-mm length of MAL or OMCD was used for a single determination of Na-K-ATPase activity or RT-PCR.

Na-K-ATPase Activity

Na-K-ATPase activity in the MAL and OMCD was determined by coupling the hydrolysis of adenosine triphosphate (ATP) to the production of a fluorescent nucleotide, as described previously (17, 26, 27). Briefly, dissected MAL or OMCD was stored in 10 μ l of solution and permeabilized by freeze-thawing. Ten μ l of incubation medium was added to each sample. The final composition of the incubation medium (pH 7.0) was as follows (in mmol/l): 120 NaCl, 10 KCl, 100 imidazole, 5 MgCl_2 , 1 ethylene glycol bis(2-aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), 10 Na_2ATP , 20 phosphoenol-pyruvate, 2 ascorbic acid, 2 nicotinamide adenine dinucleotide (NADH), 2.8 U/ml pyruvate kinase, and 4 U/ml lactate dehydrogenase. NaCl and KCl were replaced by 130 mmol/l choline Cl, and 1 mmol/l ouabain was added for the assay of ouabain-insensitive ATPase activity. The total ATPase activity was determined in the absence of ouabain, and the ouabain-insensitive ATPase activity was determined in the presence of 1 mmol/l ouabain. Na-K-ATPase activity was determined as the ouabain-sensitive ATPase activity (total ATPase activity minus ouabain-insensitive ATPase activity). Na-K-ATPase activity was also measured in a hypertonic (490 mOsm/kg H_2O) medium. One hundred mmol/l NaCl was added to the incubation medium. NaCl was replaced with 230 mmol/l choline Cl for the measurement of ouabain-insensitive ATPase activity in the hypertonic medium.

RT-PCR

RT-PCR was performed using a cDNA synthesis kit and a PCR master kit as described previously (17, 24). The sense and antisense primers for the α -1 subunit of Na-K-ATPase were defined by bases 1719–1738 (5'-GAACCCAAACG CATCGGAGC-3') and 2268–2289 (5'-GTACCGCAAAAT GTCATCCAGC-3'), respectively (11). The cDNA amplification product was predicted to be 571 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe for Southern blot analysis. The oligonucleotide (sense) included bases 1822–1842 (5'-CCCCTG GACGAAGAGCTGAAG-3') of the cDNA positioned between the sense and antisense primers.

The sense and antisense primers for the β -1 subunit of Na-K-ATPase were defined by bases 517–537 (5'-GAGAA GAAGGAGTTTTTGGGC-3') and 1209–1228 (5'-GCAGG TACTTGGGCTGCAGG-3'), respectively (12). The cDNA amplification product was predicted to be 711 bp in length. A third oligonucleotide (sense) for Southern blot analysis included bases 1049–1069 (5'-GAGACTTACCCTCTGAC GATG -3') of the cDNA. The sense and antisense primers for the β -2 subunit of Na-K-ATPase were defined by bases 74–93 (5'-TTTCCCTGCTTCTTTGCACC-3') and 510–529 (5'-TTCCACACGAACCTCCTTCCA-3'), respectively (8). The cDNA amplification product was predicted to be 456 bp in

length. A third oligonucleotide (sense) for Southern blot analysis included bases 1049–1069 (5'-GAGACTTACCCTCT GACGATG -3') of the cDNA.

RT-PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as a positive control. The sense and antisense primers were defined by bases 506–525 (5'-TCCCT CAAGATTGTCAGCAA-3') and 794–813 (5'-AGATCCA CAACGGATACATT-3'), respectively (28). The cDNA amplification product was predicted to be 308 bp in length.

PCR was performed for 28 cycles for the analysis of the α -1 and GAPDH mRNAs, and for 34 cycles for the analysis of the β -1 and β -2 mRNAs.

