

Modulation of renal CNG-A3 sodium channel in rats subjected to low- and high-sodium diets

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Abstract

In this work, we studied the mRNA distribution of CNG-A3, an amiloride-sensitive sodium channel that belongs to the cyclic nucleotide-gated (CNG) family of channels, along the rat nephron. The possible involvement of aldosterone in this process was also studied. We also evaluated its expression in rats subjected to diets with different concentrations of sodium or to alterations in aldosterone plasma levels. Total RNA isolated from whole kidney and/or dissected nephron segments of Wistar rats subjected to low- and high-sodium diets, furosemide treatment, adrenalectomy, and adrenalectomy with replacement by aldosterone were analyzed by the use of Western blot, ribonuclease protection assay (RPA) and/or reverse transcription followed by semi-quantitative polymerase chain reaction (RT-PCR). CNG-A3 sodium channel mRNA and protein expression, in whole kidneys of rats subjected to high- Na^+ diet, were lower than those in animals given a low-salt diet. Renal CNG-A3 mRNA expression was also decreased in adrenalectomized rats, and was normalized by aldosterone replacement. Moreover, a CNG-A3 mRNA expression study in different nephron segments revealed that aldosterone modulation is present in the cortical thick ascending loop (cTAL) and cortical collecting duct (CCD). This result suggests that CNG-A3 is responsive to the same hormone signaling as the amiloride sensitive sodium channel ENaC and suggests the CNG-A3 may have a physiological role in sodium reabsorption. © 2004 Elsevier B.V. All rights reserved.

Keywords: Aldosterone; CNG-A3; Sodium channel; Nephron; Sodium reabsorption; Kidney; mRNA; RPA; RT-PCR

1. Introduction

The extracellular fluid volume (ECFV) regulation is determined by the balance between ingestion and excretion of sodium, the most abundant ion in the extracellular compartment. Renal sodium excretion control is essential for sodium ion balance. Most of the sodium filtered in the glomeruli is reabsorbed along the nephron. In the proximal tubule, Henle's loop and distal tubule, sodium reabsorption is proportional to the filtered load [1]. On the other hand, in the renal collecting tubule, composed of the cortical, outer medullary and inner medullary segments, sodium reabsorp-

tion is highly regulated, in order to maintain body sodium and total ECFV [1]. The main sites of aldosterone action in the kidney are the thick ascending limb (TAL), the distal tubule (DT) and the collecting ducts (CD) [2], where aldosterone acts to increase sodium reabsorption. This hormone, released by the adrenal cortex, is dependent on the renin–angiotensin system that modulates the function and expression of different renal transporters [3–6] including the amiloride-sensitive channel ENaC [7,8].

The cyclic nucleotide-gated channel isoform A-three (CNG-A3) is a channel that is amiloride and L-*cis*-diltiazem sensitive that was originally cloned from bovine kidney [9]. This channel is a member of the cyclic nucleotide-gated (CNG) channel family that has a heteromultimer structure consisting of “principal” and “modulatory” subunits [10–12]. The principal subunits form functional channels when

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expressed alone in heterologous expression systems, whereas the modulatory subunits do not give rise to a cGMP activated current per se, but when coexpressed with principal subunits, they confer channel properties characteristic of native CNG channels such as increased sensitivity to cyclic nucleotides and channel blockers [13]. Although it is not clear if the CNG-A3 channel is also co-assembled with a modulatory subunit in the kidney, the cloning of A-three variants of CNG channel subunits in the kidney suggest that the functional properties of the CNG-A3 channel could be modulated by other CNG subunits in vivo [14]. It has been suggested that CNG-B1 forms functional hetero-oligomeric channels with CNG-A3 in vitro and may also do so in intact tissues [14].

CNG channels are expressed in several tissues. cDNA encoding the CNG-A3 cation channel have been cloned from retina cone cells, testis, colon, pineal gland, adrenal, heart, sperm, brain, and in renal epithelia [10,11,13]. Electrophysiological and biochemical approaches have demonstrated that CNG channels play a key role in sensory signal transduction including vision and smell [15–17]. When cDNA were isolated from several other tissues, it became evident what CNG channels may be involved in a variety of functions in addition to sensory signal transduction [13]. Functional transport assays suggest that these channels mediate transepithelial transport of calcium and sodium across the proximal and distal colon [18].

In the kidney, CNG-A3 channels share many biophysical and molecular properties of the retinal rod CNG-gated channel. However, unlike the retinal rod channel, one clone of the CNG channel in the kidney is inhibited by cyclic GMP and stimulated by increased calcium levels [13]. In different species, the CNG-gated channels have been identified in all nephron segments using electrophysiological methods [13]. Specifically in the inner medullary collecting duct, an amiloride-sensitive cation channel was shown to be involved in electrogenic sodium transport, which is inhibited by the atrial natriuretic peptide (ANP) via cyclic GMP [19]. These experimental results suggested that CNG-A3 might have an important physiological role in the renal function and regulation of ECVF, through the adjustment of sodium reabsorption along the nephron.

The aim of our work was to study the distribution of CNG mRNA along the nephron, focusing on the CNG-A3 principal subunit with particular attention to the expression in rats subjected to low- and high-sodium diets. We also wished to know whether this channel was regulated by aldosterone in a similar manner to ENaC.

2. Materials and methods

2.1. Animal preparation

The Ethics Committee from The Biophysics Institute, Federal University of Rio de Janeiro, previously approved

all procedures and protocols using animals mentioned in this manuscript.

