A Nonconventional Look at Ionic Fluxes in the Skin: Lessons From Genetically Modified Mice

Marjorie Guitard, Celine Leyvraz, and Edith Hummler
Département de Pharmacologie et de Toxicologie, Université de Lausanne, CH-1005 Lausanne, Switzerland

The mammalian, highly amiloride-sensitive epithelial sodium channel (ENaC) is member of the degenerin/ENaC superfamily of ion channels known to be implicated in sodium homeostasis, mechanosensation, and mechanoperception. A novel role for ENaC implicated in differentiation processes in skin reshapes our current view of this ancient transmembrane channel protein.

The highly amiloride-sensitive epithelial sodium channel (ENaC) is a membrane constituent of many salt-reabsorbing epithelia that facilitates Na⁺ movement across the tight epithelia that line the distal nephron, the distal colon, the ducts of salivary and sweat glands, and the lung. In these cells, ENaC drives Na⁺ entry apically into the cell, which then is extruded basolaterally from the cell by the Na⁺-K⁺-ATPase (see Ref. 19 for review). Three highly homologous subunits, α-, β-, and γ-ENaC, are characterized at the molecular level and are encoded by different genes (Scnn1a, Scnn1b, and Scnn1g) localized on chromosomes 6 (Scnn1a; mouse) and 12 (Scnn1a; human) and chromosomes 7 (Scnn1b, Scnn1g; mouse) and 16 (Scnn1b, Scnn1g; human). In vivo and in vitro experiments clearly demonstrate that in the absence of the α-subunit, channels made of β- and γ-subunits alone do not confer ENaC activity. Expression of each of the α-, β-, and γ-ENaC subunits is crucial for survival, as shown in gene-targeting experiments. Nevertheless, the clinical phenotype of each single knockout varies. In vivo, the constitutive inactivation of the α-subunit of ENaC leads to complete abolishment of ENaC activity. Null mutant neonates develop respiratory distress syndrome (RDS) and die soon after birth, revealing an important role of channel function in lung liquid clearance at birth (11). In contrast to the α-ENaC subunit, the β-subunit is not required for the transition from a liquid-filled to an air-filled lung but seems rather to be crucial for ENaC function in the kidney. The β-ENaC-deficient mice show normal prenatal development but also die within 40 h, most likely of hypokalemia. The γ-ENaC subunit seems to facilitate neonatal lung liquid clearance but is critical for renal sodium and potassium transport in the kidney. We propose that the variation in phenotype is due to complete abolishment of activity of ENaC in α-ENaC knockout mice, whereas β- and γ-ENaC knockout mice still retain some ENaC activity (estimated at ~15% of total ENaC activity) (2).

In human, mutations in all three ENaC subunits are reported to result in either ENaC hyper- or hypofunction associated with hypertension or salt-wasting syndrome (type 1 pseudo-hypoaldosteronism; PHA-1) (10). All PHA-1 mutations tested so far in Xenopus oocytes still confer ENaC activity. More recent investigations demonstrate that patients with systemic pseudo-hypoaldosteronism fail to absorb liquid from airway surface; the result is an increased volume of liquid in the airway. Immaturity of the liquid absorptive transport system in the very premature infant may be an important factor in the pathogenesis of RDS. In a mouse model with altered ENaC activity, diminished basal Na⁺ transport presents a predisposing factor for edema formation (18). Interestingly, increased morbidity and mortality in human male patients with RDS indicates organ immaturity other than in the lung. The ontogeny of the epidermal permeability barrier and lung occurs quite in parallel, and hormones like, for example, glucocorticoids and the sex steroids accelerate maturation. Sex steroid hormones exert opposite effects on lung maturation and on cutaneous barrier formation, with estrogens accelerating and androgens inhibiting keratinocyte differentiation. Two published reports (1, 8) describe macroscopic skin lesions like dermatitis in PHA-1 patients carrying ENaC mutations, although the skin phenotype is not further analyzed. Thus it is still unknown whether dysfunction of ENaC expression in skin contributes to and/or is indeed causative for defined skin diseases.