PCR Product Analysis

After ethanol-precipitation, the PCR products were size-fractionated by agarose gel electrophoresis. For the Southern blot analysis, gels were denatured, neutralized, and blotted onto a nitrocellulose filter (Funakoshi, Tokyo, Japan). The synthetic oligonucleotide probes were end-labelled with digoxigenin (DIG), as previously described. Pre-hybridization/hybridization washes were also performed as previously described (17).

Western Blot Analysis

The expression of the α -1, β -1, and β -2 subunits of Na-K-ATPase was examined using the membrane fraction from the outer medulla and Western blot analysis, as previously described (17, 24). The antibodies against the α -1, β -1, and β -2 subunits of Na-K-ATPase were obtained from Upstate Biotechnology (Lake Placid, USA) and were diluted to 1:1000 with buffer.

AVP-Dependent cAMP Generation

AVP-dependent cAMP generation was measured as previously described (29, 30). Microdissected MAL or OMCD was incubated with vasopressin (10^{-9} or 10^{-7} mol/l) or vehicle for 3 min at 37°C. To examine the acute effects of hyperosmolality, MAL and OMCD samples were incubated with AVP in isotonic (290 mOsm/kg H_2O) or hypertonic (490 mOsm/kg H_2O) medium. The hypertonic medium was created by adding 100 mmol/l NaCl to the isotonic medium. To determine the chronic effects of hyperosmolality, the MAL and OMCD from dehydrated rats were used. In some experiments, calphostin C, a potent protein kinase C inhibitor, was used to examine the role of protein kinase C on AVP-dependent cAMP generation. After 3 min, the reaction was stopped by the addition of trichloroacetic acid (TCA). Samples were kept at -20°C until the assay of cAMP content. TCA was extracted by the addition of water-saturated ether for 4 times. After the evaporation of the aqueous phase, the cAMP content of the samples was measured. The cAMP enzymeimmunoassay biotrak (EIA) system (Amersham) was used for the assay,

Fig. 1. Na-K-ATPase activity in the MAL and OMCD from control and dehydrated rats. Na-K-ATPase activity in the MAL and OMCD from control and dehydrated rats was measured in isotonic or hypertonic medium. Na-K-ATPase activity in the MAL from control rats was increased by incubation in the hypertonic medium. The Na-K-ATPase activity in the MAL from dehydrated rats was significantly higher than that in control rats, and incubation in hypertonic medium further increased this activity. In contrast, Na-K-ATPase activity in the OMCD was not stimulated either by dehydration or by acute exposure to hypertonic medium. C, control; D, dehydration. The values 290 and 490 indicate the osmolality of the incubation medium (mOsm/kg H₂O). The data represent mean $\pm$ SEM from 4–6 experiments. * $p < 0.05$ vs. control in isotonic or hypertonic solution (290 or 490 mOsm/kg H₂O, respectively), # $p < 0.05$ vs. control in isotonic medium, ## $p < 0.05$ vs. dehydration in isotonic solution (290 mOsm/kg H₂O).

with a range of 2–128 fmol/well.

Statistics

Four to eight measurements were averaged in order to obtain a single value for each experimental condition. The results are shown as means $\pm$ SEM. Statistical analysis was carried out by Student's *t*-test or by analysis of variance with multiple comparisons of Fisher's Protected Least Significant Difference method. Values of $p < 0.05$ were considered statistically significant.

Results

The Effect of Dehydration on Urine and Serum Osmolality

Urine and serum osmolality were measured in control and dehydrated rats. Urine osmolality in dehydrated rats was significantly higher than that in control rats (1,562 $\pm$ 183 and

Fig. 2. Changes in expression of α -1 mRNA of Na-K-ATPase in rats subjected to dehydration. The effect of dehydration on the expression of mRNA encoding the α -1 subunit of Na-K-ATPase was investigated. The upper bands show ethidium bromide-stained agarose gel. The lower bands represent an autoradiograph of the corresponding Southern blot analysis with a specific oligonucleotide probe. α -1 mRNA expression in the sMAL from control rats was considered as 1.0, and the levels of other mRNAs were expressed relative to that value (1.0). The graph indicates the results (mean $\pm$ SEM) from five experiments. α -1 mRNA expression, both in the sMAL and IMAL, was significantly stimulated by dehydration. In contrast, the corresponding types of expression in the OMCD did not change with dehydration. C, control; D, dehydration. * $p < 0.05$ vs. control.

