J Membrane Biol (2008) 223:127–139
DOI 10.1007/s00232-008-9119-3

# Epithelial Sodium Channel in a Human Trophoblast Cell Line (BeWo)

**Silvana del Mónaco · Yanina Assef · Basilio A. Kotsias**

Received: 7 April 2008 / Accepted: 1 July 2008 / Published online: 30 July 2008


**Abstract** The present study was performed to assay sodium currents in BeWo cells. These cells comprise a human trophoblast cell line which displays many of the biochemical and morphological properties similar to those reported for the in uterus proliferative cytotrophoblast. For whole-cell patch-clamp experiments, BeWo cells treated for 12 h with 100 nM aldosterone were exposed to 8Br-cAMP, a membrane-permeable cAMP analogue, to induce channel activity. Cells showed an amiloride-sensitive ion current ($IC_{50}$ of 5.77 μM). Ion substitution experiments showed that the amiloride-sensitive current carried cations with a permeability rank order of $Li^+ > Na^+ > K^+ >$ NMDG ($P_{Li}/P_{Na} = 1.3$, $P_K/P_{Na} = 0.6$, $P_{NMDG}/P_{Na} = 0.2$). In cells pretreated with aldosterone, we observed that nearly half of successful patches had sodium channels with a linear conductance of 6.4 ± 1.8 pS, a low voltage-independent $P_o$ and a $P_K/P_{Na}$ of 0.19. Using RT-PCR, we determined that control cells express the α-, but not β- and γ-, epithelial sodium channel (ENaC) mRNA. When cells were treated with aldosterone (100 nM, 12 h), all α-, β- and γ-ENaC mRNAs were detected. The presence of ENaC subunit proteins in these cells was confirmed by Western blot analysis and immunolocalization with specific ENaC primary antibodies. In summary, our results suggest that BeWo cells express ENaC subunits and that aldosterone was able to modulate a selective response by generating amiloride-sensitive sodium currents similar to those observed in other human tissues.

S. del Mónaco · Y. Assef · B. A. Kotsias (✉)
Laboratorio de Neurofisiología, Instituto de Investigaciones Médicas A. Lanari, University of Buenos Aires, C. de Malvinas 3150, Buenos Aires 1427, Argentina
e-mails: kotsias@mail.retina.ar; kotsias@yahoo.com

**Keywords** ENaC · BeWo cell · Aldosterone · Patch clamp

## Introduction

The maternal supply of minerals that are important body constituents, such as $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, phosphate and water, requires a specific placental transport mechanism to secure fetal growth. Sodium is the main extracellular cation, and its osmotic pressure is an important determinant of the extracellular fluid volume. Trophoblast plasma membranes contain various $Na^+$ transport systems that participate in nutrient transfer to the fetus and in maintaining cytosol homeostasis (Stulc 1997). We were particularly interested in the expression and functional characteristics of the epithelial sodium channel (ENaC) in human placenta as we confirmed the presence of ENaC subunit proteins in normal syncytiotrophoblasts with molecular biological techniques. We found an altered expression pattern in placentas from women suffering arterial hypertension related to pregnancy or preeclampsia (del Mónaco et al. 2006).

ENaC mediates $Na^+$ entry into the cells from the luminal fluid in many reabsorbing epithelia (Alvarez de la Rosa et al. 2000); is aldosterone-, vasopressin-, insulin- and catecholamine-inducible (Garty and Palmer 1997; Alvarez de la Rosa et al. 2000; Moretó et al. 2005); is modulated by intracellular sodium (Anantharam et al. 2006), estrogens, progesterone (Gambling et al. 2004; Quesnell et al. 2007) and membrane tension (Wei et al. 2007); and is sensitive to phosphatidylinositides (Ma et al. 2002), hypotonic stress (Taruno et al. 2008) and ClC-5 channels (Mo and Wills 2004; Bachhuber et al. 2005). ENaC is blocked by amiloride and its analogues and characterized by its high permeability for $Na^+$ over other monovalent cations (Garty

and Palmer 1997; Alvarez de la Rosa et al. 2000). The channel is composed of three homologue subunits: α, β and γ. The suggested subunit stoichiometry varies according to different studies, in which three, four and nine subunit assemblages have been proposed (Snyder et al. 1986; Canessa et al. 1994; Staruschenko et al. 2005). The latest model arises from the recent crystal structure of the homologous acid-sensing ion channel (ASIC), where it is inferred that ENaC may be an α–β–γ trimer (Jasti et al. 2007). The described sizes of ENaC subunits vary between species, tissues and cells and have been reported to be products of both differential glycosylation and proteolytic cleavage once expressed in the membrane (Masilamani et al. 1999; Wodopia et al. 2000; Alvarez de la Rosa et al. 2000; Hughey et al. 2003). The α-subunit of ENaC is present in the placenta of pigs (Page et al. 2003), whereas in human placenta the mRNA for the α- and γ-subunits was detected (Driver et al. 2003). The presence of the α-subunit, the channel-forming subunit of ENaC, raises the question about the role that ENaC and its subsequent hormone-regulated $Na^+$ transport might play in normal human placental tissues. In aldosterone-responsive epithelial cells like kidney or colon, abnormalities that upregulate its activity lead to increased $Na^+$ uptake and hypertension (as seen in Liddle syndrome), whereas diminished channel activity can result in the pseudohypoaldosteronism type I syndrome with salt loss and hypotension (Rossier et al. 2002). Furthermore, lung infection, inflammation and hypoxia are accompanied by reduced α-ENaC expression (Wodopia et al. 2000; Dagenais et al. 2004).

The present work studied sodium currents in the BeWo cell line, a human hormone-synthesizing trophoblastic cell line established in continuous culture by Pattillo and Gey (1968). These cells comprise a human trophoblast cell line which displays many biochemical and morphological properties similar to those reported for the in utero proliferative cytotrophoblast during the last trimester of pregnancy (Liu et al. 1997). Even though these cells represent an immortalized and modified cell line, the membrane transport systems expressed in BeWo cells are highly similar to those reported in normal human trophoblasts; therefore, they have been widely used as a model to investigate the placental transport mechanisms of amino acids, immunoglobulins and fatty acids (Zhao and Hundal 2000; Huang and Swaan 2001). Some channels and transporters have been studied in BeWo cells, e.g., the $\alpha_{1S}$-subunit of the L-type $Ca^{2+}$ channel, the calcium transporter protein type 1 (CaT1), plasma membrane $Ca^{2+}$-ATPases (PMCA1–4), $Na^+/Ca^{2+}$ exchangers (NCX1 and NCX3), $Na^+/H^+$ exchangers (NHE1 and NHE3) and the Na–K–Cl cotransporter (Silva et al. 1997; Zhao and Hundal 2000; Moreau et al. 2003). However, no information regarding sodium or other ion channels is presently known. In this study we performed standard molecular biological techniques and used a functional approach with electrophysiological measurements to analyze the expression and activity of sodium currents in BeWo cells. Our results indicate that this cell line expresses ENaC subunits and that aldosterone was able to modulate a selective response by generating amiloride-sensitive sodium currents similar to those observed in other human tissues (Ma et al. 2004). Some of the preliminary experiments were presented at the meeting of the Sociedad Argentina de Investigación Clínica (del Mónaco et al. 2006).

## Materials and Methods

### Cell Culture

The BeWo cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in HAM's F12 medium (GIBCO BRL, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS; Natocor Biotechnology, Córdoba, Argentina), 2 mM L-glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were harvested once a week with 0.25% trypsin-EDTA (GIBCO BRL, Life Technologies) and kept in an incubator at 37°C in humid air with constant 5% $CO_2$. Culture medium was supplemented with 100 nM aldosterone (Sigma) when needed. Human colon carcinoma T84 cells (American Type Culture Collection) were maintained in DMEM/F12 medium (GIBCO BRL, Life Technologies) supplemented with 10% FBS.

### RT-PCR

Total RNA from BeWo cells was isolated using the SV Total RNA isolation system (Promega, Madison, WI). Reverse transcription (RT) was performed for 60 min on 5 μg of total RNA (final volume 20 μl) using Moloney murine leukemia virus reverse transcriptase, oligo (dT)15 primer and 400 μM of each deoxyribonucleotide triphosphate (dNTP) at 42°C (Promega). Polymerase chain rection (PCR, 50 cycles) was performed at 94°C for 60 s, 58°C for 60 s and 72°C for 60 s, followed by a final extension of 6 min at 72°C. The reaction was carried out using 5 μM of specifically designed oligonucleotide primers. α-ENaC primers amplified an expected 257-bp fragment (sense 5′-GAACAACTCCAACCTCTG GATGTC-3′, antisense 5′-TCTTGGTGCAGTCGCCATA ATC-3′), β-ENaC primers amplified an expected 277-bp fragment (sense 5′-TGCTGTGCCTCATCGAGTTTG-3′, antisense 5′-TGCAGACGCAGGGAGTCATAGTTG-3′) and γ-ENaC primers amplified an expected 237-bp fragment (sense 5′-TCAAGAAGAATCTGCCCGTGAC-3′, antisense 5′-GGAAGTGGACTTTGATGGAAACTG-3′). To test for the presence of genomic DNA, primers for the α-subunit were

designed to span introns between exons 5 and 6 and between exons 6 and 7. Thus, the expected size for the subunit using genomic DNA was 1,023 bp, instead of the 257-bp size expected for a cDNA template (Bubien et al. 2001). PCR products were loaded in ethidium bromide–stained 2% agarose gels in Tris-borate-EDTA buffer (TBE). Images of these gels were acquired with Foto Analyst Investigator (Fotodyne, Hartland, WI). PCRs with actin primers were performed over each sample, to test cDNA integrity (data not shown).

