Original Paper

Role of Estrogen and Progesterone in the Modulation of CNG-A1 and Na⁺/K⁺-ATPase Expression in the Renal Cortex

Jones B. Gracelli^{5,*} Jackson Souza-Menezes^{1,2,*} Carolina M.L. Barbosa¹ Felipe S. Ornellas¹ Christina M. Takiya³ Leandro M. Alves³ Mira Wengert¹ Georgia da Silva Feltran² Celso Caruso-Neves¹ Margareth R. Moyses⁴ Luiz F.M. Prota¹ Marcelo M. Morales¹

¹Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ,

²Laboratório Integrado de Bioquímica Hatisaburo Masuda, Núcleo de Pesquisa em Ecologia e Desenvolvimento Sócio-Ambiental, Universidade Federal do Rio de Janeiro, Macaé, RJ, ³Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, ⁴Departamento de Fisiologia, Universidade Federal do Espírito Santo, ES, ⁵Departamento de Morfologia, Universidade Federal do Espírito Santo, ES, *Both authors contribute equally to the development of this work

Key Words

CNG-A1 • Estrogen • Progesterone • Na⁺/K⁺ ATPase • Kidney • Nephron

Abstract

The steroid hormones, estrogen and progesterone, are involved mainly in the control of female reproductive functions. Among other effects, estrogen and progesterone can modulate Na⁺ reabsorption along the nephron altering the body's hydroelectrolyte balance. In this work, we analyzed the expression of cyclic nucleotide-gated channel A1 (CNG-A1) and α1 Na⁺/K⁺-ATPase subunit in the renal cortex and medulla of female ovariectomized rats and female ovariectomized rats subjected to 10 days of 17β-estradiol benzoate (2.0 µg/kg body weight) and progesterone (1.7 mg/kg body weight) replacement. Na⁺/K⁺ ATPase activity was also measured. Immunofluorescence localization of CNG-A1 in the cortex and medulla was performed in control animals. We observed that CNG-A1 is localized at the basolateral membrane of proximal and distal tubules. Female ovariectomized rats showed low expression of CNG-A1 and low expression and activity of Na⁺/K⁺ ATPase in the renal cortex. When female ovariectomized rats were subjected to 17β-estradiol benzoate replacement, normalization of CNG-A1 expression and Na⁺/K⁺ ATPase expression and activity was observed. The replacement of progesterone was not able to recover CNG-A1 expression and Na⁺/K⁺ ATPase expression at the control level. Only the activity of Na⁺/K⁺ ATPase was able to be recovered at control levels in animals subjected to progesterone replacement. No changes in expression and activity were observed in the renal medulla. The expression of CNG-A1 is higher in cortex compared to medulla. In this work, we observed that estrogen and progesterone act in renal tissues modulating CNG-A1 and Na⁺/K⁺ ATPase and these effects could be important in Na⁺ and water balance.

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Marcelo M. Morales

Laboratório de Fisiologia Celular e Molecular, Instituto de Biofísica Carlos Chagas Filho
Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro
Ilha do Fundão, Rio de Janeiro 21941-902, RJ (Brazil)
Tel. +55-21-2562-6572, E-Mail mmorales@biof.ufrj.br

Introduction

Steroids are known to affect cell metabolism by genomic and nongenomic mechanisms [1]. Classically, the steroid hormones activate the transcription machinery in the nucleus directly [2, 3]. The nongenomic mechanisms might be initiated by activation of membrane or cytosolic receptors and result in either direct local effects (e.g., modulation of ion channel activity and cell excitability) or regulation of gene transcription secondary to activation of signaling cascades [4, 5].

Estrogen (E₂) and progesterone (P₄) are steroids hormones involved mainly in the control of female reproductive functions [4, 6]. Among other effects, E₂ and P₄ can modulate Na⁺ reabsorption along the nephron altering the body's hydroelectrolyte balance [7]. Extracellular volume increases in women during the pre-ovulatory phase of the menstrual cycle when estrogen levels are rising [8]. Salt and water retention occurs during pregnancy [7] and in postmenopausal women subjected to administration of estradiol [9]. These findings could be associated with the action of P₄ and E₂ in the kidney leading to retention of hydrosaline [10]. P₄ is known to compete with aldosterone for mineralocorticoid receptors (expressed mainly in the distal tubules) and this can lead to receptor activation and an increase in Na⁺ reabsorption in this nephron segment [11]. The action of E₂ occur mainly in the renal proximal and distal tubules [6, 12, 13].

Modulation of basolateral Na⁺/K⁺-ATPase activity and expression could be important in the control of lumen Na⁺ reabsorption in mammalian nephrons [14]. The activity of Na⁺/K⁺-ATPase creates an electrochemical gradient favorable to Na⁺ uptake across the luminal membranes of renal tubule cells by a variety of Na⁺ channels [14, 15].

Beyond Na⁺/K⁺-ATPase, ion channels such as epithelial sodium channel (ENaC) and thiazide-sensitive NaCl cotransporter (TSC) are modulated by estrogen [12, 16]. After 2 weeks recovery from ovariectomy, a reduction in ENaC mRNA [16] and TSC protein [12] expression was observed in kidneys of female rats. Replacement of E₂ normalized ENaC mRNA [16] and TSC protein expression in the same animal model [12].

Although the involvement of P₄ and E₂ in renal ion reabsorption is clear, the transporters involved in this process are not well known [7].

Cyclic nucleotide-gated (CNG) channels are nonselective cation channels first identified in retinal photoreceptors and olfactory sensory neurons (OSNs) [17]. Although their activity shows very little dependence on voltage, CNG channels belong to the superfamily of voltage-gated ion channels [18]. CNG channels form heterotetrameric complexes consisting of two or three different types of subunits [19]. Six different genes encoding CNG channels, four A subunits (A1 to A4) and two B subunits (B1 and B3), give rise to three different channels in rod and cone photoreceptors and in OSNs [20].