785 $\pm$ 82 mOsm/kg H₂O in dehydrated and control rats, respectively, $n = 6$, $p < 0.05$). Serum osmolality in dehydrated rats was also slightly higher than that in control rats (324 $\pm$ 4 and 302 $\pm$ 3 mOsm/kg H₂O in dehydrated and control rats, respectively, $n = 6$, $p < 0.05$).

The Effect of Hypertonicity on Na-K-ATPase Activity in the MAL

Na-K-ATPase activity in the MAL from dehydrated rats, as measured in isotonic medium, was significantly higher than that of the controls (59.2 $\pm$ 4.3 and 22.2 $\pm$ 1.6 pmol/mm/min, respectively, $n = 5$, Fig. 1). The Na-K-ATPase activity in MAL measured in the hypertonic medium was higher than that in the isotonic medium, both in dehydrated and control rats (84.0 $\pm$ 5.2 and 34.0 $\pm$ 1.7 pmol/mm/min measured in hypertonic medium in dehydrated and control rats, respectively, $n = 5$, Fig. 1). Na-K-ATPase activity in the OMCD did

Fig. 3. Changes in expression of β -1 (upper) and β -2 (lower) mRNAs of Na-K-ATPase by dehydration. The effects of dehydration on the expression of mRNA encoding the β -1 and β -2 subunits of Na-K-ATPase were investigated. The levels of β -1 and β -2 mRNA expression in the sMAL from control rats were considered as 1.0, and the levels of other subunits were expressed relative to that value. β -1 and β -2 mRNA expression, both in the sMAL and the IMAL, was stimulated by dehydration, as was the case with α -1 mRNA expression. In contrast, the expression in the OMCD were not altered by dehydration. The data represent mean $\pm$ SEM from five experiments. C, control; D, dehydration. * $p < 0.05$ vs. control.

not differ in the control and dehydrated rats in isotonic medium (8.5 ± 0.9 and 9.2 ± 0.9 pmol/mm/min in control and dehydrated rats, respectively) or in hypertonic medium (9.4 ± 1.0 and 8.6 ± 0.9 pmol/mm/min in control and dehydrated rats, respectively). Thus, dehydration or acute exposure to hypertonic medium stimulated Na-K-ATPase activity in the MAL, but not in the OMCD.

Changes in α -1, β -1, and β -2 mRNAs of Na-K-ATPase by Dehydration

The effect of dehydration on mRNA expression encoding the α -1, β -1, and β -2 subunits of Na-K-ATPase was investigated. The expression of the α -1 (Fig. 2), β -1 (upper panel in Fig. 3), and β -2 (lower panel in Fig. 3) mRNAs in the sMAL and

Fig. 4. Effects of dehydration on the expression of α -1, β -1, and β -2 subunits in the outer medulla. The effects of dehydration on Na-K-ATPase subunit protein expression were investigated by Western blot analysis using the membrane fraction of the outer medulla from control and dehydrated rats. The upper bands show typical examples of the Western blot analysis gel. The lower graph shows the results (mean $\pm$ SEM) of 5 experiments. The expression of each subunit in control rats was considered as 1.0, and the expression in dehydrated rats was expressed relative to that value. The expression of the α -1, β -1, and β -2 subunits was observed at 100, 50–60, and 62 kD, respectively, as previously reported (17). No expression of α -2 or α -3 protein was observed in the membrane fraction from the outer medulla. β -1 expression in the outer medulla was stimulated by dehydration, whereas the levels of expression of the α -1 and β -2 subunits were not altered by dehydration. C, control; D, dehydration.