## Immunoblotting

BeWo cell layers were thoroughly washed with phosphate-buffered saline (PBS) buffer (GIBCO BRL, Life Technologies), and cells were scraped into ice-cold PBS buffer with 0.01x Protease Inhibitor Cocktail and 0.2 mM phenylmethanesulfonyl fluoride (Sigma), centrifuged and resuspended in lysis buffer (0.3 M NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 1% Triton ×100; pH 7.4) with protease inhibitors. The homogenate was spun for 5 min at 10,000 rpm (4°C) and the supernatant collected and stored at −20°C. Total protein in each sample was quantified using the Bradford protein assay (Bradford Reagent, Sigma).

For immunoblot studies, 75 μg of BeWo proteins were dissolved in loading buffer (4% sodium dodecyl sulfate, 0.125 M Tris-HCl [pH 6.8], 0.2 M dithiothreitol, 0.02% bromophenol blue, 20% glycerol), heated to 100°C for 2 min, resolved on 8% polyacrylamide gel and electrotransferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Aylesbury, UK). Membranes were blocked for 1 h with 2% (w/v) defatted milk in Tris-buffered saline–Tween 0.1% (T-TBS) at room temperature and incubated overnight with ENaC antibodies in T-TBS buffer supplemented with 0.5% (w/v) bovine serum albumin. To detect ENaC subunits, we used rabbit polyclonal antibodies directed against amino acid residues 131–225 of human α-ENaC and residues 411–520 of human γ-ENaC at a dilution of 1:1,000 and a mouse monoclonal antibody directed against amino acid residues 271–460 of human β-ENaC at a dilution of 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with T-TBS and incubated for 1 h at room temperature with goat anti-rabbit (for α- and γ-ENaC) or horse anti-mouse (for β-ENaC) secondary antibodies conjugated to peroxidase (1:5,000; Vector, Burlingame, CA). Filters were washed, and immunoreactivity was detected using the ECL Western Blotting Analysis System (Amersham Biosciences, Buenos Aires, Argentina) according to the manufacturer's instructions. The chemiluminescence reaction was visualized on AGFA Medical X-Ray films (Agfa-Gevaert, Buenos Aires, Argentina). The same protocol was followed to obtain and process T84 protein extracts.

## Immunocytochemistry

BeWo cells were grown in coverslips and cultured with HAM's F12 medium until optimal cell concentration. Different treatments were carried out: (1) cells without stimulation, (2) cells stimulated with 100 nM aldosterone (12 h) and (3) cells stimulated with 100 nM aldosterone (12 h) and 100 μM 8Br-cAMP for 30 min at room temperature before fixation (Sigma). After washing in culture medium, cells were fixed with a solution containing 3.7% formaldehyde and 0.12 M sucrose in 50 mM PBS for 10 min at room temperature, followed by cell permeabilization with 100% methanol at −15°C for 15 min. Then, cells were washed with PBS buffer twice and incubated with 0.1% $H_2O_2$ for 40 min and with blocking solution (2% BSA in PBS buffer) for 1 h at room temperature. Coverslips were incubated overnight with the primary antibodies described above at a dilution of 1:100 (Santa Cruz Biotechnology). Sections were then washed at 5 min with 0.05 M PBS, incubated for 10 min in prediluted link antibody, washed again in PBS buffer and incubated for 30 min in a solution of streptavidin-conjugated horseradish peroxidase in PBS buffer. Color development of the antibody labeling was achieved under microscopic control by incubating slices with the substrate 3,3′-diaminobenzidine/nickel chloride (DAB/$NiCl_2$) and 0.3% hydrogen peroxide in distilled water. Control studies were performed by preincubating the antibody with its respective blocking peptide.

## Whole-Cell Recordings

The cells were grown in glass coverslips and immediately before use thoroughly washed with bath solution and transferred into the patch recording chamber mounted on the stage of an Olympus (Tokyo, Japan) inverted microscope. The electrical activity of the cells was recorded in the whole-cell configuration with standard patch-clamp technology using an amplifier (List L/M-EPC7; List Medical Electronics, Darmstadt, Germany) with a 1 GΩ feedback resistor. Electrical signals were filtered at 1 kHz and digitized at 5 kHz using Pclamp v.8 software (Axon Instruments, Union City, CA). Whole-cell configuration was established with polished standard glass micropipettes (World Precision Instruments, Sarasota, FL) with a tip resistance of 2–4 MΩ when filled with pipette solution. The series resistance ($R_s$) was compensated using the analog circuit of the amplifier. The protocol applied pulses from +60 to −140 mV in steps of 20 mV, from a holding potential of 0 mV. This protocol enabled us to prevent intracellular $Na^+$ accumulation and ENaC self-inhibition

by clamping the membrane voltage to values that minimize $Na^+$ current and reduce the electrochemical driving force for $Na^+$ entry (Segal et al. 2002). There was a 10-ms interval between each pulse. Current–voltage (*I–V*) plots were obtained from currents measured in a stationary state and expressed as current densities (current per unit cell capacitance). The capacitance of the cells ($C_m$) was measured by applying 100 ms, 10 mV, depolarizing pulses from a holding potential of 0 mV. The currents were fitted to an exponential function and the time constant ($\tau$) was measured; thus, $C_m = \tau/R_s$. All experiments were done at room temperature (20–24°C).

The amiloride-sensitive current ($I_{amil}$) was determined at −140 mV as control − amiloride at different amiloride concentrations, and the resulting concentration–response curve was fitted with a Hill equation to calculate the amiloride concentration required for half-maximal block ($IC_{50}$).

Permeability ratios ($P_X/P_{Na}$) were estimated from the shifts of the reversal potential ($\Delta E_{rev}$) induced by replacing external $Na^+$ with cation X ($K^+$, $Li^+$ or NMDG) using the modified Goldman-Hodgkin-Katz (G-H-K) equation: $\Delta E_{rev} = E_{rev}(X) - E_{rev}(Na) = (RT/zF) * \ln (P_X [X]_o / P_{Na} [Na]_o)$, where $[Na]_o$ is the extracelullar concentration of $Na^+$, $[X]_o$ is the extracellular concentration of X and *R, T, z* and *F* have their usual thermodynamic meanings.

Membrane potentials were corrected for junction potentials at the bath reference electrode for the different solutions used (software by Dr. Peter Barry, University of New South Wales, Sydney, Australia).

Pipette solution (intracellular) contained (mM) 102 K-gluconate, 6 KCl, 4 $MgCl_2$, 3 EGTA, 10 HEPES, 5 sucrose (pH 7.4). The bath solution contained (mM) 105 NaCl, 4 KCl, 3 $CaCl_2$, 3 $MgCl_2$, 10 HEPES, 40 sucrose (pH 7.4). NaCl was replaced with equimolar concentrations of NMDG-Cl, KCl and LiCl when specified. Solution osmolarity was measured with a vapor pressure osmometer (5100B; Wescor, Logan, UT): 287 ± 9 mOsm/l for intracellular solution, 305 ± 2 mOsm/l for NaCl bath solution, 312 ± 1 mOsm/l for KCl bath solution, 303 ± 2 mOsm/l for NMDG-Cl bath solution and 307 ± 3 mOsm/l for LiCl bath solution (four measurements).

Cells were exposed to 100 μM 8Br-cAMP for 30 min and to 0.01–100 μM amiloride (Sigma) (in the presence of cAMP) until maximal blockage was achieved. Drugs were diluted in distilled water and DMSO and added directly to the chamber containing the cells in solution. DMSO final concentration per se did not affect ion channel activity (data not shown).

### Single-Channel Recordings

The activity of ion channels was recorded from inside–out patches using a Warner PC-501A amplifier (Warner Instruments, Hamden, CT) with a 10 GΩ feedback resistor. Standard glass micropipettes were used (World Precision Instruments) with a tip resistance of 5–10 MΩ when filled with pipette solution. Electrical signals were filtered at 2 kHz and digitized at 5 kHz using Pclamp v.8.2 software. Single-channel records were further filtered for display purposes only. All experiments were carried out at room temperature (20–24°C). Histograms of event duration were fitted with appropriate exponential models (single or double exponentials), and the mean open or closed time was obtained from these fits. Exponential functions were fitted using a least-squares routine of Pclamp6.

To calculate the anion permselectivity ratio under Na-gluconate and K-gluconate asymmetrical conditions, current amplitudes were best fitted to the G-H-K equation:

$$I(V_h) = z_i^2 F^2 P_i V_h \{C_i/[1 - \exp(-\alpha)]\} + \left(z_j^2 F^2 P_j V_h/RT\right)\{C_j/[1 - \exp(\beta)]\}$$

where *I* is current amplitude, *i* (species in extracellular compartment) and *j* (species in intracellular compartment) represent $Na^+$ or $K^+$ depending on their location on either side of the membrane, $V_h$ is the holding potential (in mV) and $z_i$ and $z_j$ are the charges for species *i* and *j*, respectively. $P_i$ and $P_j$ represent the permeability coefficients for the species *i* and *j*, respectively, and $\alpha = z_i F V_h/RT$ and $\beta = z_j F V_h/RT$, where *R, T* and *F* have their usual meanings.