ENaC expression in skin

In mammalian skin, expression of all three ENaC subunits is demonstrated in human keratinocytes, as evidenced by RT-PCR, RNase protection assay, Northern blot, and immunocytochemistry (4, 17, 20). In situ hybridization reveals that ENaC mRNA is expressed throughout adult human epidermis but absent in 10-wk-old fetal epidermis (17). Patch-clamp recordings on human keratinocytes reveal a sodium channel conductance that is blocked by benzamil, with similar affinity and voltage dependence of the amiloride block as described previously for ENaC (4). Interestingly, expression is increased in more differentiated keratinocytes and is only found in the later stages of fetal epidermal development, suggesting that ENaC-mediated Na⁺ transport may be implicated in the control of keratinocyte differentiation. In human skin, low concentrations of amiloride (10⁻⁶ to 10⁻⁸ M), a specific inhibitor of ENaC, block the formation of cornified envelopes, inhibit the rise in intracellular Ca²⁺ seen as a result of raising extracellular Ca²⁺, and prevent the synthesis and activity of transglutaminase, a Ca²⁺-induced marker of differentiation, presumably by modulations in membrane potential. Further evidence that...
ENaC is implicated in keratinocyte and epidermal differentiation comes from the analysis of newborn α-ENaC knockout mice, which exhibit epidermal thickening, premature lipid secretion in the upper epidermis, and abnormal keratohyalin granules, suggesting that ENaC-mediated sodium fluxes control selective aspects of keratinocyte differentiation (16). These alterations may finally result in epidermal barrier dysfunction.

Epidermal barrier function

The primary function of the epidermis is to form a barrier between an organism and the outside hostile environment designed to advert the invasion of bacteria and other foreign entities while simultaneously preventing the escape of water required for terrestrial life. This role is mainly assumed by the upper layer of the epidermis, called the stratum corneum (Fig. 1). This layer is formed by totally differentiated keratinocytes embedded in a lipid matrix. The stratum corneum is maintained by the constant reproduction of the inner layer of living keratinocytes that migrate upward while undergoing a complex genetic program of terminal differentiation. This includes the formation of desmosomal adherence junctions, the accumulation of specialized keratins that provide mechanical strength to the skin, the accumulation of a complex array of structural proteins, lipids, and enzymes that constitute the cornified envelope, and the synthesis, extrusion, and processing of intercorneocyte lipids. Formation of the cutaneous permeability barrier requires both the secretion of the lipid and the hydrolytic enzyme contents of lamellar bodies and the subsequent postsecretory processing of polar lipids into their nonpolar lipid products. Lamellar body secretion is regulated by changes in extracellular ions, and lipid processing appears to be mediated by a set of hydrolytic, Ca^{2+}-dependent enzymes, which produce structural transformations within the stratum corneum interstices, leading to barrier formation.

Although transepithelial fluid-transporting properties are studied extensively in various mammalian epithelia, the regulation of fluid transport across epidermal keratinocyte layers remains poorly understood. There is evidence for a high concentration of Na^+, K^+, and Cl^- and a low concentration of water (13–35%) in the superficial stratum corneum (24). Experimental acute disruption of the epidermal permeability barrier by organic solvents, detergents, or tape stripping initiates a homeostatic repair response that rapidly restores barrier function to normal. An early and essential response to barrier disruption is the immediate (within 30 min) secretion of a pool of preformed lamellar bodies from the outermost granular cell. Acute barrier perturbations cause transepidermal water loss rates from the skin to increase, suggesting that changes in water flux per se might regulate components of the homeostatic repair response. Several studies also suggest that increased water loss that occurs secondary to barrier disruption leads to focal changes in the concentration of certain inorganic ions in the outer epidermis, alterations that in turn may initiate a repair response (15). Modified ENaC-mediated sodium transport in keratinocytes could result in abnormal epidermal differentiation, such as, for example, altered wound healing.