Electrophysiological and biochemical approaches have demonstrated that CNG channels play a key role in sensory signal transduction including vision and smell [21–23]. Rods respond to a light stimulus with a brief hyperpolarization by closing CNG channels in the surface membrane of the outer segment. In the dark, channels are activated by the binding of cGMP, allowing a steady cation current ("dark current") to flow into the outer segment. Light triggers a sequence of enzymatic reactions that leads to the hydrolysis of cGMP. When CNG channels close, the inward current ceases and the cell hyperpolarizes. The enzyme cascade comprises the photopigment rhodopsin (R), the G protein transducin (T), and a PDE. Light stimulation decreases the cytoplasmic Ca²⁺ concentration, which initiates the recovery from the light response by enhancing the synthesis of new cGMP molecules and adjusts the sensitivity of the transduction machinery, a process known as light adaptation. The CNG channel is crucially important for the control of cytoplasmic Ca²⁺ concentration, because it provides the only source for Ca²⁺ influx into the outer segment [18].

When cDNA were isolated from several other tissues, it became evident what CNG chan-

nels may be involved in a variety of functions in addition to sensory signal transduction [24]. Functional transport assays suggest that these channels mediate transepithelial transport of calcium and sodium across the proximal and distal colon [25].

The CNG family has been shown to be expressed in renal cells [15]. The CNG-A3 subunit is present in the apical membrane of the cortical thick ascending loop and cortical collecting duct from rat nephron and participates in renal Na⁺ reabsorption [26]. The role of CNG channels has not yet been established in renal tissue, but the wide distribution of CNG-A3 in the nephron seems to indicate that this channel family could participate in transepithelial ion transport. This hypothesis is sustained by the fact that rats subjected to high sodium diet showed lower expression of CNG-A3 in collecting ducts [26].

The CNG-A1 subunit was first identified in retinal rods [17]. In the kidney, activity of CNG-A1 was showed in the renal cortex [19] and expression of its mRNA in the renal medulla [17], but protein expression and function was not yet established in renal tissue [27].

Based on the knowledge that female hormones can modulate corporal body Na⁺ balance by modulation of ion channel and ion pump expression and activity, we proposed to study the possible involvement of P4 and E2 in modulation of sodium channel CNG-A1 expression and the functional relationship between this channel and Na⁺/K⁺ ATPase.

Material and Methods

Animal preparation

Adult female Wistar rats weighing 150–250 g (3 months old) were maintained under controlled lighting (12 h light/dark cycle, lights on at 07:00 h) and temperature (23/24 °C). The ethics committee for use of animals in research from The Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, previously approved all procedures and protocols using animals mentioned in this article. All female rats had a regular 4- to 5-day estrous cycle monitored by vaginal smears collected each morning for at least 2 consecutive weeks before starting the experiments. The animals were separated into two groups. The sham-operated group was kept as the control group (C) and the other groups were ovariectomized bilaterally after vaginal cytology (OVX) [28].

Surgical procedures (bilateral ovariectomy)

After anesthesia with injection of ketamine (30 mg/kg i.p.) and xylazine (3 mg/kg i.p.), females rats underwent skin incision of 1 to 1.5 cm, muscular incision to open the peritoneal cavity for posterior connection of uterine tubules and removal of the ovaries [29]. The peritoneal cavity was then sutured and cleaned.

Hormone administration

After 10 days recovering, the ovariectomized group was then further divided into three groups receiving daily subcutaneous injections for 10 days of 17β-estradiol benzoate 2.0 μg/kg body weight (OVE) (Sigma, St. Louis, MO), progesterone 1.7 mg/kg body weight (OVP) (Sigma, St. Louis, M) or corn oil as a vehicle (OVX). The animals were sacrificed on the day after the last injection and blood samples were collected for hormonal evaluation.

It was performed hormone replacement tests before choosing the correct dosage and time of treatment. In the conditions described above, the OVE and OVP animal presented no significant differences in estrogen and progesterone plasma concentration compared to their respective control animals.

Serum estradiol and progesterone concentrations

Serum 17β-estradiol benzoate (pg/mL) and progesterone (ng/mL) were measured by chemiluminescence immunoassay (Immulite: DPC Biemann GmbH, Bad Nauheim, Germany). The intra-assay coefficient of variation was 7.1% for estrogen and 5.8% for progesterone.

Renal function

To monitor the electrolyte balance and glomerular filtration rate (GFR), on the last day of hormonal/vehicle administration, the animals were kept in metabolic cages for 24 hours to allow urine collection. Under deep anesthesia, blood samples were taken and 24h urine was collected. Plasma and urine K⁺, Na⁺, Cl⁻ and urea concentrations were measured using a colorimetric assay (Dolles, Goiânia, GO, Brazil; Gold Analisa,

Belo Horizonte, MG, Brazil) to determine fractional excretion (FE) of electrolytes. The GFR was determined using the creatinine clearance method. The FE of electrolytes and the GFR were calculated using the following equations: $FE (\%) = [(U_x \times V) / (GFR \times P_x)] \times 100$ and $GFR = (U_{cr} \times V) / P_{cr}$ relative to weight in kilograms, where U_x is the urinary concentration of solute (mg/mL), P_x is the plasma concentration of solute (mg/mL), U_{cr} is urinary concentration of creatinine (mg/mL), P_{cr} is the plasma concentration of creatinine (mg/mL), and V is the urinary flow (mL/min).

