Original Article

Acute Regulation of the Epithelial Sodium Channel Gene by Vasopressin and Hyperosmolality

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The amiloride-sensitive epithelial sodium channel (ENaC) plays a key role in sodium reabsorption in the collecting ducts. We examined ENaC mRNA distribution along the nephron and acute effects of vasopressin and hyperosmolality on ENaC mRNA expression. ENaC α , β , and γ mRNA expressions were observed in cortical, outer medullary and initial inner medullary collecting ducts (CCD, OMCD and iIMCD, respectively). ENaC α mRNA expression was also observed in medullary and cortical thick ascending limbs (MAL and CAL, respectively), while ENaC β and γ mRNA expressions were not observed. Furthermore, ENaC α mRNA expression in MAL but not in collecting ducts was stimulated by acute exposure to arginine vasopressin (AVP), 8-(4-chlorophenylthio) (CPT)-cAMP and hyperosmolality. However, the physiological significance of these effects is not known, since ENaC protein is reported to be absent in MAL. These data suggest that ENaC α mRNA expression in MAL but not in collecting ducts is acutely regulated by AVP and hyperosmolality. The absence of stimulation of ENaC β and γ mRNA expression in collecting ducts suggests the physiological significance of ENaC α and γ mRNA for acute regulation by vasopressin. Determining the physiological significance of the acute effect of vasopressin in MAL will require further investigations. (*Hypertens Res* 2003; 26: 629–634)

Key Words: vasopressin, amiloride-sensitive epithelial sodium channel, hyperosmolality

Introduction

The main role of the kidney is to maintain body fluid homeostasis. Arginine vasopressin (AVP) plays a key role in urine concentration (1). AVP has two types of receptors in the kidney, V1a and V2 receptors (2). V1a receptors mediate the vasopressor effects of AVP, and V2 receptors mediate the antidiuretic action of AVP (3). V1a receptors also participate in regulation of the V2 receptor-mediated diuretic action of AVP (3, 4). V2 receptors regulate many channels and transporters, such as aquaporin 2, Na-K-2Cl cotransporters (NKCC1 and 2), chloride channels, and urea transporters in

the distal nephron. V2 receptors act as stimulatory G protein (Gs) and stimulate adenylate cyclase (5). Vasopressin stimulates Na and water reabsorption in the distal nephron (6). Sodium reabsorptions in the distal nephron are caused by NKCC2 in the thick ascending limbs, thiazide-sensitive Na-Cl cotransporter in the distal convoluted tubules, and amiloride-sensitive epithelial sodium channel (ENaC) in the collecting ducts (7, 8). ENaC is composed of three subunits, α , β and γ (9, 10). ENaC is activated by serine proteases such as trypsin and prostatic (11, 12). The genetic disturbance in ENaC is known to cause Liddle syndrome, which is characterized by hypertension, hypokalemia and metabolic alkalosis (13). Studies investigating genetic variants, including ENaC, and essential hyper-

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tension have been performed (14–16).

Sodium absorption through ENaC is regulated by aldosterone and possibly AVP (7, 8). Although it is evident that vasopressin has chronic effects on ENaC, it remains uncertain whether it also exerts acute effects on ENaC (17, 18). Sodium reabsorption through NKCC2 participates in the formation of hyperosmolality in the medullary interstitium, which in turn results in water absorption in the collecting ducts. AVP regulates NKCC2 and subsequently medullary hyperosmolality. However, it is not known whether hyperosmolality influences ENaC expression.

The purpose of the present study was to investigate whether AVP or hyperosmolality acutely influence ENaC mRNA expression. We used competitive polymerase chain reaction (PCR) in our investigation.

Methods

Materials

AVP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP), collagenase, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). The DNA synthesis kit and PCR master were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Vanadyl ribonucleotide complex (VRC) was from Gibco (Gaithersburg, USA).