Pipette and bath solution contained (mM) 140 Na-gluconate, 4 NaCl, 10 HEPES (pH 7.4), to favor the study of $Na^+$ channels. In selectivity experiments, internal $Na^+$ was replaced with an equimolar concentration of $K^+$.

### Statistical Analysis

Data were expressed as mean values ± SD (*n,* number of cells analyzed). Statistical analysis was performed using analysis of variance or Student's *t*-test for data comparisons. Differences were considered statistically significant at $P < 0.05$.

## Results

### Molecular Detection of ENaC in BeWo Cells

*RT-PCR* The presence of amiloride-sensitive ENaC mRNA in BeWo cells was evaluated by PCR amplification of the α-, β- and γ-ENaC subunits. β-Actin was amplified as a housekeeper transcript to control for cDNA integrity (data not shown). RT-PCR yielded specific α-, but not β- and γ-, ENaC products when these cells were cultivated in control conditions. However, in aldosterone-treated cells (100 nM, 12 h) α-, β- and γ-ENaC fragments were detected, with

expected band sizes (257, 277 and 237 bp, respectively), indicating a hormonal influence in channel subunit transcription (Fig. 1a).

*Western blot analysis* To assess whether ENaC subunits may be translated into proteins in BeWo cells, Western blot analysis was conducted in cells cultured with and without aldosterone (100 nM, 12 h). Figure 1b shows bands of the expected size for ENaC subunits, including band doublets for α- and γ-ENaC that apparently result from protein cleavage: ~95 and ~30 kDa for α-ENaC, ~110 kDa for β-ENaC, ~95 and ~75 kDa for γ-ENaC (Hughey et al. 2003; Harris et al. 2007). Control cells showed only α-ENaC labeling. Aldosterone-treated cells showed the corresponding bands for the three subunits. T84 cell line protein extracts were analyzed simultaneously as a positive control (Iordache and Duszyk 2007).

*Immunocytochemistry* The presence of ENaC subunit proteins in BeWo cells was also determined by immunolocalization with specific ENaC primary antibodies, as shown in Fig. 2. No β- or γ-ENaC antibody signal was detected in nonstimulated cells, whereas a subtle signal was obtained for α-ENaC. Labeling of all three subunits was observed in aldosterone-treated BeWo cells, mainly in the cytoplasm, whereas in aldosterone + cAMP–treated cells labels were observed in the plasma membrane. Methylene blue was used to counterstain cell nucleus integrity. No signal was observed when primary antibody was incubated with its corresponding blocking peptide.

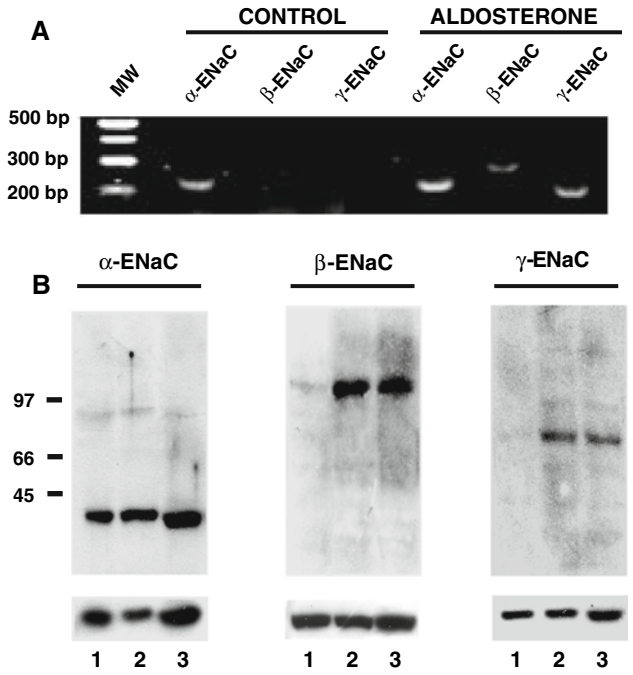


**Fig. 1** (**a**) mRNA detection of ENaC subunits in BeWo cells. Control total RNA of BeWo cells (*left*) was used along with specific templates for α-, β- and γ-ENaC subunits to RT-PCR as described in "Materials and Methods." Only α-ENaC was detected in control conditions, whereas in BeWo cells treated with aldosterone (100 nM, 12 h) the α-, β- and γ-subunit mRNAs were detected. (**b**) Western blot analysis in BeWo cells cultured in control conditions (*lanes 1*) and supplemented with 100 nM aldosterone for 12 h (*lanes 2*). Hormone-treated cells show the presence of α-, β- and γ-ENaC subunits. Protein cleavage is observed for α- and γ-subunits. T84 cell line extracts were analyzed under the same protocols as a positive control (*lanes 3*)

## Electrical Activity of BeWo Cells

*Induction of* $I_{amil}$ *by aldosterone* We determined ENaC activity in BeWo cells by measuring basal currents in response to depolarizing and hyperpolarizing pulses and registering under whole-cell conditions. A cAMP signaling pathway regulates the recruitment of ENaC from subapical storage pools to increase channel number (*N*) in the apical membrane (Butterworth et al. 2005), and the channel is blocked by the pyrazine diuretic amiloride. We used 8Br-cAMP, a membrane-permeable cAMP analogue, to induce channel exposition to the cell surface. Figure 3a shows average current densities (current per unit cell capacitance, mean $C_m = 31.3 \pm 14.8$ pF, $n = 98$) vs. voltages from cells cultured without supplementing culture medium with aldosterone. Current densities were measured before and after stimulation with 100 μM 8Br-cAMP and after adding 10 μM amiloride to the bath solution (with cAMP). No significant amiloride-sensitive current was observed under this condition. On the other hand, when cells were cultured with 100 nM aldosterone for 12 h, cAMP activation was observed with an amiloride-sensitive component (Fig. 3b). Figure 3c shows average current densities vs. voltages for aldosterone-treated cells. The difference between activated and blocked currents was significant at all tested potentials ($P < 0.05$, $n = 8$). These currents activate very fast and show time dependence only in the most hyperpolarizing pulses. Aldosterone-treated cells showed a 30-fold increase in current intensity after adding cAMP to the bath solution and a reduction of 86% of that current after amiloride treatment in the −140 mV pulse applied. The value of inward conductance (−40 to −140 mV) for the cAMP-stimulated current expressed as conductance density was 165.5 ± 43.9 pS/pF, and mean reversal potentials ($V_{rev}$) were −19.9 ± 18.3 and 4.3 ± 7.6 mV for control and cAMP-treated cells, respectively ($P < 0.05$), indicating that cAMP induces a significant shift of $V_{rev}$ to more positive values. Figure 3d shows a representative current measurement for the −140 mV pulse applied during the time course of the experiment. This analysis allowed us to estimate the time of incubation for each drug and the achievement of a maximal effect. We found that 25–30 min exposure to cAMP and 8 min exposure to amiloride resulted in no further change in current amplitude.

*Ion selectivity and amiloride sensitivity* To characterize the amiloride-sensitive current of aldosterone-treated BeWo

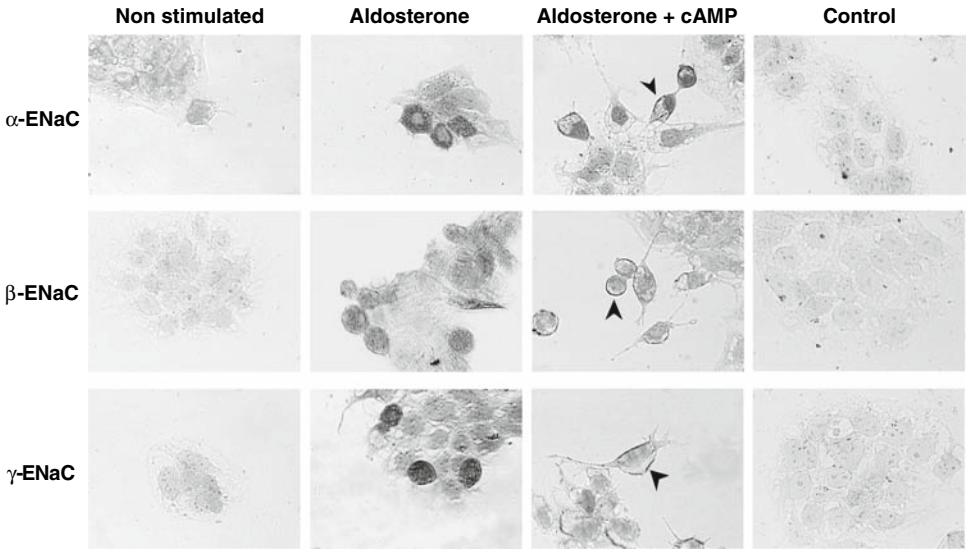


**Fig. 2** Expression of α-, β- and γ-ENaC subunits in BeWo cells. Phase contrast microscopy (×400). Different treatments were carried out: cells cultured without stimulation (*first column*), cells cultured for 12 h with aldosterone 100 nM (*second column*) and cells cultured with aldosterone 100 nM for 12 h + 100 μM 8Br-cAMP for 30 min before fixation (*third column*). Control studies were performed, incubating primary antibodies with the respective blocking peptide (*fourth column*). No β- and γ-ENaC antibody signal was detected in nonstimulated cells, while a subtle signal was obtained for α-ENaC. Labeling of all three subunits was observed in aldosterone-treated BeWo cells. No signal was observed when primary antibody was incubated with its corresponding blocking peptide. Arrows show antibody signal in the plasma membrane