IMAL was stimulated by dehydration. In contrast, the corresponding levels of expression in the OMCD did not change with dehydration (Figs. 2, 3). Within the MAL, both the sMAL and IMAL exhibited the same tendency with dehydration. GAPDH expression did not differ between control and dehydration (data not shown).

Effects of Dehydration on α -1, β -1, and β -2 Subunit Expression in the Outer Medulla

Next, the effect of dehydration on protein expression was investigated. The expression of the α -1, β -1, and β -2 subunits was observed at 100, 50–60, and 62 kD, respectively, as previously reported (17) (Fig. 4). No expression of the α -2 and

Fig. 5. Arginine vasopressin (AVP)-dependent cAMP generation in the MAL (A) and OMCD (B). The MAL and OMCD from control and dehydrated rats were incubated with AVP for 3 min. In this experiment, 10^{-9} or 10^{-7} mol/l AVP was used. The results shown here indicate mean $\pm$ SEM from 4 experiments. AVP-dependent cAMP generation in the MAL was significantly lower than that in the OMCD. The incubation of MAL from control rats in hypertonic medium (490 mOsm/kg H_2O) increased the levels of AVP-dependent cAMP generated. Higher levels of AVP-dependent cAMP were generated in the MAL from dehydrated rats than in that of the controls, but incubation in hypertonic medium did not lead to any further increase in the accumulation of AVP-dependent cAMP in the MAL. Incubation of the OMCD from control rats in hypertonic medium (490 mOsm/kg H_2O) led to an increase in the generation of AVP-dependent cAMP, as was also observed in the MAL. In contrast, the generation of AVP-dependent cAMP in the OMCD was stimulated by dehydration, and further stimulation was observed with incubation of the samples in hypertonic medium. The same results were observed with 10^{-9} or 10^{-7} mol/l AVP. Basal cAMP generation in the MAL and OMCD (0.2–0.4 fmol/mm/3 min) in either isotonic or hypertonic medium was subtracted from the levels of generation of AVP-dependent cAMP. C, control; D, dehydration. The values 290 and 490 indicate the osmolality of the incubation medium (mOsm/kg H_2O). * $p < 0.05$ vs. isotonic (290 mOsm/kg H_2O) either in control or those from rats subjected to dehydration; # $p < 0.05$ vs. control and isotonic, ## $p < 0.05$ vs. control and hypertonic.

α -3 subunits was observed in the membrane fraction from the outer medulla. β -1 expression in the outer medulla was stimulated by dehydration, whereas the levels of expression of α -1 and β -2 did not change with dehydration

AVP-Dependent cAMP Generation in the MAL and OMCD

In this experiment, 10^{-9} or 10^{-7} mol/l AVP was used. Levels of AVP (10^{-9} or 10^{-7} mol/l)-dependent cAMP generation in the MAL were significantly lower than those in the OMCD (Fig. 5A and B). Levels of AVP-dependent cAMP generation in the MAL from control rats in hypertonic medium (490 mOsm/kg H_2O) were significantly higher than those in isotonic medium (Fig. 5A). Levels of AVP-dependent cAMP generation in the MAL from dehydrated rats were higher than those from control rats, but incubation in hypertonic medium did not further increase the AVP-dependent cAMP accumulation. AVP-dependent cAMP generation in the OMCD was stimulated by hypertonicity and by dehydration, as was also observed in the MAL samples (Fig. 5B). However, AVP-

dependent cAMP generation in the OMCD from dehydrated rats was further stimulated by incubation in hypertonic medium.

To determine the mechanisms responsible for the differences in AVP-dependent cAMP in the MAL and OMCD, the role of protein kinase C was investigated using calphostin C, a potent protein kinase C inhibitor. The addition of 1.27×10^{-7} mol/l calphostin C did not affect the generation of AVP-dependent cAMP, either in the MAL or in the OMCD (Fig. 6A and B).