Renal Tubule Microdissection

Microdissection of renal tubule segments was performed as described previously (4, 19). In brief, 4-week-old male Sprague-Dawley (SD) rats were anesthetized by pentobarbital sodium (50 mg/kg). The kidney was perfused *in vivo* initially with 10 ml of ice-cold dissection solution (solution A (mmol/l): 130 NaCl, 5 KCl, 1 Na₂H₂PO₄, 1 MgSO₄, 1 Ca lactate, 2 Na acetate, 5.5 glucose and 10 Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) (pH 7.4 by NaOH)) and then with 10 ml of the same solution A containing 1 mg/ml collagenase and 1 mg/ml bovine serum albumin (solution B). The left kidney was removed, and coronal sections were transferred into tubes containing 3 ml of solution B containing 150 μ l VRC, a potent RNase inhibitor. The tubes were incubated for 30 min at 37 °C in a shaking water bath. The solutions were bubbled with 100% oxygen during the incubation. Next, tissues were transferred to a dissection dish that contained 10 mmol/l VRC. Two-millimeter lengths of tubules were microdissected for each sample. Microdissected segments were the medullary thick ascending limb (MAL), the cortical thick ascending limb (CAL), and the cortical, outer medullary and inner medullary collecting ducts (CCD, OMCD, and IMCD, respectively). In some experiments, long- and short-looped MAL were dissected (IMAL and sMAL, respectively). The IMCD was divided into an initial and a terminal part (iIMCD and tIMCD, respec-

tively). Microdissected tubules were washed with solution A in a separate wash dish.

Tubules Incubation Study

The tubules were transferred into tubes that contained various solutions for the incubation studies. The effects of AVP, CPT-cAMP, a membrane permeable cAMP analog, and hyperosmolality were examined by incubation studies. For the examination of the effect of hyperosmolality, NaCl was added to solution A. Hyperosmotic solutions of 490 mOsm/kgH₂O were made. AVP and CPT-cAMP were dissolved in solution A. Sample tubes were incubated for 1 h at 37 °C in a shaking water bath. After incubation, tubes were centrifuged and the supernatant was aspirated.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Reverse transcription (RT) was performed by adding 3.5 μ l of 2% Triton X, containing 0.4 U/ml of RNase-inhibitor and 11 mmol/l dithiothreitol (DTT), and 4.4 μ l of RT master mix. The reaction tubes were incubated at 42 °C for 60 min in the block incubator. The reaction was stopped by heating at 90 °C for 5 min, and then the reaction tubes were placed on ice until the PCR studies were performed.

Specific primers for ENaC α , β , and γ mRNA were designed. The sequences of sense and antisense ENaC α mRNA primers were defined by bases 1353–1372 (5'-TGGT AGCGATGTCCCGGTCA-3') and bases 1980–1999 (5'-AG GAGGAAGCTGGAGTGGAG-3'), respectively (9). The sequences of sense and antisense ENaC β mRNA primers were defined by bases 1214–1233 (5'-TCCAGGCCTGCCTTCAT TCC-3') and bases 1813–1832 (5'-ACACAGTTGGTGTGG GCCTC-3'), respectively (10). The sequences of sense and antisense ENaC γ mRNA primers were defined by bases 274–293 (5'-TCACGCTAACTGCAGTGGCC-3') and bases 815–834 (5'-TCTCTAGAGGCACCTGTGCC-3'), respectively (10). The predicted sizes of the PCR products of ENaC α , β , and γ were 647, 619, and 561 bp, respectively.

Competitive PCR

ENaC mRNA expressions were quantified using competitive PCR. The DNA competitor for the competitive PCR was synthesized using overlap extension PCR as described previously (4, 19). The inner antisense and inner sense primers for ENaC α were defined by bases 1511 to 1530 (5'-AGATGGC CACCGTGAGTAGCGCAATAGCCCCAGGAGCTCT-3') and bases 1628 to 1647 (5'-AGAGCTCCTGGGGCTATTG CGTACTCACGGTGGCCATCT-3'), respectively (the complementary sequence added to the 5' tail is underlined) (9). The inner antisense and inner sense primers for ENaC β were defined by bases 1521 to 1540 (5'-AATGAGGCACAGCAC CGAGCTACCCTTCCTGCTCAGGGTG-3') and bases 1661

