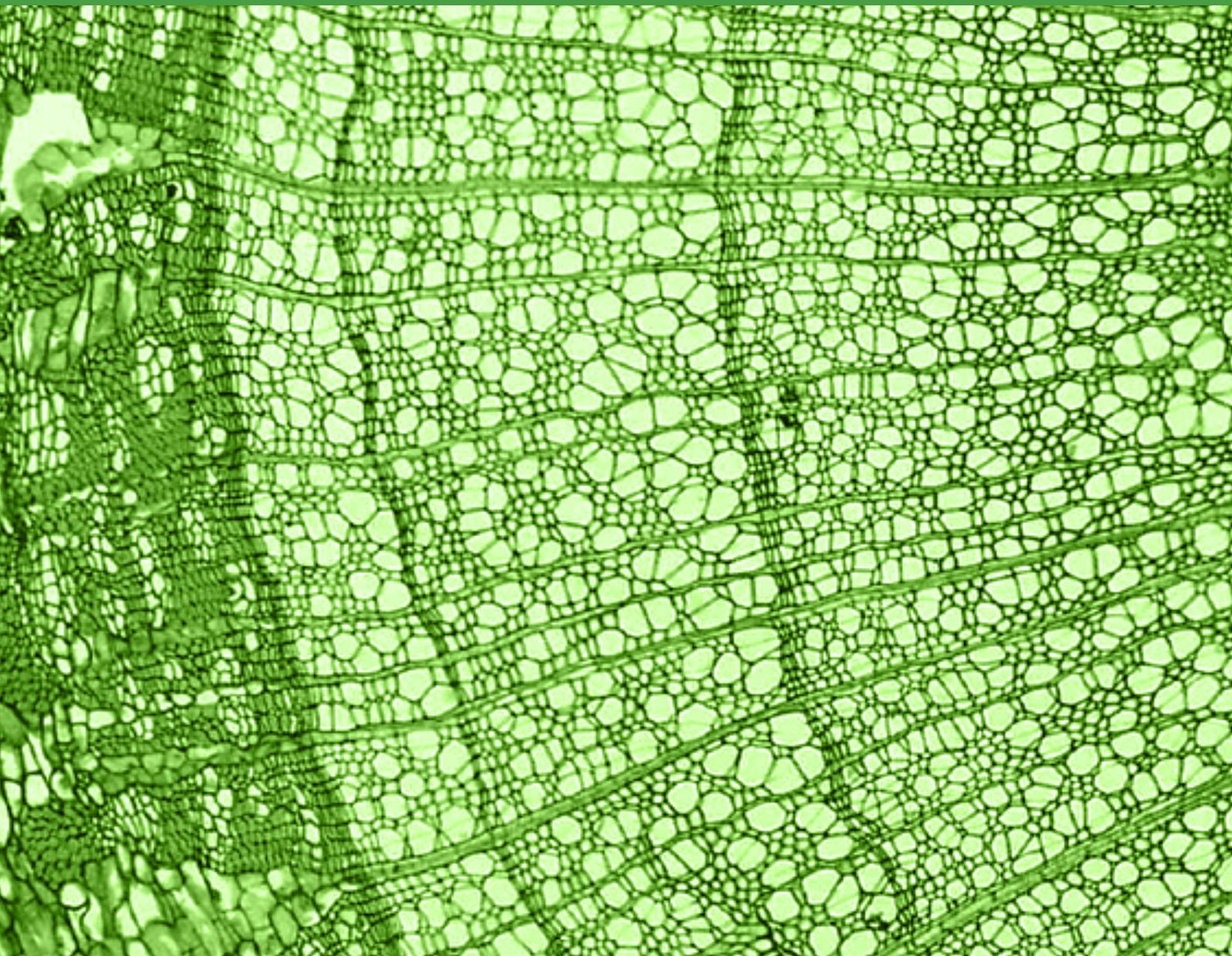


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THE EPITHELIAL SODIUM CHANNEL AND THE CELL MIGRATION

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ABSTRACT

Sodium is the main extracellular cation, and its osmotic pressure is an important determinant of the extracellular fluid volume. We were interested in the expression and functional characteristics of the epithelial sodium channel (ENaC) that mediates the Na⁺ entry into the cells from the luminal fluid in many reabsorbing epithelia and in the human placenta. Aldosterone is a key regulator of ENaC channel and stimulates protein methylation on the β -subunit of ENaC. Aldosterone and 8Br-cAMP promoted cellular migration in a wound healing model in trophoblastic BeWo cells. Amiloride blocked this effect. Electrophysiological studies showed an increase in ENaC current in the presence of aldosterone. We suggest that aldosterone positively influences wound healing through methylation of ENaC. On the other hand, we found a diminished expression of the three subunits of the ENaC in the membranes of preeclamptic placentas in comparison with the normal ones and this may have consequences for the cell migration and ion transport involved in the pathophysiology of preeclampsia.