2.1.1. Rats given a high- or low- Na^+ diet

Wistar male rats with 150–250 g body weight were divided into three groups. The first group (control) was maintained on a normal diet of 3 g/kg NaCl; the second group received a low-sodium diet of 0.02 g/kg NaCl and normal drinking water for 14 days (low- Na^+); the third group were fed a high-sodium diet containing 8 g/kg NaCl and drinking water with 9 g/l NaCl for 5 days (high- Na^+) [20–22]. To monitor the electrolyte balance and glomerular filtration rate (GFR), blood and urine samples were taken prior to sacrifice and, urine and serum concentrations of K^+ , Na^+ , Cl^- and creatinine were measured to allow the calculation of electrolytes fractional excretion (FE) and GFR in each experimental group as described before [23]. The urine was collected during the 24 h preceding the decapitation to determine the urinary flow. The electrolytes FE and the GFR were obtained from: $\text{FE}(\%) = [(U_x \times V) / (GFR \times P_x)] \times 100$ and, $GFR = (U_{cr} \times V) / P_{cr}$ relative to kg weight; where U_x : electrolyte urinary concentration (mg/ml), P_x : electrolyte plasma concentration (mg/ml), U_{cr} : creatinine urinary concentration (mg/ml), P_{cr} : creatinine plasma concentration (mg/ml) and, V : urine flow ($\text{ml} \times \text{min}^{-1}$).

2.1.2. Rats subjected to adrenalectomy

Male Wistar rats, weighing 150–250 g, were divided into three groups of four rats each for the aldosterone-induced mRNA expression studies. In group 1 (control) the animal were sham-operated. The rats of group 2 were adrenalectomized bilaterally (ADX), allowed to drink only 9 g/l NaCl solution and kept in this condition for 48 h. The rats of group 3 were bilaterally adrenalectomized and osmotic mini-pumps (Alzet 2001, Alza, Palo Alto, CA) delivering aldosterone (Sigma, St Louis, MO, USA) at 5 mg/100 g of body weight per day for 48 h were implanted (ADX+ALDO). Rats were anesthetized with pentobarbital sodium (5 mg/100 g body weight) for all procedures involving surgery. The replacement dose was chosen because it resembled the daily secretory rate in rats and results in plasma levels of aldosterone similar to those measured in awake, unstressed rats [24].

2.1.3. Rats given furosemide treatment

To study the effect of blocking transepithelial transport on the CNG-A3 mRNA expression in cortical thick ascending limbs (cTAL) we used male Wistar weighing 150–250 g given increasing doses of furosemide using the same protocol employed before [25]. The animals were divided into three groups of three rats each. The control group of animals (control) received 300 ml of 0.9% NaCl solution orally via a stomach tube. Two other groups of rats received two different concentrations of furosemide solution 10 mg/kg (F10) and 100 mg/kg (F100) orally (300 ml) via a

stomach tube. Furosemide orally at 100 mg/kg solution, but not at 10 mg/kg, is known to increase both plasma renin activity and aldosterone concentration in rats [25]. The animals were sacrificed 15 h after furosemide treatment and the kidneys were used for renal tubule dissection.

2.2. Isolation of total RNA

The kidneys were perfused with a Ringer's solution and then excised and kept at 0 °C. Thin slices of kidney were made, then the renal cortex and medulla were dissected apart. Isolation of the various rat nephron segments (3 mm each) was performed by the use of collagenase, as previously described [26,27]. These experiments were performed at least seven times for each segment dissected from different rats. The animals were prepared for surgery using pentobarbital anesthesia.

Total RNA was extracted from whole kidney and from the dissected renal cortex and medulla by the acid guanidinium thiocyanate-phenol-chloroform method [28]. The isolated RNAs were treated with ribonuclease (RNase)-free deoxyribonuclease DNase I (1 U/ml) for 1 h to eliminate contamination with genomic DNA. The purified RNA was extracted with phenol/chloroform/isoamyl alcohol (PCI, 25/24/1 v/v) and then precipitated with 100% ethanol.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

To prepare first-strand deoxyribonucleic acid (cDNA), total RNA was primed with oligodeoxythymidilic (oligo dT) primer and then reverse-transcribed with SuperScript (Gibco BRL, Grand Island, NY, USA) at 37 °C for 60 min. The cDNA was extracted with PCI alcohol and precipitation with ethanol terminated the procedure. The PCR was used to amplify the synthesized cDNA (made from 3 mm of each dissected nephron segment or five glomeruli) and the following solution was employed: 0.2 µmol/l of the primers, 0.2 µmol/l of each deoxynucleotide triphosphate (dNTP) and 50 mmol/l of KCl, 10 mmol/l of Tris-Cl (pH 8.3) and 1.5 mmol/l of MgCl₂ plus 2.5 U of Amplitaq (Perkin Elmer, New Jersey, USA). One pair of oligonucleotides was synthesized for CNG-A3 (sense: 5'-GCTAACAGGAAGACGGTGGAT-GAAAA-3' and antisense: 5'-TCCGGGTACTCCGT-GAGGGTCTCCAT-3') corresponding, respectively, to nucleotides 1112–1137 and 1528–1553 of rat CNG-A3 cDNA sequence [15]. PCR was performed with 36 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 1 min) and extension (72 °C, 1 min). PCR conditions were the same as described above. For the semi-quantitative RT-PCR rat β-actin primers, predicted to amplify the 215-bp PCR product (sense: 5'-TAGGCCAACACAGTGCTGTCTGG-3' and antisense: 5'-TACTCCTGCTTGCTGATCCACAT-3'), corresponding to nucleotides 2762–2784 and 2965–2987 of rat β-actin gene, were added into the same RT-PCR reaction tubes and their products were used as internal control.