Tissue fixation and immunofluorescence

Kidneys were obtained from adult female rats after perfusion with heparinized saline (1 mL/500 mL) followed by 4% buffered paraformaldehyde solution. The kidneys were collected and cryopreserved in sucrose solution and frozen. Sections of 6 μ m were obtained and fixed in cold acetone for 5 min. The sections were briefly rinsed in 0.01 M phosphate saline buffer (PBS; pH 7.4) and subsequently incubated in a humid chamber with blocking solution containing 5% bovine serum albumin (BSA), 3% normal goat serum in PBS 0.01 M (pH 7.4) for 1 h at room temperature, followed by avidin and biotin solutions (Vector biotin blocking kit, SP-2001, Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instructions. Sections were then incubated with the primary antibody (Product CNG11-A, Alpha Diagnostic International, San Antonio, TX; dilution 1:50 in PBS containing 3% BSA, 0.1% Triton X-100 and 0.05% Tween 20) overnight at 4 °C. After washing with PBS three times for 5 min at room temperature, the sections were incubated with goat anti-rabbit IgG conjugated to Alexa 586 (A11010, Molecular Probes, Carlsbad, CA; dilution 1:200) in PBS for 1 h at room temperature. To verify the localization of antibody staining, biotinylated lectins were used: *Dilochos biflorus* lectin (DBA) for staining proximal tubules and collector tubules [30] and *Arachis hypogaea* lectin (PNA) for distal tubules and collector tubules [31]. Biotinylated lectins 15 μ g/mL (DBA, B-1035; PNA, BA-2301-2, Vector Laboratories) were incubated for 2 h at room temperature, washed and incubated with streptavidin conjugated to fluorescein isothiocyanate (FITC; SA-1001, Caltag Laboratories, Burlingame, CA; dilution 1:100) for 1 h at room temperature. Sections were washed three times with PBS for 5 min, stained with DAPI (dilution 1 μ g/100 μ L) and mounted with Vectashield (H-1000, Vector Laboratories). Images were acquired using a Nikon E-800 fluorescence microscope equipped with an Evolution VF camera (Media Cybernetics). To obtain high-resolution fluorescence images of the entire kidney cross section, filter selection, stage movement, and image acquisition were controlled using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Total magnification is given in each figure.

Protein extraction and immunoblotting

The animals were sacrificed and the kidneys were removed and sliced sagittally. The renal medulla was manually dissected and the renal cortex was dissected using a Stadie-Riggs microtome as described previously [32]. The tissues were homogenized in lysis buffer (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 homogenizer. The homogenate was centrifuged at 1000 $\times$ g for 10 min. The supernatant was saved, the pellet was suspended in three volumes of lysis buffer, and the centrifugation was repeated. Both supernatants were mixed and centrifuged at 10,000 $\times$ g for 20 min. The resulting supernatant was centrifuged at 100,000 $\times$ g for 1 h, the supernatant was discarded and the pellet was suspended in ice-cold lysis buffer. The protein concentration was determined using the Bradford assay. Proteins were solubilized by heating at 100 °C for 1 min in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, 0.01% bromophenol blue, and 1.7% β -mercaptoethanol).

Standard SDS-polyacrylamide gel electrophoresis (PAGE) was carried out loading equal quantities of protein per lane (100 μ g) using 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in Tris-glycine transfer buffer. The following steps were performed to detect the α 1-Na⁺/K⁺-ATPase subunit as described previously [33]. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline 0.05% Tween 20 solution (TBS-T) overnight at 4 °C, washed once for 10 min in TBS-T and then incubated with rabbit polyclonal antibody generated against the cytoplasmatic domain of the N-terminal region of CNG-A1 (diluted 1:500 in blocking solution for 1 h at room temperature) (Product CNG11-A, Alpha Diagnostic International, San Antonio, TX) or mouse monoclonal antibody generated against the N-terminal domain of β -actin (diluted 1:1000 in blocking solution for 1 h at room temperature) (Novus Biologicals, Littleton, CO). After incubation with primary antibody, membranes were washed three times with TBS-T for 10 min each wash. The CNG-A1 protein was detected using a secondary anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:1000 in blocking solution for 1 h at room temperature) (Sigma Immuno-Chemicals) and the β -actin antibody was detected using an anti-mouse IgG alkaline phosphatase conjugate (diluted 1:4000 in blocking solution for 1 h at room temperature) (from Sigma Immuno-Chemicals). The blots from sodium channel CNG-A1 and their respective β -actin (used as internal control) were visualized by color development reaction using nitroblue tetrazolium chloride (NBT) and 50

Parameters	C	OVX	OVE	OVP
Plasma Estradiol (pg/ml)	98±12	Not detectable	110±5.8	Not detectable
Plasma Progesterone (ng/ml)	18±2.8	Not detectable	Not detectable	16±0.7

Table 1. Plasma estradiol and progesterone concentration. Absolute values of estradiol and progesterone concentration in plasma of Control (C), ovariectomized (OVX), ovariectomized with 17β-estradiol benzoate (OVE) and progesterone (OVP) replacement for 10 days. The results are expressed as means±SEM (n=4).

mg/mL of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (all from Life Technologies, Rockville, MD) for 5 min.

The α1-Na⁺/K⁺-ATPase, CNG-A1 and β-actin bands were analyzed by densitometry using ImageJ software. Relative expression was normalized by dividing the α1-Na⁺-K⁺ ATPase and CNG-A1 values by the corresponding internal control values (β-actin).

Na⁺/K⁺-ATPase activity

The composition of the assay medium (0.1 mL) was 5 mM MgCl₂, 20 mM Hepes-Tris (pH 7.0), 90 mM NaCl, 1 mM ouabain, 5 mM ATP (specific activity of approximately 10⁴ Bq/nmol ATP). The ATPase activity was measured according to the method described by Grubmeyer and Penefsky [34, 35]. The Na⁺-ATPase activity was assayed by incubation of kidney cortex membranes (protein concentration 0.3 mg/mL) for 20 min at 37 °C in the presence of the different compounds to be tested. The reaction was stopped by addition of charcoal activated by HCl (0.1 N). An aliquot of the supernatant was obtained after centrifugation of the HCl-activated charcoal suspension for 5 min at 2000 rpm in a clinical centrifuge. In this setting, [³²P]Pi was measured by liquid scintillation counting (Packard Tri-Carb model A2100 TR, Downers Grove, IL).

Cell culture

Immortalized rat proximal tubule (IRPT) cells were kindly provided by Dr Mello-Aires (Renal Biophysics Laboratory, Biomedical Science Institute, University of São Paulo, Brazil). IRPT cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing phenol red, supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), 25 mM glucose, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Grand Island, NY). After reaching 90% confluence, cells were incubated with DMEM, in the absence of fetal bovine serum, for 24 h containing the following concentrations of 17β-estradiol benzoate: 10⁻¹¹ M, 10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M and 10⁻⁷ M. The IRPT cells were harvested in lysis buffer (described above) and cell homogenization was carried out at 0 °C using a Potter homogenizer. The homogenate was centrifuged at 1000×*g* for 10 min. The supernatant was transferred to new tubes and centrifuged at 10,000×*g* for 10 min and the supernatant was saved. Protein concentration was assessed using the Bradford assay. All the extracts were solubilized and SDS-PAGE and immunoblotting were performed as described above.