cells, $Na^+$ from the bath solution was replaced with different cations. Figure 4a shows, on the left, an example of whole-cell currents before and after stimulation with 100 μM 8Br-cAMP and after the replacement of external (bath) $Na^+$ for equimolar concentrations of NMDG (in the presence of cAMP) and, on the right, the corresponding relationship between current densities (pA/pF) and the voltages applied. The current stimulated with cAMP was significantly reduced after the replacement in every voltage pulse applied ($P < 0.05$, $n = 7$). $V_{rev}$ values were $-33.6 \pm 21.4$ and $1.3 \pm 10.6$ mV for control and cAMP-treated cells, respectively ($P < 0.01$); and after $Na^+$ replacement, this parameter returned to a value of $-33.5 \pm 7.1$ mV ($P < 0.001$), not different from the one observed in control conditions. Figure 4b shows similar experiments but replacing external $Na^+$ for equimolar concentrations of $K^+$ (in the presence of cAMP). A representative *I–V* relationship (right) indicates that the current activated with cAMP was not altered with $K^+$ as the main extracellular cation ($n = 6$). $V_{rev}$ values were $-19.7 \pm 7.3$ and $9.3 \pm 4.5$ mV for control and cAMP-treated cells, respectively ($P < 0.001$); and after $Na^+$ replacement, the parameter did not change compared with the previous condition, being $8.8 \pm 5.1$ mV. Figure 4c shows replacement of external $Na^+$ for equimolar concentrations of $Li^+$ (in the presence of cAMP). The *I–V* curve (right) indicates that the current activated with cAMP maintained its intensity after the replacement ($n = 7$). In this case, values of $V_{rev}$ were $-6.8 \pm 11.9$ and $7.1 \pm 3.2$ mV for control and cAMP-treated cells, respectively ($P < 0.001$) and $7.0 \pm 4.5$ mV with an external solution with $Li^+$ instead of $Na^+$, not different from the previous condition.

Aldosterone and cAMP induced an increase in inward currents that reversed by adding amiloride (10 μM) to the bath solution. We measured $I_{amil}$ in aldosterone-treated cells, substituting external $Na^+$ by $K^+$, $Li^+$ and NMDG in bath solutions previously stimulated with cAMP. $V_{rev}$ values were determined from the *I–V* curves in the presence of external $Na^+$, $K^+$, $Li^+$ and NMDG (Fig. 5a). $V_{rev}$ values were $6.6 \pm 6.4$, $-8.3 \pm 11.8$, $7.4 \pm 4.8$ and $-30.9 \pm 26.4$ mV, respectively. $I_{amil}$ permeability to $K^+$, $Li^+$ and NMDG relative to $Na^+$ was calculated from the shifts of $V_{rev}$ using the G-H-K equation. The resulting permeability coefficients were 0.2 for $P_{NMDG}/P_{Na}$, 0.6 for $P_K/P_{Na}$ and 1.3 for $P_{Li}/P_{Na}$, indicating a relative permeability rank order of $Li^+ > Na^+ > K^+ >$ NMDG. $I_{amil}$ in each extracellular solution tested presented conductance densities of $48.0 \pm 15.4$, $16.7 \pm 9.3$, $70.5 \pm 26.6$ and $6.3 \pm 4.0$ pS/pF for $Na^+$, $K^+$, $Li^+$ and NMDG, respectively (Fig. 5b). Conductances in $Na^+$ and $Li^+$ were significantly different from those in $K^+$ and NMDG ($P < 0.05$), as indicated with asterisks. Figure 5c shows that amiloride induced a dose-dependent inhibition in the inward current stimulated with aldosterone. The inhibition by amiloride occurred with an $IC_{50}$ of 5.77 μM. The effect of the blocker was monitored over a period of 10 min and maintained over at least 10 more min.

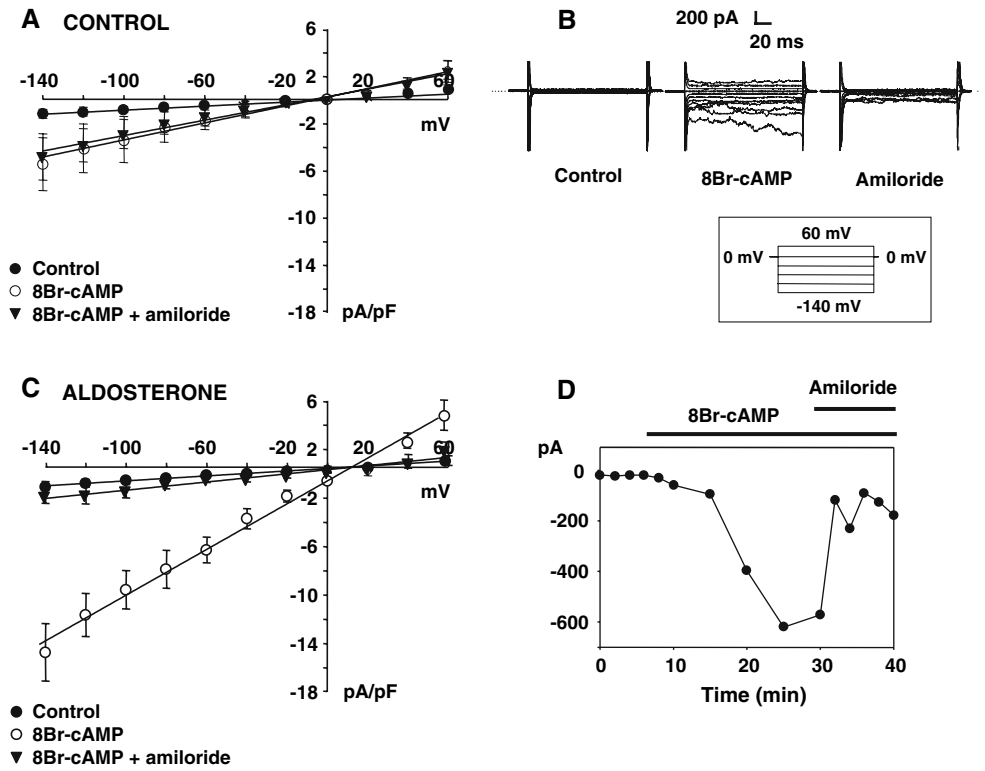


**Fig. 3** Whole-cell currents in BeWo cells. (**a**) Relationships between current density (current per unit cell capacitance, pA/pF, media ± SD) and the voltages applied, obtained culturing BeWo cells without aldosterone ($n = 8$). (**b**) Representative traces of whole-cell currents in a cells cultured with 100 nM aldosterone (12 h). *Left*, Control conditions; *middle*, after adding 100 μM 8Br-cAMP to the bath solution; and *right*, 100 μM 8Br-cAMP + 10 μM amiloride in the bath solution. *Inset*: Protocol used to study ion currents in all the experiments, consisting of pulses between +60 and −140 mV in 20-mV steps, from 0 mV holding potential. (**c**) *I–V* relationship in BeWo cells cultured with 100 nM aldosterone (12 h) ($n = 7$). Aldosterone-treated cells showed a current increase in every pulse applied when exposed to cAMP ($P < 0.05$) and that current was blocked afterward with amiloride ($P < 0.05$). (**d**) Representative current measurement (−140 mV pulse) during the time course of the experiment. After stabilization of the basal currents, the cells were treated first with cAMP for 30 min and then with amiloride until maximal blockage was achieved

## Single-Channel Activity

Single $Na^+$ channels were observed after application of depolarizing and hyperpolarizing pulses in symmetrical Na-gluconate solutions. Theoretically, ENaCs are distinguished by the slow kinetics of gating, long spontaneous open and closed times, a 4–5 pS single-channel conductance and absence of voltage dependence of gating. In inside–out patches from BeWo cells treated with aldosterone (12 h), we observed sodium channels with a linear, nonrectifying conductance of 6.4 ± 1.8 pS ($V_{rev} = 1.4 \pm 5.1$ mV, $n = 22$) over the voltage range studied (−120 to 120 mV) (Fig. 6a, b). In cells pretreated with the hormone, nearly half of successful patches had sodium channels with these characteristics, whereas in nontreated cells approximately 1/10 successful patches showed ENaC-like currents. The frequency of appearance was the only difference found between channels observed in control and aldosterone-treated cells since single-channel characteristics (conductance, open probability, permeability and kinetics) did not change (Fig. 6c, d).