Semi-quantitative RT-PCR was used to compare the expression of CNG-A3 in five different glomeruli or 3 mm segments of proximal convoluted or proximal straight tubules (PCT and PST, respectively), thin limbs of Henle's loop (HL), cortical and medullary thick ascending limbs (cTAL and mTAL, respectively), cortical collecting ducts (CCD), outer and inner medullary collecting ducts (OMCD and IMCD, respectively) dissected from low-Na⁺ diet and high-Na⁺-treated rats. CCD and cTAL were also used to compare the expression of CNG-A3 in control, ADX and ADX+ALDO-treated rats.

The semi-quantitative method of RT-PCR was validated in preliminary experiments. First, the optimal PCR conditions that yielded a single band on agarose gel electrophoresis were determined for each gene (CNG-A3 and β-actin) originated from the RT-PCR reaction made in the same reaction tube for both genes. Second, to determine whether the method was semi-quantitative, serial dissected CCD segments length (1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 mm) were used for RT-PCR amplification for both genes in the same reaction tube. CCD was chosen because it has a high expression of CNG-A3 mRNA and saturation of RT-PCR reaction in this segment would also represent maximal saturation in other segments compared to other segments that had lesser amounts of CNG-A3 mRNA. Third, experiments were performed to determine the optimal number of PCR cycles that yielded PCR products in the linear range of amplification. All reactions included a negative control (cDNA replaced with double-distilled water). The identity of the amplification was confirmed by determination of the molecular size on agarose gel electrophoresis (1.5% agarose in buffer containing 40 mmol/l of Tris-acetate plus 1 mmol/l of ethylenediaminetetraacetic acid—EDTA) and visualized by ethidium bromide staining (0.5 µg/ml) under ultraviolet light. The computer software Sigma Gel v1.1 was used (Jandel Scientific, USA) for densitometric analysis of the bands. The CNG-A3 and β-actin bands from the same sample were analyzed by densitometry and normalized by dividing the CNG-A3 values by the corresponding β-actin values.

2.4. RNase protection assay (RPA)

To make the probe DNA, the PCR product corresponding to CNG-A3 nucleotide sequences 1112 through 1553 was subcloned into pCR-Script SK (+) and used to transform Epicurian Coli XL1-blue supercompetent cells (Stratagene). It was determined that the nucleotide sequence matched previously cloned sequences using Sequenase version 2.0 (US Biochemicals, Ohio, USA). Then a probe for CNG-A3 was prepared by linearization of 1 µg template DNA by enzyme digestion with *Hind*III (Gibco BRL). The pTRI-Rat-β-actin (125 bp) (Ambion, Texas, USA) was used as a template to make an actin probes. Following the Maxscript Kit protocol (Ambion), both probes cDNA templates were transcribed in vitro in the presence of ([α-³²P] UTP NEN, 3000 Ci/mmol, Amersham) with RNA polymerase T7

generating radiolabeled antisense RNA probes. All probes were evaluated with 10 mg of yeast transfer RNA (tRNA) in the presence and absence of RNase A and T1 following the RPAII kit protocol (Ambion). Total RNA (30 mg) from rat renal tissue was mixed with a radiolabeled antisense probe to CNG-A3 (1×10^5 cpm/sample) along with a β -actin radiolabeled antisense probe to actin (internal control) (1×10^5 cpm/sample) and hybridized at 45 °C for 18 h. Both RNase A and T1 (RPAII kit, Ambion) were used to treat the samples at 37 °C for 30 min. The protected fragments were separated electrophoretically on 8 mol/l urea gels containing 50 g/l polyacrylamide. The samples were transferred to chromatography papers and exposed to X-ray films with an intensifying screen at –70 °C. After the films had been revealed, the density of the bands corresponding to the expected sizes of the probes was analyzed by computer software (Sigma Gel v1.1, Jandel Scientific). The undigested CNG-A3 and β -actin probes were 442 and 215 nucleotides larger than the digested probes, respectively.

2.5. Western blot analysis

Expression of CNG-A3 protein in whole kidneys of rats fed a high- or low-sodium diets were assessed and compared by Western blot, using rabbit polyclonal antibody generated against the cytoplasmatic domain of COOH-terminal region of CNG-A3, which specifically recognizes CNG-A3 protein (Alpha Diagnostic International, San Antonio, TX, USA) and a mouse monoclonal antibody generated against N-terminal domain of β -actin (Novus Biologicals, Littleton, CO 80160, USA). Kidneys were homogenized in a solution containing 250 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l imidazole, pH 7.2, and the following protease inhibitors: 1 mmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mmol/l benzamide, 10 mg/l leupeptin, 1 mg/l pepstatin A, 1 mg/l aprotinin, and 1 mg/l chymostatin. Homogenization was carried out at 0 °C using a Potter apparatus. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was saved, then the pellet was suspended in three volumes of the same medium, and the centrifugation was repeated. Both supernatants were mixed and centrifuged at $10,000 \times g$ for 20 min to separate mitochondria. The ensuing supernatant was centrifuged at $100,000 \times g$ for 1 h, the pellet from this centrifugation contained cell membranes that were suspended in ice-cold homogenization buffer. The protein concentration was quantified by the Bradford assay using bovine serum albumin (BSA) as the standard [29]. All the extracts were solubilized by heating at 95 °C for 2 min in buffer solution (15 g/l SDS, 10 mmol/l Tris–Cl pH 6.8, 6 g/l DTT, and 60 ml/l glycerol). The membrane proteins (100 μ g/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA). By using molecular weight markers, the membranes were cut in half to electrophoretically separate β -actin and CNG-A3 bands from the same blot. The two blots were then blocked

with 5% nonfat milk in a Tris-buffered saline with Tween 20 (T-TBS 0.05%). Then, the corresponding halves of the membrane were incubated with the polyclonal antibody against the CNG-A3 (1:500 dilution) or the monoclonal antibody against β -actin protein (1:3000 dilution) in a blocking solution at room temperature for 1 h. The CNG-A3 antibody was detected using a secondary goat-anti rabbit IgG alkaline phosphatase conjugate (1:500) (Sigma Immuno-Chemicals) and the β -actin antibody was detected using a goat-anti mouse IgG alkaline phosphatase conjugate (1:3000) (from Sigma Immuno-Chemicals). The binding was visualized by color development reaction using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Gibco). The obtained CNG-A3 and β -actin bands from a same sample were analyzed by densitometry and normalized by dividing the CNG-A3 values in each lane by the corresponding β -actin values in the same lane.