Statistical analysis

The results are presented as means±SEM. One-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test was used to compare the changes in the protein expression of CNG-A1 and Na⁺/K⁺-ATPase. Differences were assumed to be significant when *P*<0.05, n=4.

Results

Hormone plasma levels

The ovariectomized female rats (OVX), treated daily with 17β-estradiol benzoate 2.0 µg/kg (OVE) for 10 days showed no significant difference in serum estradiol concentration compared with the control group (C) (OVE, 110±5.8 pg/mL; C, 98±12 pg/mL, n=4). The OVX group was treated daily with progesterone 1.7 mg/kg (OVP) and showed no significant difference in serum progesterone concentration compared with the control group (OVP, 16±0.7 ng/mL; C, 18±2.8 ng/mL, n=4, Table 1).

Parameters	C	OVX	OVE	OVP
FE _{Na+} (%)	0.12±0.01	0.21±0.02*	0.12±0.01	0.11±0.02
FE _{K+} (%)	25.3±3.0	24.2±1.2	10.3±1.2*	11.6±1.2*
FE _{Cl-} (%)	0.41±0.01	0.61±0.01*	0.35±0.08	0.45±0.05
FE _{Urea} (%)	0.69±0.09	0.64±0.09	0.58±0.19	0.41±0.07
Urinary Flow (mL/min)	0.010±0.001	0.004±0.002*	0.009±0.002	0.013±0.001
Body Weight (g)	209.0±6.0	231.0±2.4	214.0±9.0	214.0±11.0
Water Intake (mL/day)	15.6±1.8	7.7±1.4*	24.4±3.1	24.5±6.5
GFR (mL/min)	1.70±0.40	1.50±0.20	1.70±0.40	2.10±0.04

Table 2. Urinary parameters: summary data for urinary flow, GFR, body weight, FE of K⁺, Na⁺, urea, and Cl⁻ and water intake. Control (C), ovariectomized (OVX), ovariectomized with 17β-estradiol benzoate (OVE) and progesterone (OVP) replacement for 10 days. The results are expressed as means±SEM (n=4). *P<0.05 vs control group.

Electrolytes and FE analysis

To begin evaluation of renal function in ovariectomized and hormone replacement female rats, the FE of Na⁺, K⁺, Cl⁻, urea and GFR were measured (Table 2). FE_{Na+} and FE_{Cl-} were significantly increased in the OVX group (FE_{Na+}, 0.21±0.02; FE_{Cl-}, 0.61±0.01, P<0.05, n=4), compared with the control group (FE_{Na+}, 0.12±0.01; FE_{Cl-}, 0.41±0.01, n=4), but these parameters were normalized in the OVE group (FE_{Na+}, 0.12±0.01; FE_{Cl-}, 0.35±0.08, n=4) and in the OVP group (FE_{Na+}, 0.11±0.02; FE_{Cl-}, 0.45±0.05, n=4). FE_{K+} was not changed in the OVX group (FE_{K+}, 24.2±1.2, n=4) but it was significantly reduced in the OVE group (FE_{K+}, 10.3±1.2, n=4, P<0.05) and the OVP group (FE_{K+}, 11.6±1.2, n=4, P<0.05) compared with the control group (FE_{K+}, 25.3±3.0, n=4). Urinary flow and water intake were reduced in the OVX group (0.004±0.002 mL/min and 7.7±1.4 mL/day, n=4, P<0.05, respectively) compared with the control group (0.01±0.001 mL/min and 15.6±1.8 mL/day, n=4, respectively). These parameters were normalized when 17β-estradiol benzoate (0.009±0.002 mL/min and 24.4±3.1 mL/day, n=4, respectively) and progesterone (0.013±0.001 mL/min and 24.5±6.5 mL/day, n=4, respectively) were replaced in OVX rats. No changes in FE_{urea}, GFR and body weight were observed between groups.

Modulation of α1-Na⁺/K⁺-ATPase protein expression and activity

α1-Na⁺/K⁺-ATPase protein expression and Na⁺/K⁺-ATPase activity were assessed in both renal cortex and medulla.

In renal cortex, the expression of α1-Na⁺/K⁺-ATPase protein expression in the OVX group was 0.26±0.09 (n=4, P<0.05, Fig. 1A) of the respective control. After replacement of 17β-estradiol benzoate in the OVE group, α1-Na⁺/K⁺-ATPase protein expression was restored to similar levels (1.17±0.10, n=4, Fig. 1A) compared with the respective control. After replacement of progesterone in the OVP group, α1-Na⁺/K⁺-ATPase protein expression increased but remained lower (0.55±0.11, n=4, P<0.05, Fig. 1A) than the respective control. Na⁺/K⁺-ATPase activity was lower in the renal cortex in the OVX group (9.5±2.0 mmol/mg/min, n=4, P<0.05, Fig. 1B) than the respective control (21.0±2.5 mmol/mg/min). In the OVE and OVP groups, no significant changes could be observed in this protein activity compared with the respective controls (OVE, 28.5±4.5 mmol/mg/min; OVP, 15.0±3.5 mmol/mg/min, n=4, Fig. 1B).

In the renal medulla, no significant changes in α1-Na⁺/K⁺-ATPase protein expression were observed in any of the groups (Fig. 1C) compared with the respective controls. In addi-

Fig. 1. Analysis of Na⁺/K⁺-ATPase protein expression and activity in female rat kidney of Control (C), ovariectomized (OVX), ovariectomized with 17 β -estradiol benzoate (OVE) and progesterone (OVP) replacement for 10 days. Immunoblot for $\alpha 1$ -Na⁺/K⁺-ATPase and Na⁺/K⁺-ATPase activity in renal cortex are respectively showed in panels A and B. Immunoblot for $\alpha 1$ -Na⁺/K⁺-ATPase and Na⁺/K⁺-ATPase activity in renal medulla are respectively showed in panels C and D. The results are expressed as means $\pm$ SEM (n=4). *P<0.05 vs control group.

tion, no significant changes in Na⁺/K⁺-ATPase activity were observed in the renal medulla in all groups (Fig. 1D) compared with the respective controls.