Until recently, mammalian epidermis that is characterized by multiple layers of less-polarized cells has been considered to be an epithelium with a passive transcutaneous movement of water and ions. But recently, two groups (3, 7) independently reported the presence of tight junctions within the stratum granulosum of the epidermis with typical tight junction morphology and molecular composition, characterized by colocalization of the integral membrane proteins like occludin.
and claudins (Fig. 1). Interestingly, claudin-1-deficient mice die soon after birth due to severe impairment of the epidermal barrier, demonstrating that tight junctions are crucial for the barrier function of mammalian skin (7). In these mice a subcutaneously injected tracer passes through epidermis, whereas in wild-type mice epidermis tight junctions efficiently prevent its diffusion. This clearly demonstrates that water and ions cannot move transcutaneously through the epidermis but need transporting systems like channels or pumps. Dysfunction of these transporting systems in skin may therefore have severe consequences for normal skin function by altering, for example, differentiation processes in the skin.

**Ionic fluxes in keratinocyte differentiation**

That ions may be important regulators in keratinocyte differentiation is demonstrated by the presence of a calcium gradient within the epidermis, with higher quantities of Ca$^{2+}$ in the upper than in the lower epidermis. Studied by using ion-capture cytochemistry and electron and proton probe X-ray microanalysis, the epidermis in the intact skin displays a Ca$^{2+}$ gradient, with low levels of Ca$^{2+}$ in the basal and lower spinous layers, followed by an increase in extracellular and intracellular Ca$^{2+}$ that peaks in the stratum granulosum. Thus increase of extracellular calcium may serve as a primary trigger for keratinocyte differentiation. Moreover, following acetone disruption of the barrier, this Ca$^{2+}$ gradient is lost, and the decrease in Ca$^{2+}$ levels in the outer epidermis is associated with enhanced lamellar body secretion and lipid synthesis, important components of the repair response. In contrast, if the Ca$^{2+}$ gradient is preserved by the addition of Ca$^{2+}$ to the bath solution, lamellar body secretion, lipid synthesis, and barrier recovery are inhibited. Therefore epidermal calcium regulates the mRNA expression of differentiation-specific markers, like the intermediate filament-associated proteins loricrin, profilaggrin, and involucrin (6). These findings provide direct evidence that acute and sustained fluctuations in epidermal calcium regulate events late in epidermal differentiation that together form the barrier. The passive loss of Ca$^{2+}$ and other electrolytes from the upper epidermis following barrier disruption may signal the repair response. As summarized in Table 1, only recently have mice that are deficient for ion/water channels and transporters been analyzed for their skin phenotype, revealing that indeed ions, like calcium, sodium, and protons, participate in ordered epidermal differentiation. Keratinocytes from mice deficient for the calcium-sensing receptor (CaR) no longer respond to extracellular calcium, and the mice exhibited disordered differentiation. Mice deficient for the sodium channel ENaC show altered expression of the differentiation markers keratin 1, 6, and 16, involucrin as well as premature lipid secretion, which might result in diminished lipid barrier, and mice lacking the NHE3 exchanger exhibit an impaired stratum corneum acidification.

The extracellular calcium concentration is a critical regulator of the growth and differentiation of cultured mouse epidermal cells. Addition of calcium to primary keratinocyte cultures elicits a rather complete differentiation program, including the change of biochemical markers and structural changes (5). When cultured in medium with 0.05 mM calcium (low calcium), primary mouse epidermal cells proliferate as a monolayer without desmosomal connections. When medium calcium is increased to >0.12 mM (high calcium), desmosomes rapidly form between cells, proliferation decreases to a low level, and cells begin to stratify and cornify. The increase of calcium within the culture medium also produces an elevation of both intracellular sodium and potassium within 12–24 h, suggesting that Na$^{+}$ influx could modulate Ca$^{2+}$-induced differentiation, presumably by modulations in membrane potential.

Ionic fluxes seem also to be important in wound repair.