2.6. Statistical analysis

We used an unpaired Student's *t*-test to compare the expression levels of mRNA and/or protein between the groups of high- and low- Na^+ diet. A one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test was used to compare changes in the expression levels of CNG-A3 mRNA between groups of animals (ADX, ADX with aldosterone replacement, high and low- Na^+ diet, and furosemide treatment regimens). The ANOVA followed by Newman–Keuls multiple comparison test was also used to compare the blood and urine parameters in rats subjected to different treatments. Results are presented as mean \pm S.E. Differences were assumed to be significant when $P \leq 0.05$.

3. Results

3.1. Electrolytes and fractional excretion

After the fifth day of treatment, the FE of K^+ in the group receiving low- Na^+ diet was significantly elevated compared to control and, in the high- Na^+ , ADX and ADX-ALDO groups no significant differences were found compared to the control. The FE of Na^+ and Cl^- and the urinary volume in the high- Na^+ and ADX groups showed a remarkable increase when compared to control values. The sham-operated group urinary parameters were not significantly different from control groups (data not shown). On the other hand, the FE of Na^+ and Cl^- and the urinary volume were significantly decreased in the low- Na^+ group, compared with the control group. In the ADX-ALDO group, modifications were not found in the analyzed parameters. The GFR in the high- Na^+ diet group increased, compared to controls. The GFR in the low- Na^+ diet group and in the ADX+ALDO group did not differ from the control group and, in the ADX group decreased when compared to control levels (Table 1).

3.2. CNG-A3 mRNA distribution along the nephron in animals subjected to normal diets

RT-PCR was performed to detect the CNG-A3 mRNA expression along the nephron of normal rats. CNG-A3 mRNA was expressed in the following nephron dissected segments: proximal convoluted tubules (S1/S2), proximal straight tubules (S3), cTAL segments, the mTAL, the CCD, the IMCD and OMCD. CNG-A3 was also found in the glomerulus. However, using the same experimental procedures, no CNG-A3 mRNA expression was detected in the thin segment of Henle's loop (HL) (Fig. 2).

3.3. Modulation of CNG-A3 mRNA expression in whole rat kidney by high- and low-salt diet

CNG-A3 gene mRNA expression in whole rat kidney was detected by RPA. The RNase action degraded the probes when hybridized with 30 µg of yeast total RNA, used as RNase digestion control sample.

The 442-bp protected fragments corresponding to CNG-A3 gene were detected in the different groups (Fig. 1A). Protected fragment of 215 bp, corresponded to β-actin expression (Fig. 1B). The ratio of the densitometric values of CNG-A3 and β-actin was obtained for low- and high-Na⁺ diet groups. There was a 64% decrease of the CNG-A3 sodium channel mRNA of whole rat kidneys subjected to high-Na⁺ diet compared to the CNG mRNA in kidneys with low-Na⁺ diet ($n=3$, $P<0.01$, Fig. 1C).

3.4. Modulation of CNG-A3 mRNA expression along the nephron of rats subjected to high- and low-salt diets

RT-PCR experiments showed that when the amount of CNG-A3 mRNA in each cTAL or CCD segment, was divided by the amount of mRNA of the β-actin gene in the same segment, the CNG-A3 mRNA expression decreased by 49% ($n=7$, $P<0.05$) and 37% ($n=7$, $P<0.01$), respectively, in animals on high-Na⁺ diet compared to animals fed a low-sodium diet (Fig. 2f and i). On the other hand, there was no significant variation in the other dissected segments (glomeruli, PCT, PST, HL, mTAL, OMCD, IMCD) of animals subjected to the same treatment (Fig. 2). RT-PCR experiments showed that high- or low-salt diet did not alter

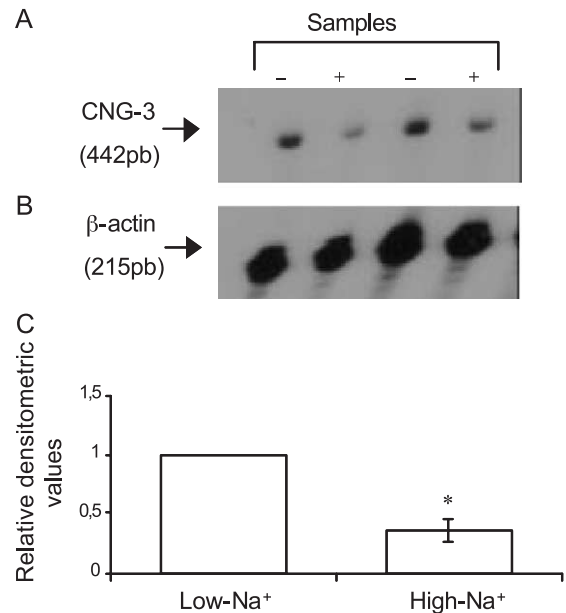


Fig. 1. RPA: (A) Bands obtained with hybridization of CNG-A3 probes and 30 mg of total RNA extracted from whole rats kidney treated with low- (–) and high- (+) Na⁺ diets; (B) β-actin bands obtained in the same hybridization described in panel A; (C) representation of the means of the ratio between densitometric values of CNG-A3 and β-actin ($n=3$, $P<0.01$).

the distribution of the CNG-A3 mRNA. No CNG-A3 mRNA was found in the thin limbs of Henle's loop of rats on high- or low-salt diet (Fig. 2d).