Fig. 1. ENaC α , β , and γ mRNA expression along the nephron. They were investigated using RT-competitive PCR. ENaC α , β , and γ mRNA expression in each nephron segment was compared with that in CCD. CCD showed the highest expressions of ENaC α , β , and γ mRNA. A low expression of ENaC α mRNA but not ENaC β or γ mRNA was observed in the thick ascending limbs. Glm, glomerulus; PCT, proximal convoluted tubule; MAL, medullary thick ascending limb; IMAL, long-looped MAL; sMAL, short-looped MAL; CAL, cortical thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; iIMCD, initial IMCD; tIMCD, terminal IMCD.

Fig. 2. Effects of vasopressin (AVP) on ENaC α mRNA expression in MAL. The microdissected MAL was incubated with various doses of vasopressin in isotonic medium (290 mOsmol/kgH₂O) for 1 h at 37°C. Top: A typical example of the ethidium bromide-stained gel. Upper and lower bands show the PCR product of ENaC α mRNA expression mRNA (647 bp) and the competitor (550 bp), respectively. Bottom: ENaC α mRNA expression in MAL after the incubation with vasopressin. ENaC α mRNA expression was stimulated by low-dose AVP (10^{-12} mol/l) and inhibited by high-dose AVP (10^{-6} mol/l). n = 5, * $p < 0.05$ vs. control (without vasopressin).

to 1680 (5'-CACCCCTGAGCAGGAAGGGTAGCTCGGTGCTGTGCCTCATT-3'), respectively (10). The inner anti-sense and inner sense primers for ENaC γ were defined by bases 521 to 540 (5'-TTTCCGCTTCCGGCCAGTGACCAA

Fig. 3. Effects of vasopressin (AVP) on ENaC α mRNA expression in CCD. The microdissected CCD was incubated with various doses of vasopressin for 1 h at 37°C. ENaC α mRNA expression in CCD was not changed by the incubation with vasopressin. n = 5.

Fig. 4. Effects of vasopressin (AVP) on ENaC α mRNA expression in OMCD. The microdissected OMCD was incubated with various doses of vasopressin for 1 h at 37°C. ENaC α mRNA expression in OMCD was not changed by vasopressin. n = 6.

CCATGCCGTCCACCTTGGTCACTGGCCGGAAGCGG AAA-3'), respectively (10). The predicted size of the competitor of ENaC α , β , and γ was 550, 499, and 480 bp, respectively.

Ethidium Bromide Staining and Southern Blotting

The PCR products were ethanol precipitated and electrophoresed in 2% agarose gel in Tris-acetate/EDTA (TAE). PCR products were visualized by ethidium bromide staining. For quantitative measurements of ENaC mRNA expressions, the intensity of the bands was measured using a densitometer (Atto, Tokyo, Japan). TAE buffer was composed (in mmol/l) of 40 Tris and 1 ethylenediamine tetraacetic acid (EDTA) (pH 8.3 by acetic acid).

Fig. 5. Effects of CPT-cAMP on ENaCα mRNA expression in MAL. The microdissected MAL was incubated with various doses of CPT-cAMP for 1 h at 37°C. Top: A typical example of the ethidium bromide-stained gel. Upper and lower bands show the PCR product of ENaCα mRNA expression mRNA (647 bp) and the competitor (550 bp), respectively. Bottom: ENaCα mRNA expression in MAL after incubation with CPT-cAMP. ENaCα mRNA expression was stimulated by low-dose CPT-cAMP (10^{-12} mol/l) and inhibited by high-dose CPT-cAMP (10^{-4} mol/l). CPT-cAMP showed dual effects on ENaCα mRNA expression in MAL, just as did AVP. $n = 4$, * $p < 0.05$ vs. control (without CPT-cAMP).