Keywords: ENaC; currents; cell migration; wound healing; BeWo cell line; preeclampsia

Ion channels are integral membrane proteins with interactions to other membrane proteins, cytoskeleton and the lipid environment. They form hydrophilic pores that extend across the lipid bilayer. These channels are nearly enzymes, specialized to catalyze movement of ions of appropriate size and charge to pass through them from one side to the other side of the membrane, with direct actions on membrane potential (V_m), action potentials (AP), synaptic current and synthesis of ATP. Currents regulate the intracellular concentration of ions such as Na^+ and Ca^{2+} which participate in several cell functions. Ion currents control the activity of related channels such as the interaction between epithelial sodium channel (ENaC) and the cystic fibrosis transmembrane regulator (CFTR). In addition, channels have an important role in cellular differentiation, apoptosis and cellular migration (**Figure 1**).

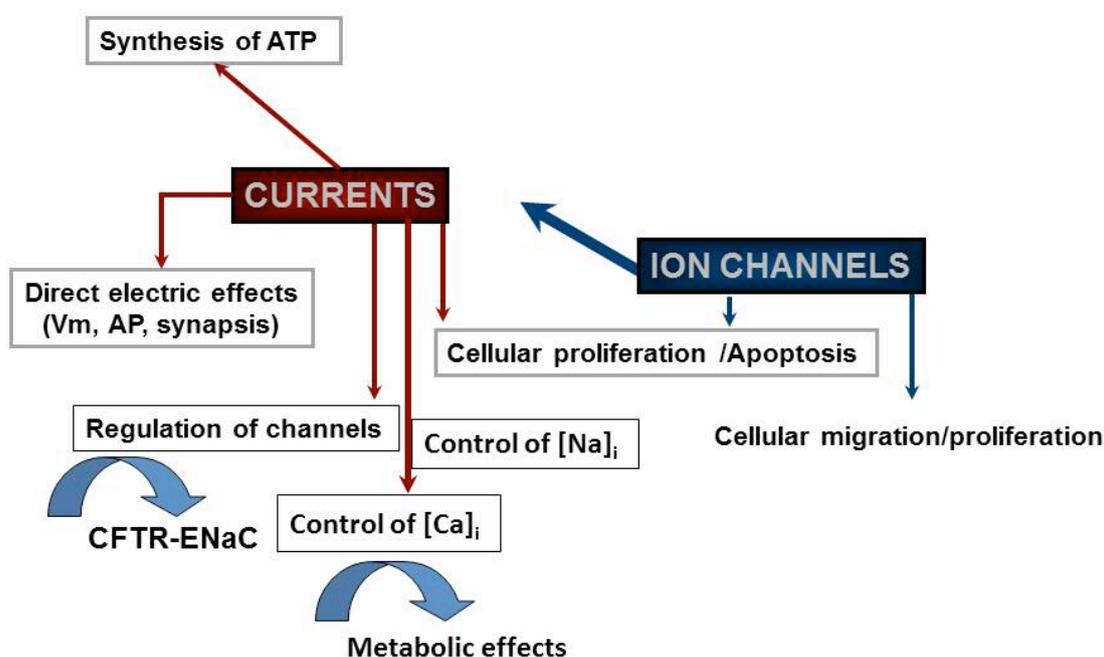


Figure 1. Scheme of the general function of ion channels.