---

**Table 1. Skin abnormalities due to dysfunction of ion and water fluxes**

<table>
<thead>
<tr>
<th>Protein, Gene</th>
<th>Ion/Water</th>
<th>Type of Genetic Modification</th>
<th>Skin Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENaC, Scnn1a</td>
<td>Na$^+$ channel</td>
<td>Knockout</td>
<td>Hyperplasia, disordered differentiation, fewer keratohyalin granules, premature lipid secretion</td>
</tr>
<tr>
<td>ENaC, Scnn1a</td>
<td>Na$^+$ channel</td>
<td>Transgenic rescue</td>
<td>Premature lipid secretion</td>
</tr>
<tr>
<td>NHE3, Slc9a3</td>
<td>Na$^+$/H$^+$ exchanger</td>
<td>Knockout</td>
<td>Slower barrier recovery, impaired stratum corneum acidification</td>
</tr>
<tr>
<td>CaR, Gprc2a</td>
<td>Ca$^{2+}$-sensing receptor</td>
<td>Knockout</td>
<td>Disordered differentiation, abnormal polarity and flattening of nucleated epidermal cells, thinning of the granular layer</td>
</tr>
<tr>
<td>AQP3, Aqp3</td>
<td>H$_2$O channel</td>
<td>Knockout</td>
<td>Reduced superficial skin conductance (impaired stratum corneum hydration)</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPCA1, ATP2C1</td>
<td>Ca$^{2+}$/Mn$^{2+}$</td>
<td>Point mutations</td>
<td>Hailey-Hailey disease, persistent blisters, erosions</td>
</tr>
<tr>
<td>SERCA2, ATP2A2</td>
<td>Ca$^{2+}$</td>
<td>Missense mutations</td>
<td>Darier disease, impaired intracellular adhesion, epidermal blistering</td>
</tr>
</tbody>
</table>

ENaC, epithelial sodium channel; NHE, sodium/hydrogen exchanger; CaR, calcium-sensing receptor; AQP, aquaporin; SPCA, secretory pathway Ca$^{2+}$/Mn$^{2+}$-ATPase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase.
Wound healing is a relatively simple but important developmental process that begins with the migration of epithelial cells to cover the wound. There is some indirect evidence that intrinsic factors play a role in promoting the epithelization of wounds. A transepidermal voltage gradient, created by the sodium ion pumps of the epithelium, acts as a driving force for an electrical current that flows through the low-resistance pathway of the wound. When the epidermis’s ability to generate wound currents is impaired by interfering with its Na⁺ transport capacity, epithelization is retarded. Directed migration of keratinocytes is essential for wound healing. This is strongly influenced by the presence of a physiological electric field, and in vitro these cells migrate toward the negative pole of such an electric field (galvanotaxis). Calcium channel blockers like Ni²⁺ and Gd³⁺ can inhibit this galvanotaxis, suggesting that calcium influx through Ca²⁺ channels is required for directed migration of keratinocytes (23). Galvanotaxis may be one of the mechanisms used by keratinocytes to guide wound closure, but the channel(s) involved in those processes is still unknown.

**Consequences of ENaC deficiency on epidermal differentiation**

To determine the importance of ENaC function in epidermal differentiation, we analyzed skin from newborn mice in which the α-ENaC subunit, and as a consequence the whole channel function, had been deleted. Newborn α-ENaC knockout mice demonstrate epithelial hyperplasia, and all suprabasal levels are thickened, suggesting that absence of ENaC function results in defects in epidermal differentiation (16). In addition, the epidermis demonstrates focal abnormalities in epidermal maturation, including a failure of suprabasal cells to flatten progressively as well as nuclear atypia. Electron microscopy reveals additional defects. Keratohyalin granules are decreased in number and premature lipid secretion is noted in the mid-stratum granulosum in α-ENaC knockout mice. We therefore conclude that normal lipid secretion requires full channel activity, since α-ENaC transgenic rescue mice with ~15% of residual ENaC activity retain this premature lipid secretion (12, 16). Epidermal differentiation is not influenced by a reduction of ENaC activity, as demonstrated in the mouse model for pseudohypoaldosteronism. Only complete abolishment of ENaC function in α-ENaC null mutant mice results in altered epidermal differentiation (16).