3.5. Protein expression variation of the CNG-A3 channel

The Western blot showed a decrease of 49% in renal CNG-A3 protein expression in rats subjected to high-Na⁺ diet compared to the rats subjected to a low-Na⁺ diet (Fig. 3, $n=3$, $P<0.05$).

3.6. Evaluation of the possible participation of aldosterone

Other experiments were performed in order to verify if the decrease in CNG-A3 mRNA found in cTAL and CCD segments occurred through the action of aldosterone, using control, high- and low-Na⁺, adrenalectomized bilaterally with and without aldosterone replacement. When compared

Table 1

Urinary parameters: summary data of the urinary flow, GFR, body weight, FE of K⁺, Na⁺, and Cl[−] comparing control rats, adrenalectomized rats with (ADX+ALDO) or without (ADX) aldosterone replacement, rats fed a high-Na⁺ diet for 5 days and rats fed a low-Na⁺ diet

	Control	High-Na ⁺	Low-Na ⁺	ADX	ADX+ALDO
K ⁺ FE (%)	48.47±0.85 ^a	46.13±3.12 ^a	56.23±2.69 ^b	45.76±3.02 ^a	47.87±0.63 ^a
Na ⁺ FE (%)	1.17±0.35 ^a	3.60±0.20 ^b	0.59±0.02 ^c	2.1±0.18 ^d	1.02±0.26 ^a
Cl [−] FE (%)	2.40±0.36 ^a	6.20±2.34 ^b	1.82±0.09 ^c	3.8±0.22 ^d	2.07±0.56 ^a
Urinary flow (ml/day)	9.5±0.5 ^a	15±1.0 ^b	8.5±0.11 ^c	16.7±0.6 ^b	10.6±0.7 ^a
GFR (ml/kg/min)	5.84±0.45 ^a	9.16±1.29 ^b	4.98±0.52 ^a	3.7±0.56 ^c	4.97±0.35 ^a
Body weight (g)	205.4±15 ^a	201.3±5 ^a	202.7±2.73 ^a	198.7±9 ^a	207.1±9 ^a

Data represent mean±S.E. There is no statistical difference between groups with the same letters.