Modulation of sodium channel CNG-A1 protein expression

CNG-A1 protein expression was analyzed in both renal cortex and medulla.

In the renal cortex, the expression of CNG-A1 in the OVX group was 0.60 ± 0.10 (n=4, P<0.05, Fig. 2A) compared with the control group. When estradiol was administered in the OVE group, the CNG-A1 protein expression (1.05 ± 0.25 , n=4) was restored to levels similar to the control group (Fig. 2A). After replacement of progesterone in the OVP group, CNG-A1 protein expression was 0.70 ± 0.15 (n=4, p<0.05) compared with the control group (Fig. 2A).

In the renal medulla, no significant changes in CNG-A1 protein expression were observed in any of the groups (Fig. 2B) compared with the respective controls.

Expression of CNG-A1 protein in renal cortex and medulla

It was observed that the expression of CNG-A1 protein in renal medulla of control female rats was lower (0.52 ± 0.11 , n=3, P<0.05) compared to renal cortex (Fig. 3).

Modulation of CNGA-1 protein expression by 17 β -estradiol in IRPT cells

CNG-A1 protein was observed in IRPT cells when they were assayed using the immunoblotting technique (Fig. 4). The cells were treated with a range of concentrations of 17 β -estradiol and all concentrations (10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M and 10^{-7} M) increased the

Fig. 2. Analysis of CNG-A1 protein expression in female rat kidney of Control (C), ovariectomized (OVX), ovariectomized with 17 β -estradiol benzoate (OVE) and progesterone (OVP) replacement for 10 days. Immunoblots for CNG-A1 sodium channel in renal cortex and medulla is showed respectively in panels A and B. The results are expressed as means $\pm$ SEM ($n=4$). * $P<0.05$ vs control group.

Fig. 3. Western blot analysis for CNG-A1 protein in renal cortex and medulla of female control rats. The results are expressed as means $\pm$ SEM. * $P<0.05$ vs cortex.

Fig. 4. Western blot analysis for CNG-A1 in IRPT cells treated with different concentrations of 17 β -estradiol benzoate. C, control; V, vehicle. The results are expressed as means $\pm$ SEM ($n=3$). * $P<0.05$ vs control group.

expression of CNG-A1 protein (1.70 ± 0.20 , 1.80 ± 0.20 , 1.80 ± 0.15 , 1.90 ± 0.3 and 2.15 ± 0.4 , respectively, $n=4$, $P<0.05$, Fig. 4). The enhancement of CNG-A1 expression does not occurs in dose dependent manner for the concentrations of 17 β -estradiol used to perform this experiment.

Modulation of Na⁺/K⁺-ATPase protein expression and activity by 17 β -estradiol in IRPT cells

As observed for CNG-A1, Na⁺/K⁺-ATPase activity and $\alpha 1$ -Na⁺/K⁺-ATPase expression were observed in IRPT cells. The cells were treated with 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M and 10^{-7} M 17 β -estradiol for 24 h before expression and activity were assessed. Expression of $\alpha 1$ -Na⁺/K⁺-ATPase was enhanced, compared with the respective control, when cells were treated only with 10^{-9} M, 10^{-8} M and 10^{-7} M 17 β -estradiol (1.30 ± 0.15 , 1.3 ± 0.10 and 2.20 ± 0.30 , respectively, $n=4$, $P<0.05$, Fig. 5A). No significant changes were observed after treatment with 10^{-11} M and 10^{-10} M 17 β -estradiol. Only concentrations of 10^{-9} M, 10^{-8} M and 10^{-7} M 17 β -estradiol were able to increase the activity of Na⁺/K⁺-ATPase (0.78 ± 0.06 ,

Fig. 5. Analysis of Na⁺/K⁺-ATPase expression and activity in IRPT cells treated with different concentrations of 17β-estradiol benzoate. Immunoblot for α1-Na⁺/K⁺-ATPase in IRPT cells is shown in panel A. Na⁺/K⁺-ATPase activity in IRPT cells is shown in panel B. C, control; V, vehicle. The results are expressed as means ± SEM (*n*=3). **P*<0.05 vs control group.

0.76 ± 0.08 and 0.73 ± 0.07 mmol/mg/min, respectively, *n*=4, *P*<0.05, Fig. 5B) compared with the control (0.35 ± 0.07 mmol/mg/min). As observed for protein expression, 10⁻¹¹ M and 10⁻¹⁰ M 17β-estradiol did not increase Na⁺/K⁺-ATPase activity (Fig. 5B).

Immunofluorescence for CNG-A1 in renal cortex

After the kidneys were collected and cryopreserved, 6-μm sections were obtained for immunolocalization of the CNG-A1 channel along the nephron. Indirect immunofluorescence microscopy performed in female adult rat kidney using a polyclonal antibody against sodium channel CNG-A1 revealed that CNG-A1 was expressed in the basolateral membrane of proximal and distal tubules (Fig. 6C and F). To verify the localization of antibody staining in the tubules of the nephron, biotinylated lectins were used: *Dilochos biflorus* lectin (DBA) stains proximal tubules (Fig. 6B) and *Arachis hypogaea* lectin (PNA) stains distal tubules (Fig. 6E). The reactivity to this antibody was seen only in the kidney cortex. No immunofluorescence was detected in the kidney medulla (data not shown).

Discussion

There are several studies showing that estrogen and progesterone increase body fluids and lead to sodium retention [7, 9, 10, 36]. Recently, it has been shown that female sex hormones regulate the expression of different transporters in different tissues. Estrogen combined with progesterone leads to an increase in expression of the ENaC and the CFTR chloride channel in the lungs [35], and regulates the expression of ENaC in fertile female rat kidneys, acting on proximal tubules [17]. This effect in proximal tubules could be mediated directly, via its receptors, or it could also act indirectly through the renin-angiotensin-aldosterone system [37, 38]. Estrogen, *in vitro*, increases the expression of NaCl cotransporter and Na⁺/K⁺-ATPase in renal distal tubules cells in culture [12]. Fifteen minutes incubation with 17β-estradiol (10⁻⁸ M) in renal distal and proximal tubules of rabbit increased sodium reabsorption, which demonstrates that the direct effects of estrogen [4] occur in the same way as NaCl co-transporters [12] and Na⁺/H⁺ exchange in fertile female rat kidneys [39] by nongenomic mechanisms.