ENaC has evolved as a channel that is thought to be primarily in an active state, facilitating the bulk movement of Na^+ from the luminal fluid into the cells in many reabsorbing epithelia. From the crystal structure of the homologous acid-sensing ion channel (ASIC) it is inferred that ENaC is a heterotrimer of homologue subunits α , β and γ [1] in accordance with the electrophysiological studies of Staruschenko et al. [2]. The regulated insertion and retrieval of channels in the membrane have crucial roles in modulating Na^+ transport. The subunits are synthesized in the reticulum endothelium and after processing and assembling they are inserted in the cell membrane; at the end they are polyubiquitinated and degraded in

the proteasome or in the lysosomes (**Figure 2**). 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 (see [3] for a review of the literature). The channel is blocked by the diuretic amiloride and is highly regulated. In particular, aldosterone produces an increase in ENaC protein trafficking and its apical membrane expression [4, 5], and an increase in the time that ENaC is open (open probability of the channel). The latter is due to an activation of channel by proteolysis [6, 7] and to a protein methylation process that has been linked to genomic and non-genomic activation of ENaC [8, 9].

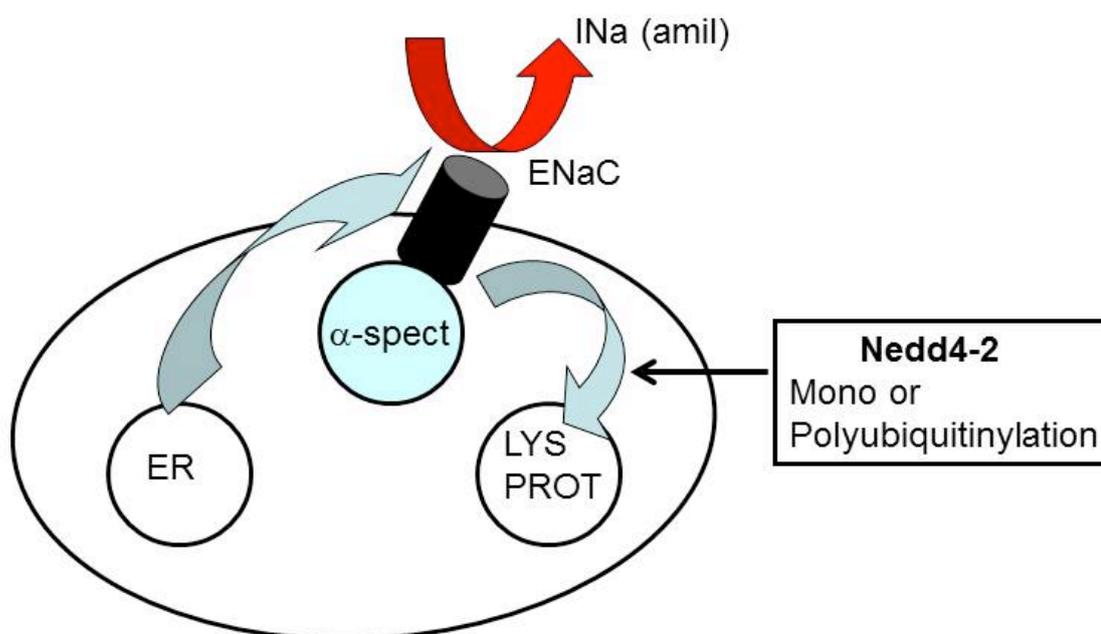


Figure 2. Schematic diagram of ENaC degradation. Subunits are synthesized in the reticulum endothelium (RE) and after processing and assembling they are inserted in the cell membrane. Alpha-spectrin, an apical cytoskeleton protein, binds to actin cytoskeleton and ENaC. In cells expressing endogenous ENaC, subunits are polyubiquitinated and degraded in the proteasome (PROT). In cells transfected with ENaC subunits, monoubiquitin-conjugated ENaC are degraded in lysosomes (LYS) [10]. Depending on the type of cell, this process may be dependent or not of Nedd4-2-mediated ENaC ubiquitination [11]. INa (amil): Na^+ currents mediated by ENaC.