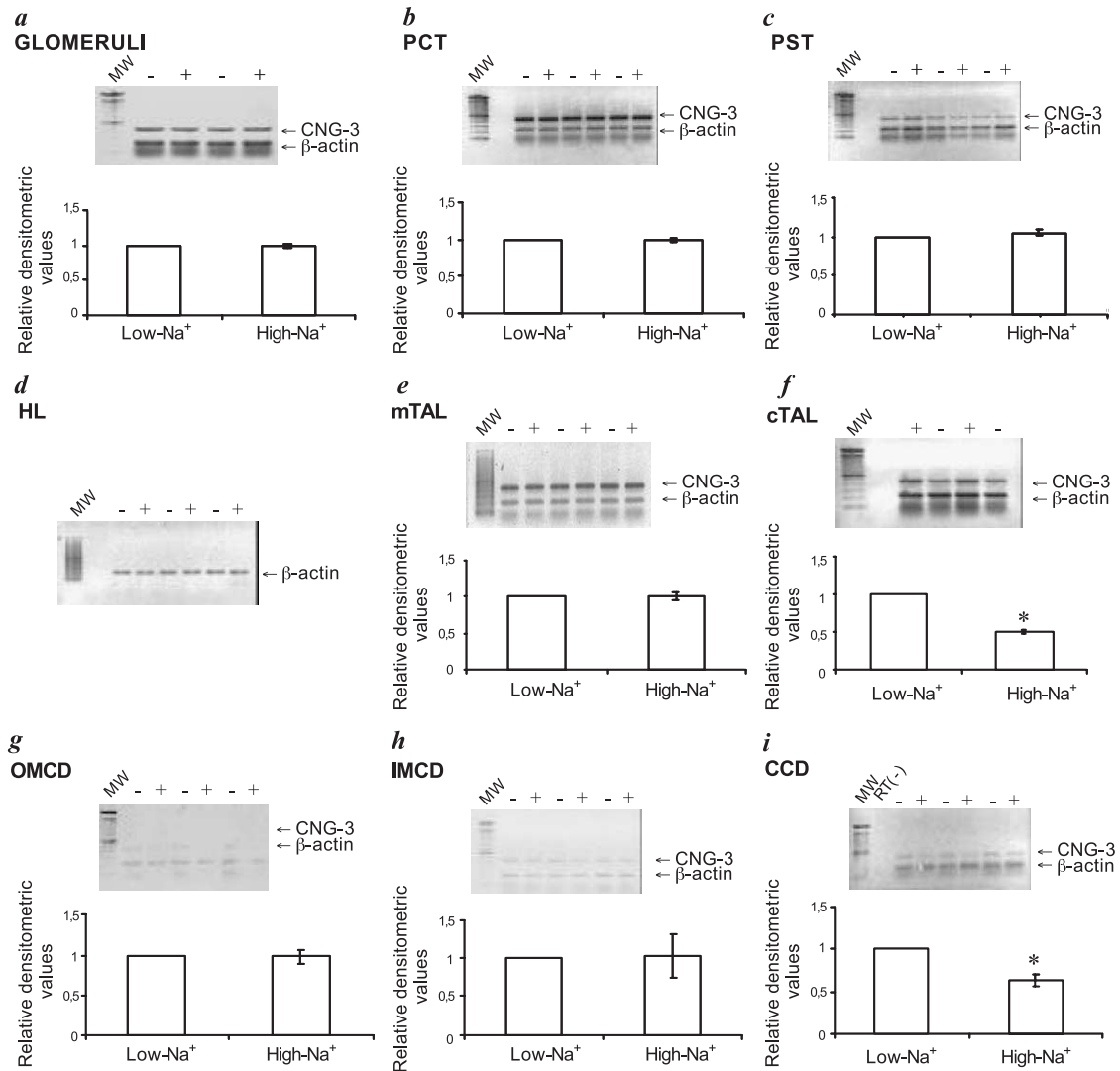


Fig. 2. Representative 1.5% agarose gels of CNG-A3 (442 bp) and β-actin (215 bp) genes semi-quantitative RT-PCR. Low-Na⁺ diet represented by (–) and high-Na⁺ diet represented by (+). In the first lane of each picture, the 100 bp ladder molecular weight (MW) is shown. Graphics present the reason between densitometric values of CNG-A3 and β-actin in the following segments: (a) glomerulus ($n=10$), (b) segments S1/S2 (PCT) of proximal tubule ($n=9$), (c) segment S3 (PST) of proximal tubule ($n=7$), (e) segments of the mTAL ($n=4$), (g) outer medullary collecting duct (OMCD, $n=3$), (h) IMCD ($n=3$), where no significant difference between low- and high-Na⁺ diet groups were found ($P>0.05$). (d) The thin segment of Henle's loop (HL) does not express the channel mRNA, in any of the conditions presented here. In the cortical thick segment of Henle's loop (cTAL, $n=7$) (f) and CCD ($n=10$) (i), the mRNA expression decreases in high-Na⁺ diet condition than in low-Na⁺ diet ($P<0.05$).

to low-Na⁺ animals, rats subjected to high-Na⁺ diet have a 49% diminished CNG-A3 expression in the cTAL segment ($n=7$, $P<0.001$, Fig. 4a), and a 37% decrease in CCD ($n=7$, $P<0.01$, Fig. 4b). In animals on a low-Na⁺ diet, there was no significant variation of the CNG-A3 sodium channel expression in the same segments compared to control group. In the cTAL, adrenalectomy leads to a decrease of 25% ($n=5$, $P<0.05$, Fig. 4a) in the CNG-A3 mRNA expression and a 58% decrease in CCD ($n=5$, $P<0.001$, Fig. 4b), when compared to control values. Rats adrenalectomized and receiving aldosterone showed no difference from controls in their CNG-A3 expression. CNG-A3 mRNA expression, evaluated by semi-quantitative PCR,

was not different comparing control animals and sham-operated animals (data not shown).

3.7. Evaluation of the possible role of transepithelial transport

When animals were treated with 10 mg/kg of furosemide (a dose that blocks the Na⁺/K⁺/2Cl[–] co-transporter but does not lead to an increase of plasma aldosterone concentration in rats) [25], we found no change in CNG-A3 mRNA expression in cTAL compared to animals not treated with furosemide. However, the treatment with a higher dose of 100 mg/kg of furosemide, enough to block the Na⁺/K⁺/2Cl[–]

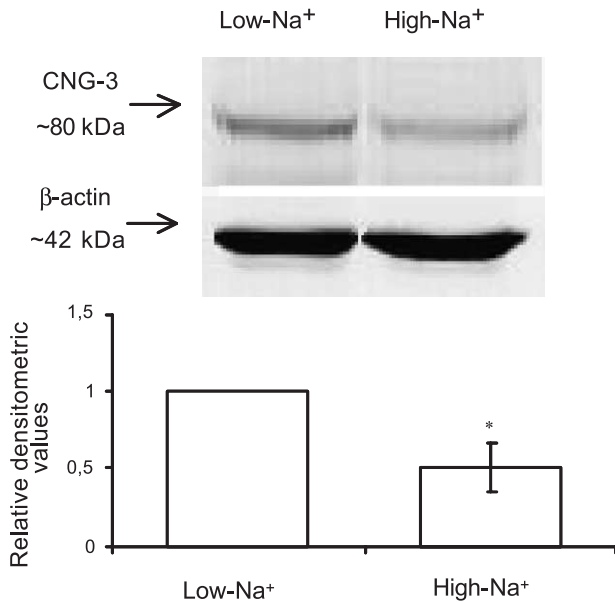


Fig. 3. Detection of CNG-A3 protein by Western blot analysis in the whole kidney of rats fed a high- and low-Na⁺ diet. Mean \pm S.E. ($n=3$) values for the densitometric analysis of CNG-A3 and β -actin proteins bands obtained in the Western blot experiments. The β -actin band served as internal control for all samples and its densitometric values were used to normalize the corresponding CNG-A3 values. * $P<0.05$ vs. low-Na⁺ group. Insert: a representative immunoblot for CNG-A3 and β -actin in membrane extracts prepared from rat kidneys; rat fed a low-Na⁺ diet (lane 1) and rat fed a high-Na⁺ diet (lane 2); 100 μ g protein loaded per lane.

co-transporter and increase both plasma rennin activity and aldosterone concentration in rats, caused a 44% increase in the CNG-A3 expression in cTAL ($n=3$, $P<0.001$, Fig. 5).