CNG channels are nonselective cation channels first identified in retinal photoreceptors and olfactory sensory neurons (OSNs) [18]. CNG channels, in general, have a heteromultimeric structure consisting of principal and modulatory subunits [26]. The principal subunits

Fig. 6. Immunolocalization of CNG-A1 in distal and proximal tubules of control female rats. (A) CNG-A1 was revealed with a rabbit polyclonal specific antibody followed by a secondary antibody conjugated to ALEXA 586. Immunoeexpression was detected in the basal region of proximal tubule (red). (B) Biotinylated DBA lectin followed by streptavidin conjugated to FITC revealed the luminal pole of proximal tubules (green). (C) This panel shows the overlaying of images from panel A and B. (D) CNG-A1 immunolocalized at the basal pole of a tubule revealed with ALEXA 586 (red). (E) Distal tubule stained with PNA biotinylated lectin, revealed with streptavidin-FITC (green). (F) This panel shows the overlaying of images from panel D and E. The rectangle shows low magnification image of the kidney stained only with DAPI as nuclei marker. Bars: 20 μ m.

form functional channels when expressed alone in heterologous expression systems, whereas the modulatory subunits do not give rise to a cGMP activated current per se, but when co-expressed with principal subunits, they confer channel properties characteristic of native CNG channels such as increased sensitivity to cyclic nucleotides and channel blockers [40]. The CNG family has been shown to be expressed in renal cells [15]. CNG-A3, an isoform of the CNG channels, is present in cortical thick ascending loop and cortical collecting duct; it is modulated by aldosterone and has a physiologic role in Na⁺ reabsorption [27].

Our data support the notion that the CNG-A1 subunit is present in renal tissue. According to Western blot analysis, the CNG-A1 subunit is likely to be present in renal cortex and medulla. However, localization of the CNG-A1 subunit in renal medulla has so far not been consistently confirmed by immunohistochemical experiments. Despite the negative staining of this channel in renal medulla tissue, it is localized in the basolateral membrane of proximal and distal convolute tubules, indicating that the renal cortex is the predominant site of the CNG-A1 subunit in rat kidney. The distribution of CNG-A1 in the nephron seems to indicate that this channel could be involved in transepithelial ion transport in this nephron segment. The discrepancy in detection of CNG-A1 in renal medulla between Western blot and immunohistochemical experiments could be due to intrinsic factors of each technique or CNG-A1 may be present in structures other than nephron segments.

The role of CNG-A1 in renal tissue has not yet been established. Our results show that CNG-A1 protein expression is increased by estrogen *in vivo* and *in vitro*. In ovariectomized female rats, we observed a reduction in CNG-A1 in the renal cortex but this reduction in expression was not observed in the renal medulla, showing that the mechanisms involved in regulation of CNG-A1 expression by female hormones are specific to renal cortex structures. When 17 β -estradiol was replaced in ovariectomized rats, we observed normalization

of CNG-A1 expression. These data suggest that estrogen and progesterone are physiologic modulators of renal CNG-A1 expression.

In order to verify if modulation of CNG-A1 by estrogen is due to direct action in renal proximal tubule, a rat proximal tubule cell line (IRPT) was treated with a range of concentrations of 17 β -estradiol for 24 h and an increase in CNG-A1 expression was observed for all concentrations. These data suggest that estrogen modulates CNG-A1 expression directly by association with its receptor. There are many papers already published showing the expression of estrogen receptor in proximal tubules of rat, mouse and opossum kidney cells. These articles showed that the kidneys have the expression of nuclear estrogen receptor (ER- α and ER- β) and transmembrane G-protein couple estrogen receptor (GPR30) [41-45].

Once the IRTP is a cell line established from rat proximal tubule and we showed, in this work, that estrogen has the same effect in IRPT cells as observed in rat renal cortex (increasing expression of CNG-A1 and expression and activity of Na⁺/K⁺-ATPase), we considered that IRPT cells has estrogen receptor. In this way, we do not consider necessary a set of experiments characterizing the expression and localization of estrogen receptor in this cell line.

Because ovariectomy reduced CNG-A1 expression and hormonal replacement normalized or increased CNG-A1 expression, it was hypothesized that this downregulation could reduce Na⁺ reabsorption by the nephron and hormonal replacement could normalize this possible reduction in Na⁺ reabsorption. An increase in FE_{Na+} was observed and hormonal replacement normalized it. These data suggest the involvement of CNG-A1 in transepithelial transport of Na⁺ in renal cortex. In addition, FE_{Cl-} was reduced in ovariectomized female rats and normalized by hormonal replacement. This finding could be partially explained by low ClC-2 chloride channel expression in ovariectomized female rats and normalization of expression during hormonal replacement shown by us previously [37]. FE_{K+} did not show significance differences after ovariectomy but the replacement of estradiol benzoate and progesterone, alone, decreased this value. These data suggest that one of these hormones inhibits the action of the other in the modulation of K⁺ excretion by the kidney. This hypothesis can be supported by the fact that 17 β -estradiol increases BK(Ca) potassium channel current in a concentration-dependent manner in *Xenopus oocytes*, and progesterone reduces BK(Ca) current and inhibits 17 β -estradiol current stimulation of BK(Ca) potassium channel [46].