Discussion

Dehydration is known to stimulate the renin-angiotensin-aldosterone system and to increase plasma levels of AVP, which occurs in order to restore the decreased circulating blood volume (1, 21). Angiotensin II stimulates sodium reabsorption in proximal tubules. In contrast, aldosterone and AVP as well as dopamine, insulin, and glucocorticoid regulate Na-K-ATPase activity in the distal nephron (3, 4). Osmolality is also a regulator of Na-K-ATPase in the renal medulla.

Fig. 6. Effects of calphostin C on AVP-dependent cAMP generation in the MAL (A) and OMCD (B). The role played by protein kinase C in hyperosmolality-induced cAMP generation was investigated. MAL and OMCD from control and dehydrated rats were incubated with AVP in either isotonic or hypertonic medium with (+) or without (-) calphostin C for 3 min. The data show mean $\pm$ SEM from 4–5 experiments. The presence of 1.27×10^{-7} mol/l calphostin C, a potent protein kinase C inhibitor, did not affect 10^{-9} mol/l AVP-dependent cAMP generation either in the MAL (A) or the OMCD (B). C, control; D, dehydration. The values 290 and 490 indicate the osmolality of the incubation medium (mOsm/kg H_2O).

AVP stimulates hyperosmolality in the renal medulla in dehydration. Thus, AVP regulates Na-K-ATPase activity both directly and indirectly. Therefore, we investigated the effects of hyperosmolality on Na-K-ATPase and AVP-dependent cAMP generation in the MAL and OMCD. Our findings revealed that Na-K-ATPase activity is increased in the MAL, but not in the OMCD by dehydration. Incubation in hypertonic medium increased Na-K-ATPase activity in the MAL, but not in the OMCD. It was also demonstrated that acute and chronic hyperosmolality led to increases in Na-K-ATPase activity in the MAL, but not in the OMCD. It was of note that Na-K-ATPase activity in the MAL is upregulated in response to acute increases in osmolality. Na-K-ATPase is one known route for the release of Na from the MAL, and the activation of Na-K-ATPase provides a driving force for NaCl reabsorption *via* NKCC2 (20). Therefore, Na-K-ATPase is involved in the acute regulation of sodium transport in the MAL. Since AVP-dependent cAMP generation increases in response to acute increases in osmolality in the MAL, increased protein kinase A activity is thought to stimulate Na-K-ATPase activity (4). Our findings (Fig. 6) demonstrate that protein kinase C does not participate in the hyperosmolality-stimulated generation of AVP-dependent cAMP. Thus, AVP regulates both short- and long-term Na-K-ATPase activity directly *via* activation of protein kinase A and indirectly resulting from hyperosmolality in the MAL.

Although the expression of the α -1, β -1, and β -2 subunits of Na-K-ATPase mRNA was stimulated, Western blot analysis revealed that stimulation of the β -1 subunit of Na-K-

ATPase may participate in the stimulation of Na-K-ATPase activity in the MAL with dehydration. The increase in expression of the α -1 subunit was reported in ouabain-hypertensive rats (31). We did not examine the effects of hyperosmolality on the γ -subunit of Na-K-ATPase due to difficulties in obtaining an effective antibody against that subunit. The γ -subunit of Na-K-ATPase is reported to be present in the MAL, but not in the OMCD (18, 19). The differences in the distribution of the γ -subunit may play some role in this context.

The MAL and OMCD are located in the outer medulla. Therefore, the osmolality in the interstitium around the MAL and OMCD should be almost same. However, the regulation of Na-K-ATPase by hyperosmolality differed between the MAL and OMCD in our experiments. We reported that Na-K-ATPase in the IMCD is down-regulated by dehydration, primarily due to the decrease in the expression of the α -1 and β -1 subunits (17). AVP has two types of receptors in the distal nephron segments (32). Although the main receptor responsible for the antidiuretic action of vasopressin is the V2 receptor, we reported that the V1a receptor might regulate V2 activity in the collecting ducts (33). The V1a receptor is most abundant in the collecting ducts. In contrast, few V1a receptors are present in the MAL. This difference in the localization of these two types of vasopressin receptors in the MAL and OMCD may play some role in the differences in Na-K-ATPase activity under conditions of dehydration.