ENaC open probability ($P_o$) can vary from near 0 to near 1 in standardized patch-clamp conditions, due at least in part to previous history of the cells in terms of hormonal and feedback regulation (Garty and Palmer 1997). In this study, the sodium channels observed in aldosterone-treated cells showed a low $P_o$ (0.09 ± 0.10 at −80 mV), and this $P_o$ was voltage-independent ($P > 0.05$) (Fig. 7a). Figure 7b shows a histogram of open and closed current amplitudes, where the low $P_o$ can also be observed (−100 mV pulse in the patch pipette). The mean open and closed times of single-channel activity were determined from histograms of event duration. In Fig. 7c, d, a pair of such histograms are presented for the case when a −100 mV potential was applied to the patch pipette. The sodium channels studied presented two open time constants, 1.4 ± 0.5 and 27.9 ± 18.6 ms, and a closed time constant of 932.4 ± 177.3 ms ($n = 16$). We substituted internal (bath) $Na^+$ by equimolar concentrations of $K^+$ and observed a significant reduction in outward currents, from membrane potential of 40 to 120 mV ($P < 0.05$, $n = 12$) (Fig. 8a). $V_{rev}$ values were determined from the *I–V* curves, shifting from 2.0 ± 5.2 to

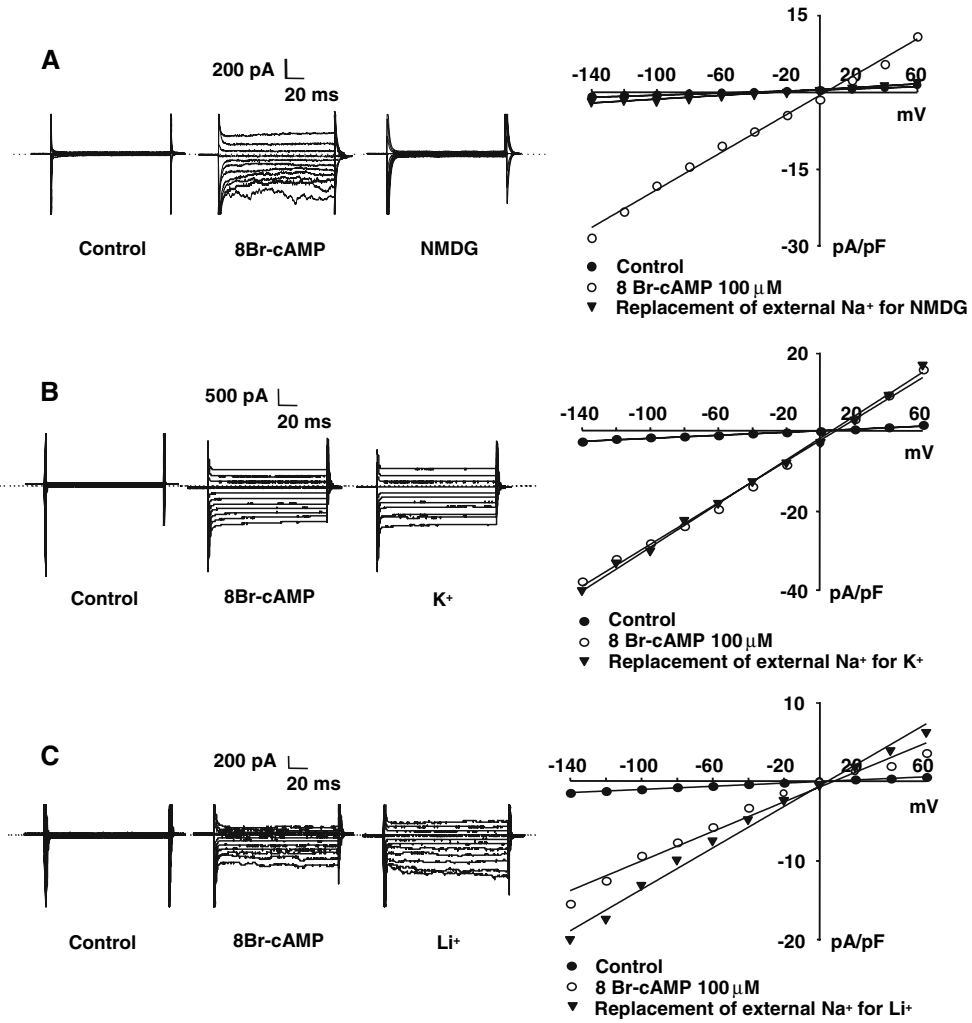


**Fig. 4** Ion selectivity of aldosterone-induced currents in BeWo cells. Aldosterone treatment = 100 nM, 12 h. Each row depicts the results obtained from a single cell. *First column* displays whole-cell currents in control conditions. *Second column* displays whole-cell currents from the same cells after adding 100 µM 8 Br-cAMP. *Third column* displays whole-cell currents after replacing external $Na^+$ with NMDG (*top row*), $K^+$ (*middle row*) or $Li^+$ (*bottom row*) in the presence of cAMP (representative of $n = 7$). Cells showed a current increase in every pulse when exposed to cAMP ($P < 0.05$), and that current diminished after the Na replacement with NMDG ($P < 0.05$) but not with $K^+$ or $Li^+$. *Fourth column* shows the corresponding current density–voltage relationships. NMDG induced a positive shift on the reversal potential. In contrast, $K^+$ and $Li^+$ were less effective at altering the reversal potential

46.0 ± 19.9 mV in the presence of internal $Na^+$ and $K^+$, respectively ($P < 0.0001$). The resulting permeability coefficient $P_K/P_{Na}$ was 0.19, indicating a high permeability for $Na^+$ over $K^+$ for these channels. $P_o$ was not altered after $K^+$ substitution ($P > 0.05$), values remaining lower than 0.2 in every applied pulse (Fig. 8b).

## Discussion

In human placental chorionic villi, a layer of syncytiotrophoblasts lies over the cytotrophoblasts and surrounds the internal mesoderm and fetal capillaries. BeWo cells may serve as an in vitro model for studying the regulation of transplacental transport and uptake mechanisms, although to our knowledge there are no reports on studies of sodium channels with electrophysiological techniques in these cells. Here, we show the expression of the three subunit components of ENaC in BeWo cells with molecular biological techniques and the functional and pharmacological properties of the amiloride-sensitive current using standard electrophysiological techniques.

The expression and activity of ENaC are regulated by several factors, including long-term regulation by aldosterone and short-term regulation by cAMP (Butterworth et al. 2005). An intracellular cAMP rise may be generated by different stimuli in human cells, such as vasopressin, glucagon, parathyroid hormone, calcitonin, prostaglandin $E_2$ and ß-adrenergic analogues, among others. We used 8Br-cAMP, a membrane-permeable cAMP analogue, to stimulate ENaC-transported sodium currents. This drug permeates the plasma membrane and increases intracellular cAMP levels, directly activating protein kinase A. A body of evidence has accumulated to suggest that channels are recruited from intracellular stores and inserted into the apical membrane on cAMP stimulation, action that has been demonstrated previously using electrophysiological, biochemical and imaging methods (Snyder 2000; Perrotti et al. 2001; Morris and Schafer 2002; Butterworth et al. 2005; Yang et al. 2006). Taking this into consideration, we

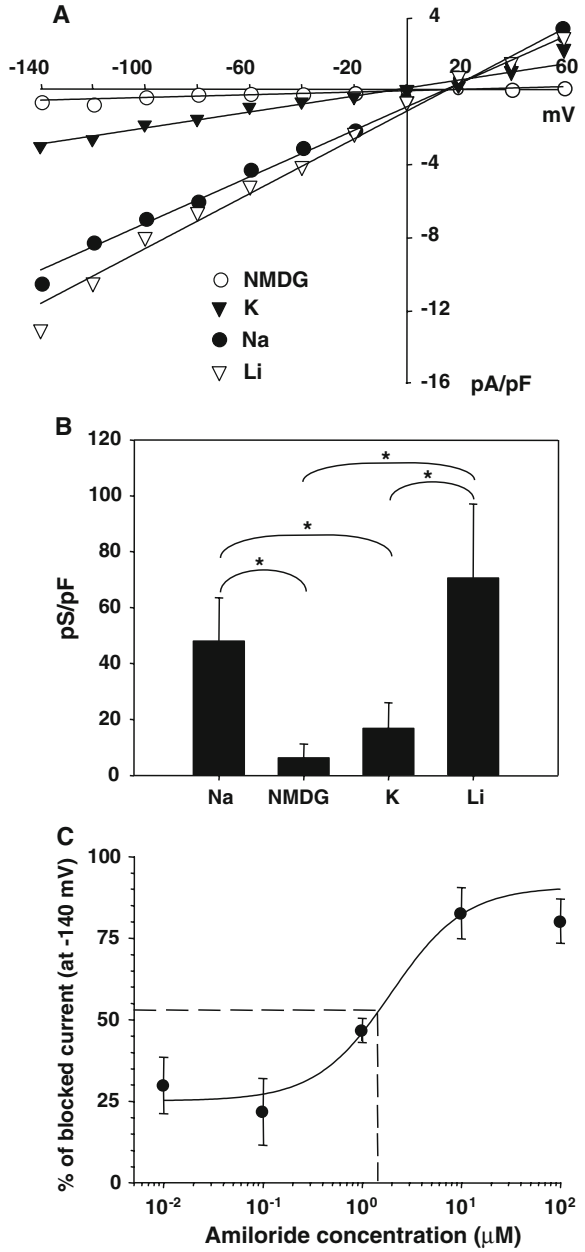


**Fig. 5** Ion selectivity sequence and blockade by amiloride in BeWo cells. (**a**) *I–V* relationships in the presence of the indicated external cations. (**b**) Conductance densities (conductance per unit cell capacitance, pS/pF) observed in BeWo cells in the presence of $Na^+$, $K^+$, $Li^+$ and NMDG in the bath solution ($n = 7$, for each condition). *Asterisks* represent statistical significance between conditions ($P < 0.05$). (**c**) Dose–response relationship for the amiloride block of ENaC in BeWo cells cultured with 100 nM aldosterone (12 h). Cells were incubated with 8Br-cAMP for 25 min, and then different amiloride concentrations for 8 min were used to block sodium currents. Results are means ± SD of $n = 4$–10 experiments