Transepithelial Na⁺ transport in nephron segments is not only dependent on the expression and activity of ions channels but is also dependent on Na⁺K⁺-ATPase activity and expression present in the basolateral membrane of the nephron, which establishes the electrochemical driving forces necessary for luminal reabsorption and secretion of Na⁺ and K⁺, respectively [14]. Estrogen is able to increase Na⁺K⁺-ATPase activity and expression in rat kidneys, which increase renal epithelial Na⁺ transport capacity [47, 48]. In ovariectomized female rats, we observed a reduction in Na⁺K⁺-ATPase expression and activity in renal cortex but this reduction was not observed in renal medulla, showing that the mechanisms involved in the regulation of Na⁺K⁺-ATPase expression by female hormones are specific to renal cortex structures. When 17 β -estradiol was replaced in ovariectomized rats, we observed a normalization of Na⁺K⁺-ATPase expression and activity. When progesterone was replaced in ovariectomized rats, we observed that Na⁺K⁺-ATPase expression remained lower but activity was re-established. The Na⁺K⁺-ATPase data suggest that the increase in FE_{Na+} observed in the absence of female hormone and its normalization by hormonal replacement is a coordinate action between Na⁺ channels (e.g. CNG-A1) and Na⁺K⁺-ATPase.

As shown by CNG-A1, 17 β -estradiol is able to modulate Na⁺K⁺-ATPase in IRPT cells demonstrating the direct effects of this hormone in the modulation of Na⁺K⁺-ATPase probably by the association with its receptor. In this experiment, 17 β -estradiol was able to increase expression and activity of this pump only at concentrations higher than 10⁻¹⁰ M (10⁻⁹ M, 10⁻⁸ M and 10⁻⁷ M).

In this study, we did not observe significant changes in urea excretion and GFR suggesting that estrogen and progesterone are not involved in modulation of the mechanisms of urea transport and control of GFR. We observe that urinary flow increased in ovariectomized

female rats. This can be explained by the possible osmotic effect created by increased luminal concentration of this ion induced by low expression of Na⁺K⁺-ATPase and CNG-A1. In this way, lower amounts of water will be reabsorbed specially in proximal tubule where water reabsorption is directed associated with Na⁺ reabsorption.

In this work, we have shown that the CNG-A1 sodium channel is present in renal cortex, specifically in proximal and distal convolute tubules, and it is modulated by estrogen and progesterone. Estrogen and progesterone act in renal tissues modulating CNG-A1 and Na⁺K⁺-ATPase and these effects could be important in Na⁺ and water balance.

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References

- 1 Mendelsohn ME, Karas RH: The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999;340:1801–1811.
- 2 Lonard DM, O'Malley BW: A contemporary understanding of progesterone receptor function. *Mech Ageing Dev* 2004;125:669–78.
- 3 Nadal A, Díaz M, Valverde MA: The estrogen trinity: membrane, cytosolic, and nuclear effects. *News Physiol Sci* 2001;16:251–255.
- 4 Brunette MG, Leclerc M: Effect of estrogen on calcium and sodium transport by the nephron luminal membranes. *J Endocrinol* 2001;170:441–450.
- 5 Morales A, Diaz M, Ropero AB, Nadal A, Alonso R: Estradiol modulates acetylcholine-induced Ca²⁺ signals in LHRH-releasing GT1–7 cells through a membrane binding site. *Eur J Neurosci* 2003;18:2505–2514.
- 6 Brunette MG, Leclerc M: Renal action of progesterone: effect on calcium reabsorption. *Mol Cell Endocrinol* 2002;194:183–190.
- 7 Stachenfeld NS, Keefe DL, Taylor HS: Responses to a saline load in gonadotropin-releasing hormone antagonist-pretreated premenopausal women receiving progesterone or estradiol-progesterone therapy. *J Clin Endocrinol Metab* 2005;90:386–394.
- 8 Stephenson LA, Kolka MA: Plasma volume during heat stress and exercise in women. *Eur J Appl Physiol Occup Physiol* 1988;57:373–381.
- 9 Stachenfeld NS, Taylor HS: Exogenous estradiol and progesterone administration does not cause edema in healthy young women. *Clin Endocrinol* 2007;66:410–418.
- 10 Stachenfeld NS, Taylor HS, Leone CA, Keefe DL: Oestrogen effects on urine concentrating response in young women. *J Physiol* 2003;552:869–880.
- 11 Myles K, Funder JW: Progesterone binding to mineralocorticoid receptors: in vitro and in vivo studies. *Am J Physiol* 1996;270:E601–E607.
- 12 Verlander JW, Tran TM, Zhang L, Kaplan MR, Hebert SC: Estradiol enhances thiazide-sensitive NaCl cotransporter density in the apical plasma membrane of the distal convoluted tubule in ovariectomized rats. *J Clin Invest* 1998;101:1661–1669.
- 13 Sharma PK, Thakur MK: Estrogen receptor expression in mice kidney shows sex differences during aging. *Biogerontology* 2004;5:375–381.
- 14 Vinciguerra M, Deschenes G, Hasler U, Mordasini D, Rousselot M, Doucet A, Vandewalle A, Martin PY, Féraillé E: Intracellular Na⁺ controls cell surface expression of Na,K-ATPase via a camp-independent PKA pathway in mammalian kidney collecting duct cells. *Mol Biol Cell* 2003;14:2677–2688.
- 15 Vandoorpe DH, Ciampolillo F, Green RB, Stanton BA: Cyclic nucleotide-gated cation channels mediate sodium absorption by IMCD (mIMCDK2) cells. *Am J Physiol* 1997;272:C901–C910.
- 16 Gambling L, Dunford S, Wilson CA, McArdle HJ, Baines DL: Estrogen and progesterone regulate α , β , and γ ENaC subunit mRNA levels in female rat kidney. *Kidney Int* 2004;65:1774–1781.
- 17 Kaupp UB, Niidome T, Tanabe T, Terada S, Bönigk W, Stühmer W, Cook NJ, Kangawa K, Matsuo H, Hirose T, et al: Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature* 1989;342:762–766.
- 18 Kaupp UB, Seifert R: Cyclic nucleotide-gated ion channels. *Physiol Rev* 2002;82:769–824.
- 19 Ahmad I, Korbmacher C, Segal AS, Cheung P, Boulpaep EL, Barnstable CJ: Mouse cortical collecting duct cells show nonselective cation channel activity and express a gene related to the cGMP-gated rod photoreceptor channel. *Proc Natl Acad Sci USA* 1992;89:10262–10266.