Cell motion mechanisms are related to cytoskeleton reorganization, where cortical actin filaments are involved in the generation of locomotive structures and in the interaction with other signalling and structural proteins. Ion channels and transporters actively participate in this process, being regulated by cytoskeleton components and cell volume [12, 13]. Thus, there is an interaction between ENaC and the membrane cytoskeleton [14,

15]. In this regard Na^+ currents mediated by ENaC channels are involved in cell migration, well documented in epithelial and vascular cells [16, 17] and in the proliferation of tumour cells [18].

Cell migration is vital for embryonic development and adult tissue remodelling. Its mechanisms determine the physiopathology of processes such as neuronal targeting, immunity, inflammation, wound healing, angiogenesis and metastatic spread [12, 13, 19]. Movement of cells requires a sequence of cell protrusions and retractions that mainly depends on a high regulation of the actin cytoskeleton and volume changes. These processes are triggered and modulated by a combination of signals. External stimuli are sensed and integrated by membrane receptors, including integrins, which transduce these signals into cellular signalling pathways, such as the small GTPase proteins belonging to the Rho family. These pathways regulate the coordinated cytoskeletal rearrangements adhesion, contraction and detachment at the front and rear side of cells allowing them to move into the extracellular space. The overall process involves continuous modulation of cell motility, shape and volume, in which ion channels play major roles (**Figure 3**).

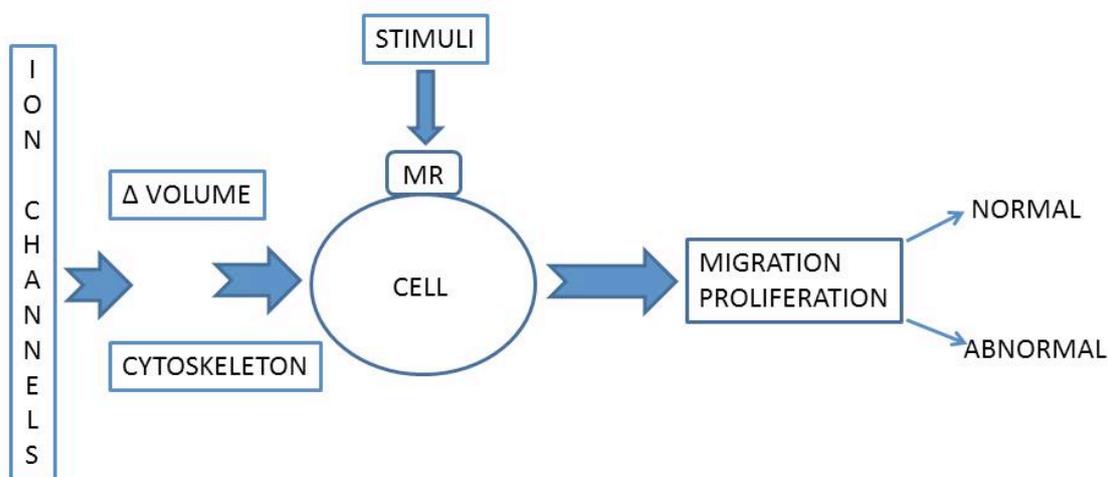


Figure 3. Scheme of cell migration and proliferation. Movements of cells requires the participation of the actin cytoskeleton and volume changes of the cell; they are triggered by a combination of stimuli sensed by membrane receptors (MR).

The BeWo cell line

Investigations on ENaC are difficult because of the low abundance of channels in hormone-unstimulated tissues. A useful alternative to overcome this problem is the employment of cell lines that we can incubate with the selected hormones. In many of our studies we employed the BeWo cell line, a human hormone-synthesizing trophoblastic cell line established in continuous culture by Pattillo and Gey [20]. 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 [21]. 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 trophoblast; therefore, they have been used as a model to investigate the placental transport mechanisms [22- 27].