pretreated BeWo cells with aldosterone for 12 h and then stimulated them with 8Br-cAMP. With $Na^+$ and $K^+$ as the main cation in the bath and pipette solutions, respectively, the cells were held at 0 mV and clamped sequentially to membrane potentials from +60 to −140 mV, to avoid intracellular $Na^+$ accumulation and ENaC self-inhibition by $Na^+$. Unstimulated BeWo cells do not show amiloride-sensitive $Na^+$ currents, even though there is a current increase with cAMP, in concordance with what was previously observed for these cells attributed to the activation of amiloride-insensitive nonselective cation channels (Ramos et al. 2008). On the other hand, BeWo cells treated with aldosterone did show amiloride-sensitive currents, demonstrating that the channels expressed in the cells are hormone-regulated. These currents induced by aldosterone, activated with cAMP and measured as the amiloride-sensitive component were nonrectifying and reversed at positive potentials, suggesting that cAMP allows sodium ions to move along its electrochemical gradient, shifting the *I–V* plot toward positive potentials. In many of the cells with the most negative pulses, the developing inward currents become noisy, as found in renal collecting duct principal cells and lymphocytes (Zhou and Bubien 2001).

We determined the cation selectivity of the channels in aldosterone-treated cells by substituting external $Na^+$ with $K^+$, $Li^+$ and NMDG in bath solutions following previous stimulation with cAMP. Permeability coefficients were 0.2 for $P_{NMDG}/P_{Na}$, 0.6 for $P_K/P_{Na}$ and 1.3 for $P_{Li}/P_{Na}$, indicating a selectivity sequence of $Li^+ > Na^+ > K^+ >$ NMDG. The blocking effect of amiloride in whole-cell experiments seen in the present study had an $IC_{50}$ value of 5.77 μM, in concordance with earlier observations for amiloride-sensitive sodium channels from cells cultured on nonpermeable supports (Garty and Palmer 1997; Kellenberger and Schild 2002). Amiloride inhibited a greater amount of whole-cell currents when $Na^+$ was the extracellular predominant cation than in the presence of the $K^+$ solution, in agreement with the results obtained by Palmer and Andersen (1989), who found an increase in the amiloride apparent binding constant in the presence of $K^+$.

Although the general properties of these channels are well known (4–5 pS single-channel conductance, $P_{Na}/P_K$ ratio >10 and blockage by submicromolar concentrations of amiloride), there are other amiloride-sensitive $Na^+$ channels that do not display these prototypical characteristics, with conductances ranging 3–40 pS and $P_{Na}/P_K$ ratios of >50 to 1 (Benos et al. 1996; Garty and Palmer 1997). Hamilton and Eaton (1985) analyzed single-channel recordings from cultured epithelial kidney cells (A6) and found amiloride-sensitive ENaCs with conductances between 7 and 10 pS and $P_{Na}/P_K$ selectivity of approximately 3–4:1. In this research, we observed $Na^+$ channels in symmetrical Na-gluconate solutions with a conductance of about 7 pS, which may participate in the whole-cell amiloride-sensitive sodium current studied and influence the overall characteristics of the total current measured. As the binding site for amiloride is located in the extracellular side of the channel (see

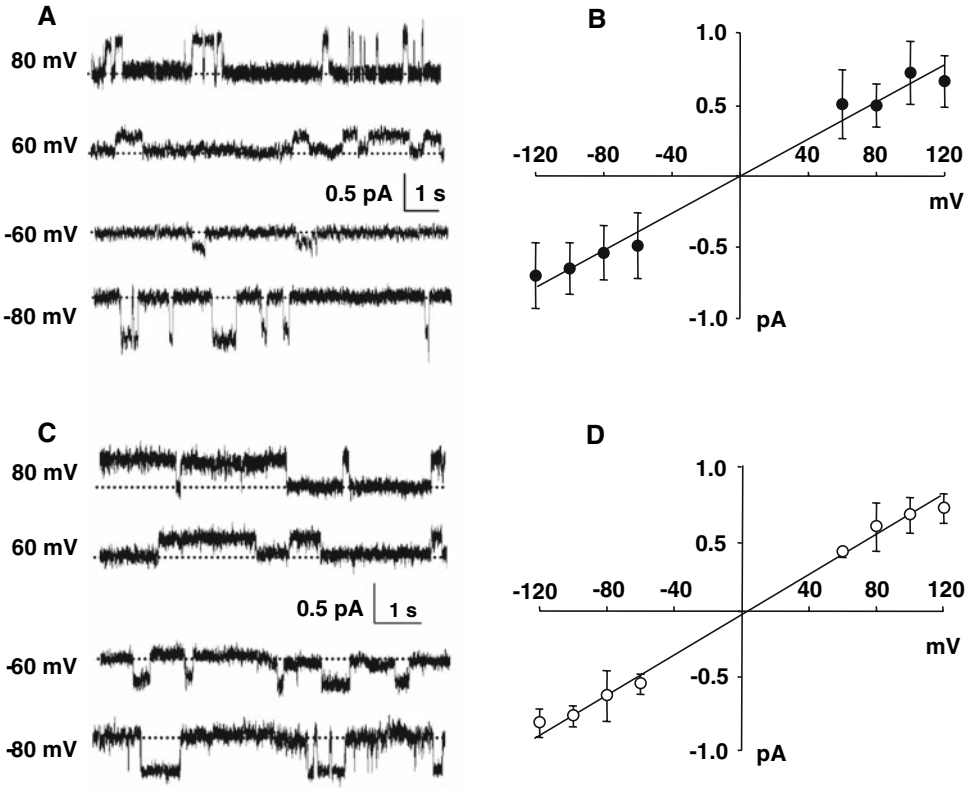


**Fig. 6** Single sodium channels in BeWo cells. (**a**) Representative recordings in an excised inside–out patch in a BeWo cell treated with aldosterone (12 h). Different positive and negative potentials in symmetrical 140 mM Na-gluconate solution. *Dotted lines* indicate the closed state of the channels. (**b**) *I–V* relationship in Na-gluconate solution in both pipette and bath from cells treated with aldosterone (mean ± SD, $n = 22$). (**c**) Representative recordings in an excised inside–out patch in a BeWo cell cultured in control conditions. Positive and negative potentials in symmetrical 140 mM Na-gluconate solution. *Dotted lines* indicate the closed state of the channels. (**d**) *I–V* relationship in Na-gluconate solution in both pipette and bath from cells cultured in control conditions (mean ± sd, $n = 4$)

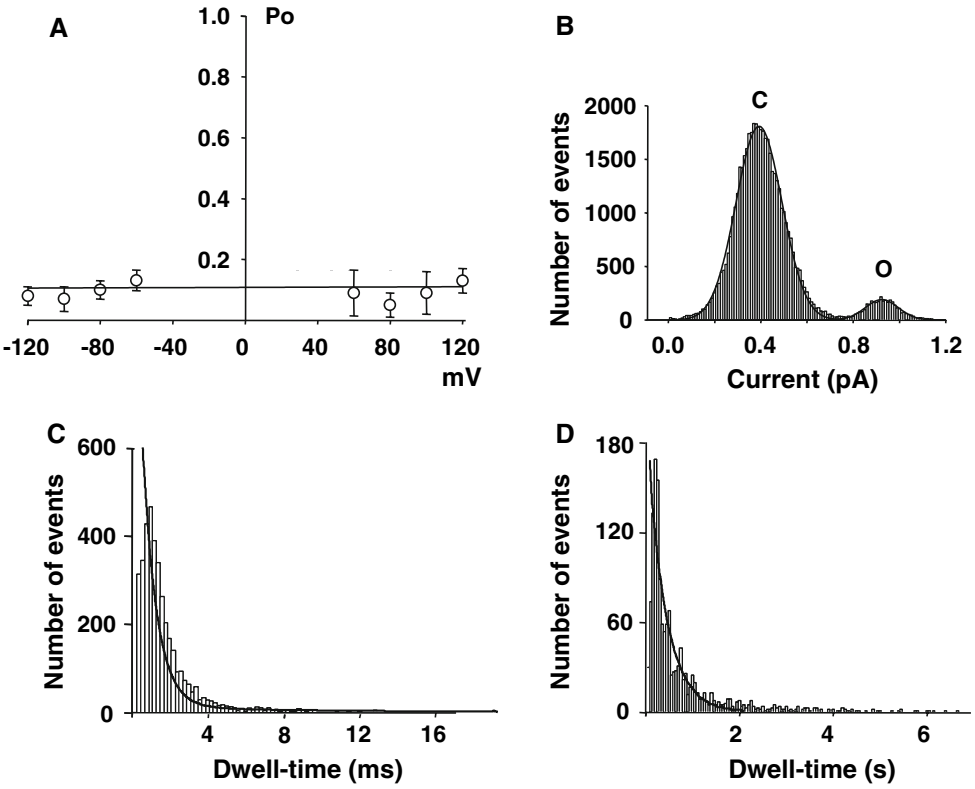


**Fig. 7** Single-channel activity in aldosterone-treated cells. (**a**) Relationship between $P_o$ and voltage when the patch was bathed in symmetrical Na-gluconate solutions (mean ± SD). (**b**) A typical amplitude histogram demonstrating gaussian distribution of open and closed channel current amplitudes (−100-mV pulse on patch pipette). C, closed channel state; O, open channel state. (**c**) Open-time histogram of event duration. Data were obtained at −100 mV in patch pipette. *Solid line* is the best fit of a double exponential function with time constants 1.4 ± 0.5 and 27.9 ± 18.6 ms. (**d**) Closed-time histogram of event duration. Data were fitted to a single exponential function with time constant 932.4 ± 177.3 ms ($n = 15$)