Statistics

Results are expressed as the mean $\pm$ SEM. Statistical analysis was performed using Student's *t*-test for two groups or analysis of variance (or Kruskal Wallis analysis) followed by Dunnett's multiple comparison for more than three groups, as appropriate. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

ENaC α , β , and γ mRNAs Distribution along the Nephron

Quantitative examination of ENaCα, β, and γ mRNA expression was performed using RT-competitive PCR. Figure 1 shows the distribution of ENaC mRNA expressions. The highest expressions of ENaCα, β, and γ mRNAs were observed in CCD. Although ENaCβ and γ mRNA expressions were observed only in CCD, OMCD and iIMCD, ENaCα expression was also seen in MAL, CAL and tIMCD. Among the thick ascending limbs, CAL showed higher levels of ENaCα mRNA expression. ENaCα, β, and γ mRNA expressions were not observed in the glomeruli or proximal tubules.

Fig. 6. Effects of hyperosmolality on ENaCα mRNA expression in MAL. MAL was incubated in isotonic (290 mOsm/kgH₂O) or hypertonic (490 mOsm/kgH₂O by adding NaCl) medium for 30–120 min. Top: A typical example of the ethidium bromide-stained gel. Upper and lower bands show the PCR product of ENaCα mRNA expression mRNA (647 bp) and the competitor (550 bp), respectively. Bottom: ENaCα mRNA expression in MAL after the incubation in isotonic or hypertonic medium. ENaCα mRNA expression was transiently stimulated by hyperosmolality. $n = 5$, * $p < 0.05$ vs. isotonic medium.

Acute Effects of AVP on ENaC mRNA Expression

The acute effect of AVP *in vitro* was examined by the incubation of MAL, CCD or OMCD in isotonic medium for 1 h. Low-dose AVP (10^{-12} mol/l) stimulated ENaCα mRNA expression, while high-dose AVP (10^{-6} mol/l) decreased it (Fig. 2) in MAL. Such effects were not observed in CCD (Fig. 3) or OMCD (Fig. 4).

Acute Effects of CPT-cAMP on ENaC mRNA Expression in MAL

Since AVP affected ENaCα mRNA expression in MAL, the effects of CPT-cAMP, a membrane permeable cAMP analog, on ENaCα mRNA expression were examined. Low-dose CPT-cAMP (10^{-12} mol/l) stimulated ENaCα mRNA expression, whereas high-dose CPT-cAMP (10^{-4} mol/l) decreased it (Fig. 5).

Effects of Hyperosmolality on ENaC mRNA Expression

The effects of hyperosmolality induced by NaCl on ENaC mRNA expression were examined in MAL and OMCD. Incubation of MAL in hypertonic solution (490 mOsm/kgH₂O) stimulated ENaCα mRNA expression at 30 to 60 min (Fig. 6). After 120 min incubation, the expression returned to almost the control level. In contrast, hyperosmolality did not stimulate ENaCα mRNA expression in OMCD (Fig. 7).

Fig. 7. Effects of hyperosmolality on ENaC α mRNA expression in OMCD. OMCD was incubated in isotonic or hypertonic medium for 30–120 min. Top: A typical example of the ethidium bromide-stained gel. Upper and lower bands show the PCR product of ENaC α mRNA expression mRNA (647 bp) and the competitor (550 bp), respectively. Bottom: ENaC α mRNA expression in OMCD after the incubation in isotonic or hypertonic medium. ENaC α mRNA expression in OMCD was not changed by hyperosmolality. $n = 5$.

Discussion

Our study showed that ENaC α , β , and γ mRNAs were expressed in the distal nephron. It is quite interesting that ENaC α mRNA expression was observed in MAL and CAL, while ENaC β and γ mRNA expressions were not seen. Furthermore, ENaC α mRNA expression was stimulated by acute exposure to low doses of AVP and CPT-cAMP, and by hyperosmolality in MAL but not in collecting ducts. However, the physiological significance of these effects will need to be examined further.