4. Discussion

Most sodium filtered through the glomeruli is reabsorbed along different nephron segments, where the transport of this ion is highly regulated. However, the regulation of sodium reabsorption occurs mainly in the cortical, outer medullary and inner medullary segments. Sodium is well-known to be important for the maintenance of extracellular volume [1] and this ion balance depends on several hormones, which act in the kidney. One of the most important hormones, aldosterone, leads to an increase in renal sodium reabsorption. This transepithelial sodium transport is a result of the function of several membrane proteins, which have altered function and/or expression regulated by hormones, including aldosterone. Sodium transport modulated by aldosterone through amiloride-sensitive sodium channel (ENaC) in renal epithelia was demonstrated before and those findings emphasize the importance of sodium channels in the renal regulation of ECFV and blood pressure [7,8]. It is well-known that aldosterone increases the accessory subunits of the epithelial sodium channel (ENaC) in the kidney. The alpha, beta and gamma rabbit ENaC subunits (rENaC) mRNA levels were

found to be increased twofold in primary cultures of immunodissected rabbit kidney connecting tubule and CCD cells after aldosterone treatment [30] (Fig. 6). In addition, several other sodium channels are abundant in renal cells, including CNG-A3, and they could be also modulated in conditions of extracellular volume variation. The expression of CNG cation channels have been demonstrated in all nephron segments using electrophysiological methods [13] and, specifically in the IMCD cells, it was demonstrated that CNG channels are localized at the

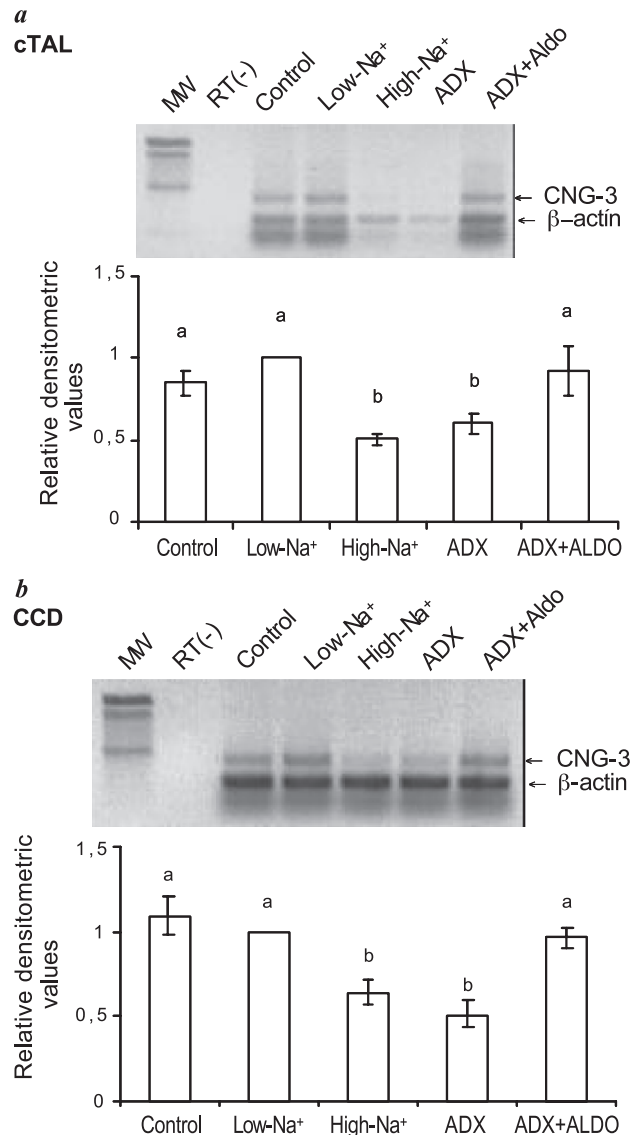


Fig. 4. Representative 1.5% agarose gels of CNG-A3 (442 bp) and β -actin (215 bp) genes semi-quantitative RT-PCR. The results above correspond to: (a) analysis of CNG-A3 expression modulation in the cTAL of Henle's loop (a different from b, $n=5-7$, $P<0.01$) and (b) analysis of CNG-A3 expression modulation in the CCD (a different from b, $n=5-7$, $P<0.001$) in control group, low-Na⁺ diet group, high-Na⁺ diet group and adrenalectomized with and without aldosterone replacement groups. In the first lane of each picture, the 100 bp ladder molecular weight is shown. Graphics present the ratio between densitometric values of CNG-A3 and β -actin, obtained from the agarose gel.

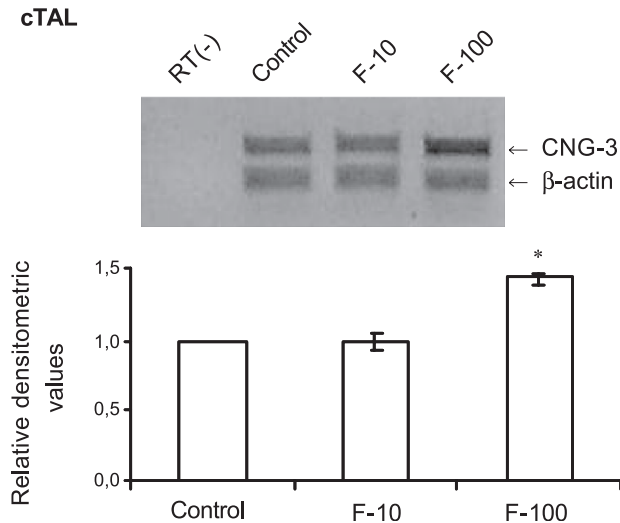


Fig. 5. Representative 1.5% agarose gel of CNG-A3 (442 bp) and β-actin (215 bp) genes semi-quantitative RT-PCR. The results correspond to analysis of CNG-A3 expression modulation in the cTAL of Henle's loop in control rats and treated with 10 mg/kg (F10) and 100 mg/kg (F100) of furosemide ($n=3$, $P<0.001$). Graphics present the ratio between densitometric values of CNG-A3 and β-actin, obtained from the agarose gel.

apical membrane [36]. It was suggested that, in the kidney, those channels can be inhibited by the ANP [19], a very well-known hormone involved in the extracellular volume regulation.