Therefore, we investigated the effects of AVP on cAMP generation in the MAL and OMCD. Acute exposure to a hypertonic medium stimulated AVP-stimulated cAMP gener-

ation, both in the MAL and the OMCD. However, AVP-stimulated cAMP generation in the MAL from dehydrated rats was not stimulated by acute exposure to the hypertonic medium. Interestingly, the generation of AVP-stimulated cAMP in the OMCD from dehydrated rats was further stimulated by acute exposure to the hypertonic medium. Calphostin C, a potent inhibitor of protein kinase C, did not alter the levels of AVP-stimulated cAMP generated in either the MAL or the OMCD, thus suggesting that protein kinase C does not participate in this difference between the MAL and OMCD. The adaptation of the OMCD to hypertonic stress appears to be much greater than that in the MAL. The absence and presence of water channels in the MAL and OMCD, respectively, may play important roles in this difference in adaptation. MAL and CAL are completely unique segments and they do not pass water and ammonia (NH_3) (34). AVP-sensitive water channels in the collecting ducts may facilitate rapid volume regulatory decreases in order to counter hypertonic stress. The accumulation of organic osmolytes also participates in this process. Since the OMCD is an important segment for water reabsorption, the capacity for acute adaptation must be preserved, even after a period of chronic dehydration. The adaptation to hypertonic stress is more rapid, but lower in capacity in the MAL than in the OMCD. The adaptive capacity in the OMCD remains, even after a period of continued chronic hypertonic stress. Since the collecting duct is the final segment in the nephron, such an adaptive capacity must be retained for urine concentration, even after sustained dehydration.

We also examined differences between the sMAL and lMAL in terms of the stimulation of Na-K-ATPase mRNA expression due to hyperosmolality. The long-loop nephron plays a more important role in the concentration of urine than does the short-looped nephron (25, 35). Our results revealed that there is no difference in Na-K-ATPase mRNA expression between the sMAL and lMAL. Previously, we reported that Na-K-ATPase activity in the lMAL is slightly higher than that in the sMAL (25). However, the regulation of Na-K-ATPase activity in the sMAL and lMAL appears to take place in a similar manner.

Na-K-ATPase activity is increased in some models of hypertension. The activation of Na-K-ATPase has been reported in spontaneously hypertensive rats and in rats with obesity-induced hypertension (36, 37). Since the induced subunit was different from that examined here, the mechanisms of stimulation may vary according to the subunit examined (31). The proximal tubules are the main site of activation and sodium retention in such hypertensive models. Such regulation of sodium reabsorption by Na-K-ATPase in the proximal tubules plays a key role in the long-term regulation of the sodium balance. Although sodium reabsorption in the MAL is less extensive than that in the proximal tubules, the rapid upregulation of Na-K-ATPase activity induced by dehydration and vasopressin demonstrates the importance of Na-K-ATPase activity in the short-term regulation of the sodium

balance.

In summary, our findings demonstrate that Na-K-ATPase activity in the MAL, but not in the OMCD, was stimulated by acute and chronic hyperosmolality. Although the expression of the α -1, β -1, and β -2 mRNAs was stimulated by dehydration in the MAL but not in the OMCD, only the expression of the β -1 subunit protein was increased in the outer medulla, suggesting that the β -1 subunit plays a key role in the regulation of Na-K-ATPase activity in the MAL. The generation of AVP-dependent cAMP was stimulated by dehydration, both in the MAL and the OMCD. However, the generation of AVP-dependent cAMP was further stimulated by acute exposure to a hypertonic medium in the OMCD, but not in the MAL. These results suggest that Na-K-ATPase activity is upregulated by hyperosmolality (and probably by AVP) in both the short- and long-term in the MAL, but not in the OMCD.

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