ENaC is composed of three subunits and is regulated by aldosterone and proteases

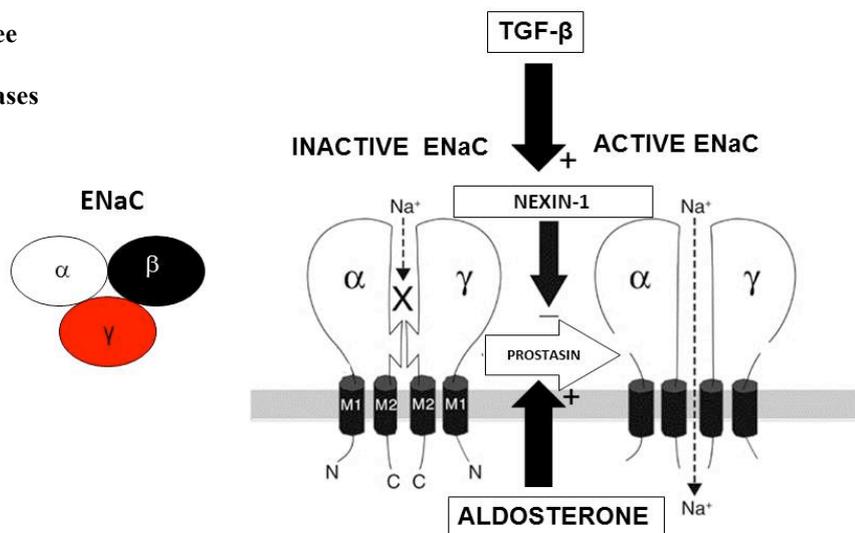


Figure 4. Regulation of ENaC. Although the channel is composed of three homologue subunits, α , β and γ (left), as stated above, only two subunits are shown here for clarity with their transmembrane segments (M1 and M2) (right).

To study the functional expression of ENaC and its relation with cell migration, three techniques were employed: 1) Standard molecular biology techniques for the expression of the ENaC subunits with antibodies directed toward each of the subunits (see 3 for details); 2) Cell migration measurement with a wound healing method [28] and 3) Recordings of cell currents through ENaC with a patch clamp technique.

ENaC protein expression studies shows in BeWo cells with and without different treatments, bands of the expected size for ENaC subunits, including band doublets for α - and γ -ENaC that apparently result from protein cleavage: ~ 95 (corresponding to the full-length) and ~ 30 kDa (cleavage product) for α -ENaC, and ~ 95 and ~ 75 kDa, respectively for γ -ENaC [3, 29]. Multiple mechanisms such as hormones, intracellular factors, and other regulatory factors contribute to regulation of ENaC activity. Proteolytic cleavage of γ -ENaC by serine proteases, including trypsin, furin, elastase, and prostatic, has been shown to increase channel activity. For example, prostatic, a glycosylphosphatidylinositol-anchored serine protease is an activator of ENaC that increases its open probability. On the other hand furin cleaves α -ENaC at two sites and γ -ENaC at one site. Dual cleavage of α - and γ -subunit releases inhibitory segments from ENaC, leading to channel activation. On the other hand, protease nexin-1, an endogenous prostatic inhibitor, inhibits ENaC activity through suppression of prostatic activity. Aldosterone and transforming growth factor- β 1

(TGF- β) reciprocally regulate expression of prostasin and ENaC. In BeWo cells we showed that aldosterone increases the expression of the three subunits of the channel with respect to control [3] (**Figure 4**). Thus, it is important to emphasize that extracellular proteases enhance ENaC activity a mechanism that may be relevant to proteinuric states when proteases, not normally found in urine, can enter the urinary space across damaged glomeruli [6].

Sodium currents through the ENaC

With the patch clamp technique in the whole cell configuration the electrode records a macroscopic current, whereas in the inside out configuration the record corresponds to the current flowing through one channel; the values are expressed in nanoamperes and picoamperes respectively.

From the current-voltage curves, the conductance (G or g) can be calculated as the current (I) divided by the voltage (V) applied and expressed in micro, nano or pico Siemens (S).