Kellenberger and Schild 2002, for a review), we could not test the effect of amiloride on the same inside–out patches. The frequency of appearance of these channels is considerably higher in aldosterone-treated BeWo cells than in nontreated cells. Internal $K^+$ replacement evidenced a marked selectivity of these channels for sodium over

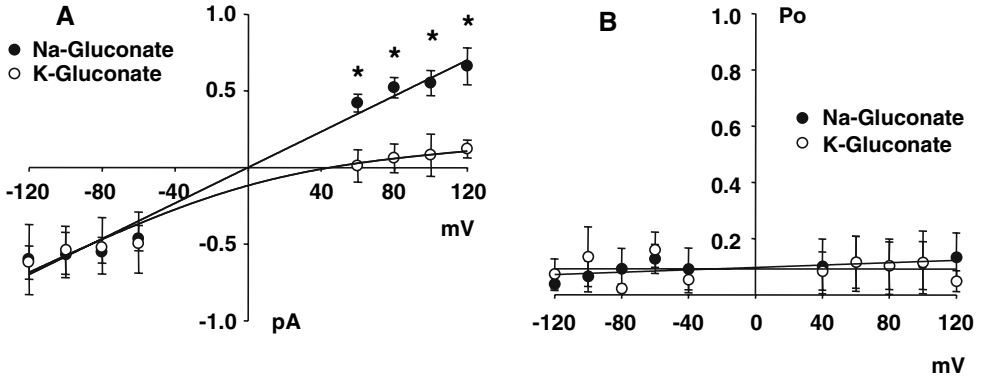


**Fig. 8** Ion selectivity of single channels observed in aldosterone-treated BeWo cells. (**a**) *I–V* relationships before (●) and after (○) internal (bath) $Na^+$ replacement for $K^+$ (means ± SD, $n = 12$). Lines were best fits to experimental points using the G-H-K equation. (**b**) Relationship between $P_o$ and voltage when the patch was bathed in symmetrical Na-gluconate solutions (●) and after $Na^+$ replacement (○). No difference was observed between treatments for every pulse applied (means ± SD, $n = 12$)

potassium ($P_K/P_{Na} = 0.19$). The difference between cation selectivity in whole-cell and single-channel studies could explain the presence of alternative ion currents in BeWo cells that may influence sodium transport while performing whole-cell analysis.

The stimulation of macroscopic amiloride-sensitive sodium current ($I_{amil}$) by aldosterone could be explained by a proportional stimulation of the open channel density. With a pulse of −140 mV, $I_{amil}$ density is 48.0 pS/pF when Na is the main cation and there is a $C_m$ of 1 μF/cm$^2$, a mean channel conductance of 6.4 pS and a calculated number of active channels of about 7/100 μm$^2$, a value similar to that reported previously for ENaCs of A6 cells (Alvarez de la Rosa et al. 2000).

ENaCs are also distinguished by the slow kinetics of gating, long spontaneous open and closed times and absence of voltage dependence of gating (Garty and Palmer 1997). In addition, $P_o$ can vary from near 0 to near 1 due in part to a hormonal effect and feedback regulation (Garty and Palmer 1997). It also has been proposed that proteolysis of ENaC subunits is associated with a significant change in ENaC $P_o$, studies that likely address the highly variable $P_o$ values described for the channel (Hughey et al. 2003; Caldwell et al. 2004; Adebamiro et al. 2007). In BeWo cells, we observed that values never exceeded a $P_o$ of 0.3 for each channel registered and that $P_o$ was voltage-independent. Although aldosterone has been shown to stimulate the expression of endogenous proteases and consequently sodium transport through ENaC (Narikiyo et al. 2002), we did not observe changes in our sodium channel $P_o$ from cells treated with or without aldosterone (data not shown).

Different subunit composition and expression of other internal regulatory proteins may be the cause of the variations from the canonical channel characteristics observed in the BeWo cell line. In fact, ENaCs may form the core conducting element in many of the amiloride-sensitive sodium channels (Benos et al. 1996), and altering the relative levels of α-, β- and γ-subunit mRNA leads to channels with different regulatory characteristics, ion selectivity, conductance and amiloride sensitivity (McNicholas and Canessa 1997; Jain et al. 2001). Here, we show that after stimulation with aldosterone, β- and γ-subunit mRNAs were detected in addition to constitutive levels of the α-subunit mRNA, so it is likely that ENaC channels in BeWo cells are made of these three subunits. Western blot studies yielded the same results. This regulation of ENaC by aldosterone is tissue-specific since aldosterone increase α-ENaC mRNA and protein levels but not β and γ mRNA in the rat kidney (Stokes and Sigmund 1998; Masilamani et al. 1999; see also Salleh et al. 2005). The opposite effect is seen in distal colon (Asher et al. 1996) and may be applied to placental tissue as well.

Our results concerning ENaC protein expression revealed that the channel was actually translated in BeWo cells. Western blot studies also showed the occurrence of subunit proteolysis since we observed band doublets in α- and γ-ENaC that apparently result from protein cleavage (Hughey et al. 2003; Harris et al. 2007). However, these subunit processes did not induce changes in the $P_o$ of the channel; therefore, the increases registered by us in cellular ionic currents, in frequency of appearance of sodium single channels and in the molecular expression of the channel subunits imply that the stimulation by aldosterone in these cells is due to other mechanisms besides active proteolysis.

Immunocytochemical studies showed that aldosterone induces an increase in labeling of the three subunits, mainly in the cell cytoplasm, and that labeling is shifted to plasma membranes when aldosterone stimulus is complemented with 8Br-cAMP incubation.

In conclusion, our data suggest the presence of an amiloride-sensitive sodium current in aldosterone-treated BeWo cells. The characteristics of this current are consistent with the presence of ENaC protein expression. ENaC may participate in the maintenance of normal sodium transport and ionic homeostasis, for both mother and fetus, to carry on with a successful pregnancy. We found that ENaC expression in human syncytiotrophoblasts is reduced in preeclamptic women compared with normal pregnancies and that this difference may have consequences for ion and nutrient transport as well as for cell migration (del Mónaco et al. 2006). Further studies will be required to assess the role(s) of a functional ENaC channel in BeWo cells and in human placenta.

**Acknowledgments** We thank Dr. Ted Begenisich (University of Rochester, Rochester, NY) and Dr. Jorge Arreola (Universidad Autonóma de San Luis Potosí, San Luis Potosí, Mexico) for helpful comments and Dr. Cristina Ibarra and Dr. Alicia Damiano (University of Buenos Aires, Buenos Aires, Argentina) for facilities. S. d.M. is a research fellow from the National Council of Research of Argentina (CONICET). Y. A. and B. A. K. are established members of the CONICET. This work was supported in part from grants from the Secretaría de Ciencia y Tecnología (PICT 38181) and the University of Buenos Aires.