About 60–70% of sodium reabsorption has been reported to take place in the proximal tubules. However, the distal nephron participates in the precise regulation of sodium reabsorption in the kidney (1). Vasopressin plays a major role in the regulation of sodium reabsorption in the distal nephron. In fact, V1a and V2 receptors of vasopressin are located in the distal nephron (2–5). Under normal conditions, sodium reabsorption in the collecting ducts accounts for only 2–3% of filtered sodium (1). However, AVP and aldosterone are thought to stimulate sodium reabsorption through ENaC (7, 8). Chronic effects of vasopressin on ENaC abundance and sodium transport have been reported (17, 18). In the present study, AVP did not show acute effects on ENaC α mRNA expression either directly or through hyperosmolality in the collecting ducts. Our findings are compatible with the report by Ecelbarger *et al.* that acute *in vivo* exposure to [deamino-Cys¹, D-Arg⁸]-vasopressin (dDAVP) led to an increase in both ENaC β and γ mRNA expression (17). They

observed a very small and acute effect of AVP on ENaC α mRNA expression. ENaC β and γ but not ENaC α subunit may play a key role in the acute stimulation by vasopressin. It is quite interesting that low-dose AVP, low-dose CPT-cAMP, and hyperosmolality stimulated ENaC α mRNA expression in MAL, since ENaC has been thought to be present only in collecting ducts. Our finding that AVP and CPT-cAMP showed the same biphasic effects on ENaC α mRNA expression in MAL are compatible with the idea that the effects of AVP on ENaC α mRNA expression are mediated by its second messenger, cAMP. The presence of ENaC α mRNA in CAL may be due to the presence of connecting tubules.

Although vasopressin has been reported to have an acute effect on the abundance of ENaC, cAMP has been reported to show no effect on sodium current in *Xenopus* oocytes that expressed rat ENaC (20). The substitution of a guinea pig ENaC α subunit for the rat ENaC α subunit coexpressed with the rat β and γ subunit resulted in the stimulation of sodium current by permeable cAMP (21). These data suggest the complexity of the mechanisms of sodium transport by ENaC. The regulation of the α subunit appears to be different from that of the β and γ subunits. Recently, a novel vasopressin-induced transcript was shown to induce downregulation of ENaC (22).

AVP and CPT-cAMP showed dual effects on ENaC α mRNA expression in MAL in our study. Dual effects of AVP on water permeability in collecting ducts have also been reported (23). The inhibition of water permeability by high-dose AVP is caused by the increase of intracellular calcium or by the stimulation of protein kinase C (PKC) (24). These data suggest that high-dose AVP may inhibit ENaC α mRNA expression by stimulating PKC. The same mechanisms could be responsible for the inhibition of ENaC α mRNA expression by high-dose CPT-cAMP, since the activation of protein kinase A by a cAMP analog has been shown to increase intracellular calcium and to stimulate PKC (25). It is also possible that high-dose CPT-cAMP may reduce the stability of ENaC α mRNA (26).

Hyperosmolality also did not change ENaC α mRNA expression in OMCD in our study, a finding that was also compatible with the report by Ecelbarger *et al.* (17). Although acute exposure to vasopressin caused an increase in ENaC α mRNA expression, water restriction did not cause any change in ENaC α mRNA expression in their study. This result indicates that hyperosmolality did not cause changes in ENaC α mRNA expression. They also reported that water restriction caused an increase in both ENaC β and γ mRNA expression. ENaC β and γ may thus be important for the acute regulation of ENaC by hyperosmolality.

In summary, ENaC β and γ mRNA expressions were observed only in collecting ducts, while ENaC α mRNA expression was seen also in MAL and CAL. Acute exposure to low doses of AVP and CPT-cAMP, and hyperosmolality caused the stimulation of ENaC α mRNA expression in MAL

but not in collecting ducts. The physiological significance of the changes of ENaC α mRNA expression in MAL should be examined further. The β and γ subunits of ENaC may play a key role in the acute regulation of sodium transport by vasopressin or hyperosmolality in collecting ducts.

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