- 20 Richards MJ, Gordon SE: Cooperativity and cooperation in cyclic nucleotide-gated ion channels. *Biochemistry* 2000;39:14003–14011.
- 21 Misaka T, Kusakabe Y, Emori Y, Gono T, Arai S, Abe K: Taste buds have a cyclic nucleotide-activated channel, CNG gust. *J Biol Chem* 1997;272:22623–22629.
- 22 Dhallan RS, Yau KW, Schrader KA, Reed RR: Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* 1990;347:184–187.
- 23 Dhallan RS, Macke JP, Eddy RL, Shows TB, Reed RR, Yau KW, Nathans J: Human rod photoreceptor cGMP-gated channel: amino acid sequence, gene structure, and functional expression. *J Neurosci* 1992;12:3248–3256.
- 24 McCoy DE, Guggino SE, Stanton BA: The renal cGMP-gated cation channel: its molecular structure and physiological role. *Kidney Int* 1995;48:1125–1133.
- 25 Qiu W, Lee B, Lancaster M, Xu W, Leung S, Guggino SE: Cyclic nucleotide-gated cation channels mediate sodium and calcium influx in rat colon. *Am J Physiol Cell Physiol* 2000;278:C336–C343.
- 26 Novaira HJ, Botelho BF, Goldenberg RC, Guggino SE, Morales MM: Modulation of renal CNG-A3 sodium channel in rats subjected to low- and high-sodium diets. *Biochim Biophys Acta* 2004;1665:101–110.
- 27 Ding C, Potter ED, Qiu W, Coon SL, Levine MA, Guggino SE: Cloning and widespread distribution of the rat rod-type cyclic nucleotide-gated cation channel. *Am J Physiol* 1997;272:C1335–C1344.
- 28 Marcondes FK, Bianchi FJ, Tanno AP: Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol* 2002;62:609–614.
- 29 Moysés MR, Barker LA, Cabral AM: Sex hormone modulation of serotonin-induced coronary vasodilation in isolated heart. *Braz J Med Biol Res* 2001;34:949–958.
- 30 Zolotnitskaya A, Satlin LM: Developmental expression of ROMK in rat kidney. *Am J Physiol Renal Physiol* 1999;276:825–836.
- 31 Shepard M, Dhulipala P, Kabaria S, Abraham NG, Lianos EA: Heme oxygenase-1 localization in the rat nephron. *Nephron* 2002;92:660–664.
- 32 Souza-Menezes J, Morales MM, Tukaye DN, Guggino SE, Guggino WB: Absence of CIC5 in knockout mice leads to glycosuria, impaired renal glucose handling and low proximal tubule GLUT2 protein expression. *Cell Physiol Biochem* 2007;20:455–464.
- 33 De Souza AM, Carvalho TL, Sabino PM, Vives D, Fontes CF, Lopes AG, Caruso-Neves C: Characterization and partial isolation of ouabain-insensitive Na⁺-ATPase in MDCK I cells. *Biochimie* 2007;89:1425–1432.
- 34 Grubmeyer C, Penefsky HS: Cooperatively between catalytic sites in the mechanism of action of beef heart mitochondrial adenosine triphosphatase. *J Biol Chem* 1981;256:3728–3734.
- 35 Grubmeyer C, Penefsky HS: The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphatase. *J Biol Chem* 1981;256:3718–3727.
- 36 Stachenfeld NS, Hugh ST: Effects of estrogen and progesterone administration on extracellular fluid. *J Appl Physiol* 2004;96:1011–1018.
- 37 Johannesson M, Lúdvíksdóttir D, Janson C: Lung function changes in relation to menstrual cycle in females with cystic fibrosis. *Respir Med* 2000;94:1043–1046.
- 38 Nascimento DS, Reis CU, Goldenberg RC, Ortiga-Carvalho TM, Pazos-Moura CC, Guggino SE, Guggino WB, Morales MM: Estrogen modulates CIC-2 chloride channel gene expression in rat kidney. *Pflugers Arch Eur J Physiol* 2003;446:593–599.
- 39 Baiardi G, Macova M, Armando I, Ando H, Tyurmin D, Saavedra JM: Estrogen upregulates renal angiotensin II AT1 and AT2 receptors in the rat. *Regul Peptides* 2005;124: 7–17.
- 40 Gerstner A, Zong X, Hofmann F, Biel M: Molecular Cloning and Functional Characterization of a New Modulatory Cyclic Nucleotide-Gated Channel Subunit from Mouse Retina. *J Neurosci* 2000;20:1324–1332.
- 41 Lindsey SH, Yamaleyeva LM, Brosnihan KB, Gallagher PE, Chappell MC: Estrogen receptor GPR30 reduces oxidative stress and proteinuria in the salt-sensitive female mRen2.Lewis rat. *Hypertension* 2011;58:665–671.
- 42 Stock JL, Coderre JA, Burke EM, Danner DB, Chipman SD, Shapiro JR: Identification of estrogen receptor mRNA and the estrogen modulation of parathyroid hormone-stimulated cyclic AMP accumulation in opossum kidney cells. *J Cell Physiol* 1992;150:517–525.
- 43 Sharma PK, Thakur MK: Estrogen receptor alpha expression in mice kidney shows sex differences during aging. *Biogerontology* 2004;5:375–381.
- 44 Lane PH: Estrogen receptors in the kidney: Lessons from genetically altered mice. *Gender Medicine* 2008;5: S11–S18.
- 45 Esqueda ME, Craig T, Hinojosa-Laborde C: Effect of ovariectomy on renal estrogen receptor-alpha and estrogen receptor-beta in young salt-sensitive and -resistant rats. *Hypertension* 2007;50:768–772.
- 46 Giorgetti A, Nair AV, Codega P, Torre V, Carloni P: Structural basis of gating of CNG channels. *FEBS Lett* 2005;579:1968–1972.
- 47 Wong CM, Tsang SY, Yao X, Chan FL, Huang Y: Differential effects of estrogen and progesterone on potassium channels expressed in *Xenopus* oocytes. *Steroids* 2008;73:272–279.
- 48 Aperia A, Larsson L, Zetterström R: Hormonal induction of Na-K-ATPase in developing proximal tubular cells. *Am J Physiol* 1981;241:F356–F360.