As we demonstrated in this manuscript, CNG-A3 channel mRNA is present in all dissected renal tubules except in the thin limbs of Henle's loop (Fig. 2). In addition, the wide distribution of CNG-A3 in the nephron seems to indicate that this channel could be poised for transepithelial transport.

Besides the majority of sodium channels that were found mostly in the distal nephron, we also found a relatively high mRNA expression level of CNG-A3 in the proximal tubules, which could suggest a role of CNG-A3 in the renal proximal transport of sodium once about 60% of sodium filtered in the glomeruli is reabsorbed along the proximal tubules.

The role of CNG-A3 channels has not yet been established in renal tissue. Our results showed an increased CNG-A3 mRNA (Fig. 1) and protein (Fig. 3) expression in the kidney of rats treated with a low-sodium diet compared to animals treated with a high-sodium diet. These changes were a result of modulation of CNG-A3 mRNA expression in cTAL and CCD nephron segments (Fig. 2). The cTAL and CCD are known to be sites of action of hormones involved in the extracellular volume regulation such as angiotensin-II, ANP, vasopressin and aldosterone [1,8].

The decrease of renal CNG-A3 mRNA and protein expression in rats subject to a high- Na^+ diet could be due to the influence of different hormones related to the extracellular volume regulation or by the changes in renal tonicity secondary to this procedure [31]. The decrease in renal CNG-A3 mRNA expression in ADX rats, which is reversed by aldosterone replacement, in both cTAL and CCD segments, suggest that this hormone might be involved in CNG-A3 gene expression (Fig. 4), although there were no significant changes in the renal expression of CNG-A3 in cTAL or CCD of the animals in low- Na^+ diet (Fig. 4). We conclude that the absence of aldosterone promotes a decrease in the CNG-A3 sodium channel expression especially in cTAL and CCD segments where aldosterone receptors are present [32,33]. However, changes in ion

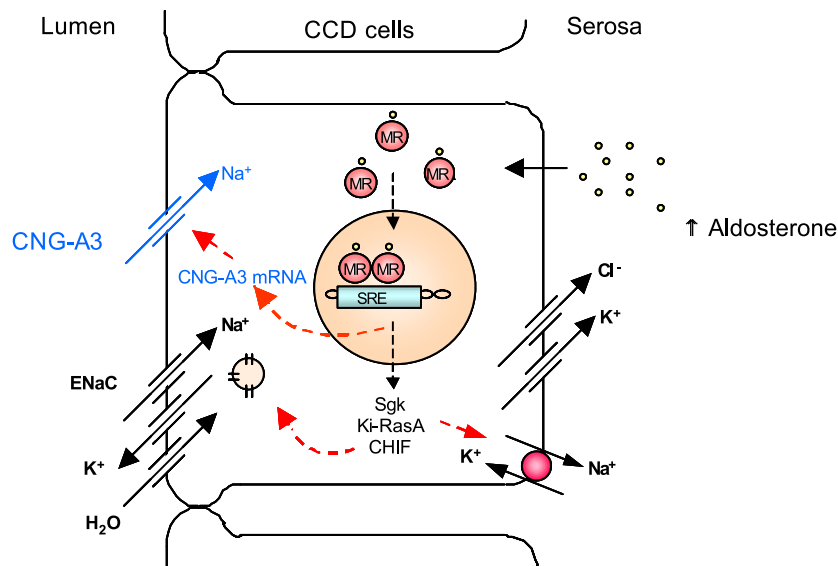


Fig. 6. Model of the genomic actions of aldosterone in renal epithelia. Final effectors of aldosterone are involved in Na^+ reabsorption and K^+ secretion. Aldosterone-induced proteins such as serum and glucocorticoid-inducible kinase (Sgk), corticosteroid hormone-induced factor (CHIF), and Kirsten Ras (Ki-Ras) increase the activity and/or number of these transport proteins during the early phase of action [37]. During the late phase of aldosterone action, expression levels of ion transporters proteins, including CNG-A3, increase. ENaC, epithelial sodium channel; MR, mineralocorticoid receptor; SRE, steroid response element (adapted from Booth et al. [37]).

transport of epithelial cells can lead to changes in mRNA expression of different genes [34,35]. In order to investigate if the modulation observed in CNG-A3 mRNA is not caused by altered transport through these epithelia, but by direct action of aldosterone, we used rats treated with furosemide [34]. The animals treated with this drug in amounts that are able to partially block the renal $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ transporter without producing increased aldosterone plasma levels (10 mg/kg) [25] did not show changes in CNG-A3 mRNA expression in cTAL (Fig. 5). In contrast, treatment with a furosemide at 100 mg/kg, a concentration able to increase serum levels of aldosterone [25], generated an increase in CNG-A3 expression at cTAL, suggesting the direct action of aldosterone in this process (Fig. 5).

The transport model in CCD is well-known and it is dependent on the Na^+/K^+ ATPase present at the basolateral membrane, which establishes the electrochemical driving forces necessary for luminal entry and exit of Na^+ and K^+ , respectively. Aldosterone also increases Na^+/K^+ ATPase activity to increase the epithelial transport capacity [40]. However, the apical channel activity is limiting for trans-cellular ion movement and aldosterone can enhance this transport in an early and in a late phase. In the early phase, aldosterone could increase the open probability of apical ion channels and/or promote channel insertion to increase the number of functional channels [38,39]. In the late phase, aldosterone could stimulate ion transporter gene expression [38,39]. Our results, as proposed in the adapted model represented in Fig. 6, suggest that CNG-A3 would be included in the family of transporters stimulated by aldosterone in the kidney and it might have an important role in renal function.

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