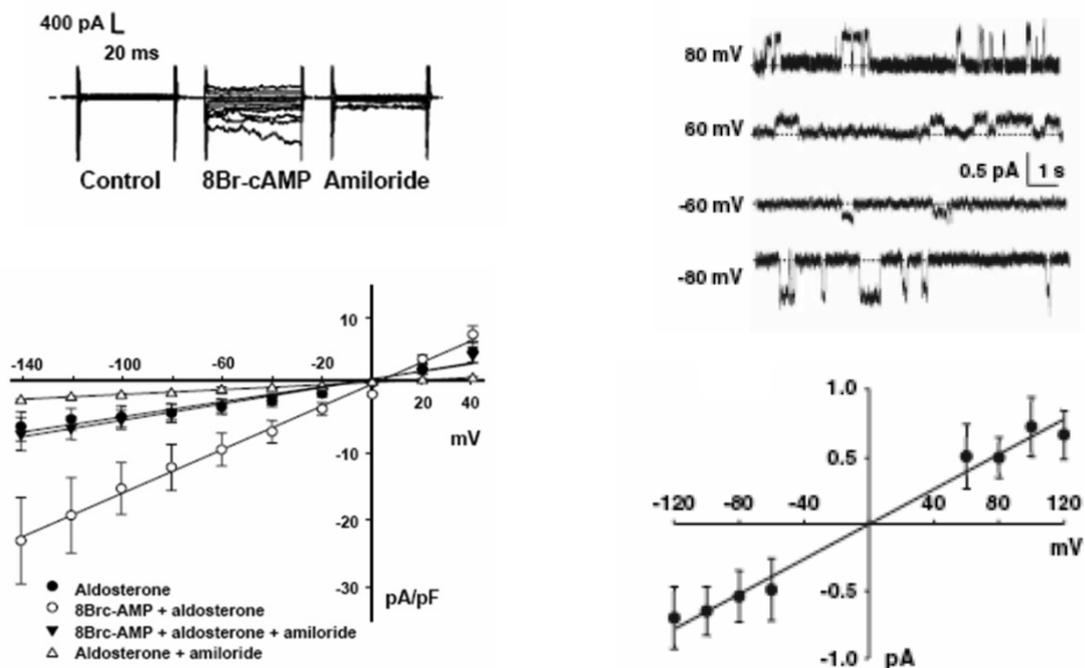


Figure 5. Macro and microscopic examples of currents (pA) through ENaC channels. Upper left: Whole-cell currents in aldosterone before and after 8Br-cAMP and the blocking effect of amiloride. Bottom left. Average current densities (current per unit cell capacitance vs. voltages) from cells cultured with 100 nM aldosterone. The current densities were measured before and after stimulation with 100 μ M 8Br-cAMP and after stimulation of 10 μ M amiloride. Upper right: Single channels (inside-out patch) in a BeWo cell with aldosterone. Different positive and negative potentials (in mV) in symmetrical were used. Dotted lines indicate the closed state of the channels. Lower right: I-V relationship from cells treated with aldosterone

with a linear, nonrectifying conductance of about 6 pS [From del Mónaco et al., 2008 [24] and Marino et al., 2013 [3].