## References

Adebamiro A, Cheng Y, Rao US, Danahay H, Bridges RJ (2007) A segment of $\gamma$ ENaC mediates elastase activation of $Na^+$ transport. J Gen Physiol 130:611–629

Alvarez de la Rosa D, Canessa CM, Fyfe GK, Zhang P (2000) Structure and regulation of amiloride sensitive sodium channels. Annu Rev Physiol 62:573–594

Anantharam A, Tian Y, Palmer LG (2006) Open probability of the epithelial sodium channel is regulated by intracellular sodium. J Physiol 574:333–347

Asher C, Wald H, Rossier BC, Garty H (1996) Aldosterone-induced increase in the abundance of $Na^+$ channel subunits. Am J Physiol 271:C605–C611

Bachhuber T, König J, Voelcker T, Mürle B, Schreiber R, Kunzelmann K (2005) $Cl^-$ interference with the epithelial $Na^+$ channel ENaC. J Biol Chem 280:31587–31594

Benos DJ, Awayda MS, Berdiev BK, Bradford AL, Fuller CM, Senyk O, Ismailov II (1996) Diversity and regulation of amiloride-sensitive $Na^+$ channels. Kidney Int 49:1632–1637

Bubien JK, Watson B, Khan MA, Langloh AL, Fuller CM, Berdiev B, Tousson A, Benos DJ (2001) Expression and regulation of normal and polymorphic epithelial sodium channel by human lymphocytes. J Biol Chem 276:8557–8566

Butterworth MB, Edinger RS, Johnson JP, Frizzell RA (2005) Acute ENaC stimulation by cAMP in a kidney cell line is mediated by exocytic insertion from a recycling channel pool. J Gen Physiol 125:81–101

Caldwell RA, Boucher RC, Stutts MJ (2004) Serine protease activation of near-silent epithelial $Na^+$ channels. Am J Physiol 286:C190–C194

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC (1994) Amiloride-sensitive epithelial $Na^+$ channel is made of three homologous subunits. Nature 367:463–467

Dagenais A, Frechette R, Yamagata Y, Yamagata T, Carmel JF, Clermont ME, Brochiero E, Masse C, Berthiaume Y (2004) Downregulation of ENaC activity and expression by TNF in alveolar epithelial cells. Am J Physiol 286:L301–L311

del Mónaco S, Assef Y, Damiano A, Zotta E, Ibarra C, Kotsias BA (2006) Caracterización del canal epitelial de sodio en sinciciotrofoblasto de placenta humana pre-eclámptica. Medicina (B Aires) 66:31–35

Driver PM, Rauz S, Walker EA, Hewison M, Kilby MD, Stewart PM (2003) Characterization of human trophoblast as a mineralocorticoid target tissue. Mol Hum Rep 9:793–798

Gambling L, Dunford S, Wilson CA, McArdle HJ, Baines DL (2004) Estrogen and progesterone regulate $\alpha$, $\beta$, and $\gamma$ENaC subunit mRNA levels in female rat kidney. Kidney Int 65:1774–1781

Garty H, Palmer LG (1997) Epithelial sodium channels: function, structure and regulation. Physiol Rev 77:359–396

Hamilton KL, Eaton D (1985) Single-channel recordings from amiloride-sensitive epithelial sodium channel. Am J Physiol 249:C200–C207

Harris M, Firsov D, Vuagniaux G, Stutts MJ, Rossier BC (2007) A novel neutrophil elastase inhibitor prevents elastase activation and surface cleavage of the epithelial sodium channel expressed in *Xenopus laevis* oocytes. J Biol Chem 282:58–64

Huang SS, Swaan P (2001) Riboflavin uptake in human trophoblast-derived BeWo cell monolayers: cellular translocation and regulatory mechanisms. J Pharmacol Exp Ther 298:264–271

Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Pol PA, Harkleroad KL, Carattino MD, Kleyman TR (2003) Maturation of the epithelial $Na^+$ channel involves proteolytic processing of the $\alpha$- and $\gamma$-subunits. J Biol Chem 278:37073–37082

Iordache C, Duszyk M (2007) Sodium 4-phenylbutyrate upregulates ENaC and sodium absorption in T84 cells. Exp Cell Res 313:305–311

Jain L, Chen XJ, Ramosevac S, Brown LA, Eaton DC (2001) Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. Am J Physiol 280:L646–L658

Jasti J, Furukawa H, Gonzales EB, Gouaux E (2007) Structure of acid-sensing ion channel 1 at 1.9 Å resolution and low pH. Nature 449:316–323

Kellenberger S, Schild L (2002) Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol Rev 82:735–767

Liu F, Soares MJ, Audus KL (1997) Permeability properties of monolayers of the human trophoblast cell line BeWo. Am J Physiol 273:C1596–C1604

Ma HP, Saxena S, Warnock DG (2002) Anionic phosphoinositides regulate native and expressed epithelial sodium channel (ENaC). J Biol Chem 277:7641–7644

Ma HP, Al-Khalili O, Ramosevac S, Saxena S, Liang Y, Warnock DG, Eaton DC (2004) Steroids and exogenous gamma-ENaC subunit modulate cation channels formed by alpha-ENaC in human B lymphocytes. J Biol Chem 279:33206–33212

Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA (1999) Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. J Clin Invest 104:R19–R23

McNicholas CM, Canessa CM (1997) Diversity of channels generated by different combinations of epithelial sodium channel subunits. J Gen Physiol 109:681–692

Mo L, Wills NK (2004) ClC–5 chloride channel alters expression of the epithelial sodium channel (ENaC). J Membr Biol 202:21–37

Moreau R, Simoneau L, Lafond J (2003) Calcium fluxes in human trophoblast (BeWo) cells: calcium channels, calcium-ATPase,

and sodium-calcium exchanger expression. Mol Reprod Dev 64:189–198

Moretó M, Cristià E, Pérez-Bosque A, Afzal-Ahmed I, Amat C, Naftalin RJ (2005) Aldosterone reduces crypt colon permeability during low-sodium adaptation. J Membr Biol 206:43–51

Morris RG, Schafer JA (2002) cAMP increases density of ENaC subunits in the apical membrane of MDCK cells in direct proportion to amiloride-sensitive $Na^+$ transport. J Gen Physiol 120:71–85

Narikiyo T, Kitamura K, Adachi M, Miyosi T, Iwashita K, Shiraishi N, Nogoguchi H, Chen L, Chai KX, Chao J, Tomita K (2002) Regulation of prostasin by aldosterone in the kidney. J Clin Invest 109:401–408

Page KR, Ashworth CJ, McArdle HJ, Finch AM, Nwagwu MO (2003) Sodium transport across the chorioallantoic membrane of porcine placenta involves the epithelial sodium channel (ENaC). J Physiol 547:849–857

Palmer LG, Andersen OS (1989) Interactions of amiloride and small monovalent cations with the epithelial sodium channel. Inferences about the nature of the channel pore. Biophys J 55: 779–787

Pattillo RA, Gey GO (1968) The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. Cancer Res 28:1231–1236

Perrotti N, He RA, Phillips SA, Haft CR, Taylor SI (2001) Activation of serum- and glucocorticoid-induced protein kinase (Sgk) by cyclic AMP and insulin. J Biol Chem 276:9406–9412

Quesnell RR, Han X, Schultz BD (2007) Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium. Am J Physiol 292:C1739–C1745

Ramos AJ, Cantero MR, Zhang P, Raychowdhury MK, Green A, MacPhee D, Cantiello HF (2008) Morphological and electrical properties of human trophoblast choriocarcinoma, BeWo cells. Placenta 29:492–502

Rossier BC, Pradervand S, Schild L, Hummler E (2002) Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors. Annu Rev Physiol 64:877–897

Salleh N, Baines DL, Naftalin RJ, Milligan SR (2005) The hormonal control of uterine luminal fluid secretion and absorption. J Membr Biol 206:17–28

Segal A, Cucu D, Van Driessche W, Weber WM (2002) Rat ENaC expressed in *Xenopus laevis* oocytes is activated by cAMP and blocked by $Ni^{2+}$. FEBS Lett 515:177–183

Silva NL, Wang H, Harris CV, Singh D, Fliegel L (1997) Characterization of the $Na^+/H^+$ exchanger in human choriocarcinoma (BeWo) cells. Pfluegers Arch 433:792–802

Snyder PM (2000) Liddle's syndrome mutations disrupt cAMP-mediated translocation of the epithelial $Na^+$ channel to the cell surface. J Clin Invest 105:45–53

Snyder PM, Cheng C, Prince LS, Rogers JC, Welsh MJ (1986) Electrophysiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. J Biol Chem 273:681–684

Staruschenko A, Adams E, Booth RE, Stockand JD (2005) Epithelial $Na^+$ channel subunit stoichiometry. Biophys J 88:3966–3975

Stokes JB, Sigmund RD (1998) Regulation of rENaC mRNA by dietary NaCl and steroids: organ, tissue, and steroid heterogeneity. Am J Physiol 274:C1699–C1707

Stulc J (1997) Placental transfer of inorganic ions and water. Physiol Rev 77:805–836

Taruno A, Niisato N, Marunaka Y (2008) Intracellular calcium plays a role as the second messenger of hypotonic stress in gene regulation of SGK1 and ENaC in renal epithelial A6 cells. Am J Physiol 294:F177–F186

Wei SP, Li XQ, Chou CF, Liang YY, Peng JB, Warnock DG, Ma HP (2007) Membrane tension modulates the effects of apical cholesterol on the renal epithelial sodium channel. J Membr Biol 220:21–31

Wodopia R, Ko HS, Billian J, Wiesner R, Bartsch P, Mairbaurl H (2000) Hypoxia decreases proteins involved in epithelial electrolyte transport in A549 cells and rat lung. Am J Physiol 279:L1110–L1119

Yang LM, Rinke R, Korbmacher C (2006) Stimulation of the epithelial sodium channel (ENaC) by cAMP involves putative ERK phosphorylation sites in the C termini of the channel's $\beta$- and $\gamma$-subunit. J Biol Chem 281:9859–9868

Zhao H, Hundal HS (2000) Identification and biochemical localization of a Na–K–Cl cotransporter in the human placental cell line BeWo. Biochem Biophys Res Commun 274:43–48

Zhou Z-H, Bubien JK (2001) Nongenomic regulation of ENaC by aldosterone. Am J Physiol 281:C1118–C1130