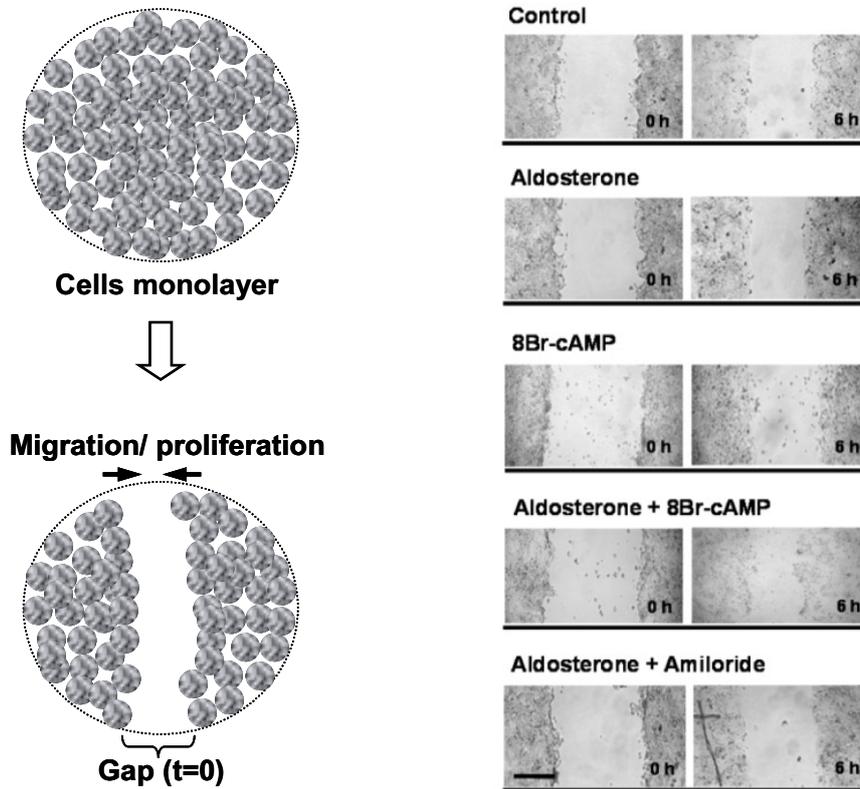
To study ENaC channels, cells were cultivated in the absence and presence of 100 nM aldosterone for the last 12 h. On the other hand a cAMP signaling pathway regulates the recruitment of ENaC from subapical storage pools to increase channels in the apical membrane or by increasing the number of channels in the membrane by inhibiting endocytosis [30], and activates ENaC increasing its open probability [31]. Thus, we employed both treatments in these experiments using aldosterone and 8Br-cAMP, an analogue of cAMP. Figure 5 shows examples of macro and microscopic examples of currents through ENaC channels. In the left panel (up) there are currents traces in the whole cell incubated in aldosterone before and after 8Br-cAMP and the blocking effect of amiloride. In the lower panel the average current densities (current per unit cell capacitance vs. voltages) from cells under different treatments are shown. We have shown with this procedure that cells in aldosterone elicited larger currents than in the absence of the hormone and this difference is increased in the presence of 8Br-cAMP. The blocking effect of amiloride is also evident. Amiloride-sensitive currents ($I_{Na\ (amil)}$) were defined as the difference of the current in the absence versus the presence of amiloride; this represents the current transported by ENaC. Single Na^+ channels were observed after application of depolarizing and hyperpolarizing pulses. Theoretically, ENaCs are distinguished by the slow kinetics of gating, long spontaneous open and closed times, a 4–12 pS single-channel conductance and absence of voltage dependence of gating. In inside-out patches from BeWo cells treated with aldosterone, we observed channels with a linear, nonrectifying conductance of about 6 pS over the voltage range studied (**Figure 5**, right panel).

Wound healing model to test the cell migration and ENaC participation

To test the hypothesis that the ENaC channel is required for the migration of BeWo cells, we performed a series of experiments measuring cell migration/proliferation processes associated with wound healing in these cells. The scratch wound assay studies the migration of a sheet of cells in two dimensions in response to formation of a wound. This initiates migration from a standing start in a defined direction. The assay requires imaging of the cells and subsequent analysis with software. In our experiments a section of the cell layer was removed with the tip of a micropipette. This action left a clear space on the plastic for the cells to fill and the wound border served as a migratory start line. This cleared space was subsequently visualized under the microscope and photographed to assess the ability of the cells to migrate and fill the wounded area.

When BeWo cells were treated with aldosterone we observed a significant increase in wound healing in comparison with the non-treated cells, as expected by our previous results [25] (**Figure 6**). To see if 8Br-cAMP would affect synergistically with aldosterone the wound healing, we studied BeWo cells with aldosterone plus 8Br-cAMP. The migratory behavior of the cells in aldosterone plus the cAMP analogue was higher in

comparison with the cells treated only with aldosterone. 8Br-cAMP by itself had a similar effect as with aldosterone alone. In all cases amiloride blocked the positive effect of the hormone (**Figure 6**). We employed, in this work, 100 nM aldosterone, in the range studied by others working on ENaC, the lowest 10 nM and 1.5 μ M the highest (references in 22). In some experiments we observed that a lower concentration (10 nM) influenced the wound healing only 12 h after injury in comparison with the control treatment.



Cellular proliferation could mask the contribution of migration to wound healing [17]. Thus, we tested wound healing at 6 h, when mitosis is still scarce, and used culture medium with 1% serum, to minimize proliferation. In addition we measured cell proliferation with the MTT assay. In a homogenous sample of cells (like the BeWo cell line), MTT reduction is proportional to the number of metabolically active cells and is used as an indicator of cell proliferation or viability. Our results show that neither aldosterone nor amiloride increases the proliferation of BeWo cells cultured in 1% FBS concentration, although it has been observed that aldosterone stimulates proliferation of cardiac fibroblasts [32].

ENaC in preeclampsia

Preeclampsia is a high incidence heterogeneous multisystem disorder of unknown aetiology that may cause foetal injury and growth retardation as well as endothelial cell activation/dysfunction in a susceptible mother. One of the proposed mechanisms could be an impaired trophoblast invasiveness and defects in spiral arterial remodelling as is observed in some cases of preeclampsia [33, see references in 27]. In preeclampsia there is a lower plasma volume expansion, lower cardiac output and an increase in total peripheral vascular resistance. Plasma levels of renin, angiotensin II and aldosterone increase during

normal pregnancy, although in preeclampsia for reasons that are unclear, renin activity, angiotensin II, and aldosterone decrease and the ratio aldosterone/renin is increased [34]. According with this, we tested the hypothesis that if aldosterone regulates ENaC expression, the amount of ENaC should be decreased in preeclamptic placentas. The abundance of the subunits of ENaC protein (ENaC/actin relative ratio) was decreased in preeclamptic placental explants when compared with the normal ones. In addition, we found by immunohistochemistry that the three ENaC subunits are present in the polarized syncytium but the intensity of the channel protein staining in preeclampsia is lower compared with that in normal samples.

Conclusions

We demonstrated that aldosterone and 8Br-cAMP influenced wound healing in BeWo cells through its effect upon ENaC. In response to injury, epithelial cells migrate across the wound and this response requires a range of processes acting from the inside and out of the cell such as directional motility, chemotaxis and mechanotransduction. A growing body of evidence suggests that ENaC may function as a mechanosensor, interacting with the cytoskeleton and the extracellular matrix [14, 15] generating a mechanically gated Na^+ influx which triggers a secondary signal transduction pathway [35]. According to this, Na^+ entering the cells through ENaC could be a signal before cells start to migrate. This movement in conjunction with the transport of anions and the consequent water movements, may favor the cell swelling required for *lamellipodium* expansion [36, 37]. Chiflet et al. [16] suggested an alternative explanation with the idea that the depolarization generated by ENaC stimulates cytoskeleton reorganization, thus promoting wound healing. BeWo cells treated with aldosterone show amiloride-sensitive currents regulated by aldosterone and a cAMP sensitive component. In a number of studies of the long-term action of aldosterone, significant increase in the expression and the redistribution from the intracellular localization to the membrane of any ENaC subunit was detected only after 15 h [38]. The higher increment in wound healing observed when both aldosterone and 8Br-cAMP are present may be due to an increase in channel open probability combined with an increase in channel surface expression, the two mechanisms by which cAMP leads to enhanced ENaC currents [30, 31]. The open probability and the number of channels inserted in the membrane are the key components of the hormone regulation with incubation time used in our experiments. Acute activation of ENaC by aldosterone not depending on the mineralocorticoid receptor was reported in *Xenopus* A6 kidney cells [39] and in rabbit but not in rat principal cells [40]. In addition, biochemical methods support the observation that ENaC mRNA and ENaC protein do not increase in the presence of aldosterone in the first 2-4 h when the increase in Na^+ transport and open probability is most dramatic [39].

Cellular proliferation could mask the contribution of migration to wound healing. To test this, we measured cell proliferation with the MTT assay. In a homogeneous sample of cells (like the BeWo cell line), MTT reduction is proportional to the number of metabolically active cells and is used as an indicator of proliferation or viability. Our results show that none of the treatments induced the proliferation of BeWo cells cultured in 1 % FBS concentration for 6 h.

Finally, plasma levels of aldosterone increase during normal pregnancy although in preeclampsia for reasons that are unclear aldosterone decrease and the ratio aldosterone/renin is increased (see [34] for references). We found a diminished expression of the three subunits of the ENaC in the membranes of preeclamptic placentas in

comparison with the normal ones. Although the role of ENaC in placental tissues is poorly understood, these differences may have consequences for the cell migration and ion transport involved in the pathophysiology of preeclampsia.

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