**How ENaC may trigger normal differentiation of keratinocytes**

Increased extracellular calcium may serve as a primary trigger for keratinocyte differentiation. Calcium-responsive cells like parathyroid cells, express on their surface a low-affinity transmembrane calcium receptor. Binding of calcium and/or other divalent or trivalent cations to this receptor triggers downstream signaling pathways, which include phospholipase C (PLC) activation and increase of intracellular calcium (5). Calcium may enter keratinocytes via the CaR, because keratinocytes from CaR-deficient mice did not respond to extracellular calcium. This suggests that the full-length CaR is required to mediate calcium signaling in keratinocytes (Table 1) (14).

We propose that calcium enters the keratinocyte via the CaR that activates phosphoinositide-specific PLC. This leads to the formation of membrane diacylglycerol and soluble inositol 1,4,5-triphosphate (IP₃). IP₃ diffuses rapidly in the cytoplasm and binds to the IP₃ receptor, which stimulates the release of Ca²⁺ from internal stores (5). ENaC might be activated by a Ca²⁺-sensitive process, e.g., via MAPK. Sodium entry might depolarize the membrane, thereby allowing Ca²⁺ influx. For example, voltage-gated calcium channels. Although Ca²⁺-permeable channels in the granular-layer keratinocytes have not yet been characterized by molecular, electrophysiological, and pharmacological methods, ion substitution experiments suggest that these keratinocytes express T- or L-type voltage-sensitive Ca²⁺ channels (6). Moreover, raised intracellular Ca²⁺ blocks lipid secretion in a manner that is reversible by L-type channel blockers (15).

Thus abolishing Na⁺ influx through the ENaC may hyperpolarize the membrane and decrease Ca²⁺ influx, thereby allowing unregulated lipid secretion. Decreased Ca²⁺ influx also inhibits the posttranslational processing of profilaggrin to filaggrin, which are major constituents of these granules. This might explain the presence of abnormal keratohyalin granules in α-ENaC knockout mice.

**Perspectives and conclusion**

Ionic fluxes might play an important role in skin differentiation processes, as shown by identification of mutations in two calcium pumps leading to severe skin pathologies (Hailey-Hailey disease and Darier disease). In Hailey-Hailey disease, a mutation in the calcium pump ATP2C1 leads to a skin disease characterized by persistent blisters and erosions of the skin (9). Impaired intercellular adhesion and epidermal blistering occurs in patients with Darier disease, which is caused by mutations in a gene encoding a sarco(endo)plasmic reticulum-Golgi calcium pump (SERCA2) (22). The analysis of ENaC-deficient mice revealed that ENaC expression is required for normal epidermal differentiation, suggesting a novel role of ENaC in skin. Since constitutive inactivation of the α-ENaC subunit leads to early postnatal death, functional consequences of ENaC deficiency in adult skin could not be addressed. We have now generated mice exhibiting a conditional (“floxed”) allele of the α-ENaC gene locus that can be used to induce a tissue-specific knockout of this sodium channel (13, 21). These mice can now be crossed with mice expressing the Cre recombinase in basal keratinocytes either in an inducible or a constitutive manner (e.g., through a keratin 5 promoter).
atin 14-driven Cre recombinase). This should lead to inactivation of the ENaC in cells expressing keratin 14, like basal keratinocytes, and consequently to absence of ENaC in all epidermal keratinocytes.

The sodium channel ENaC controls selective aspects of epidermal differentiation, including synthesis of markers of differentiation, keratinohyalin granule formation, and lipid secretion, but it is still an open question whether ENaC is implicated in wound repair processes. Tissue-specific gene targeting of this channel will help to clarify its functional role in skin function.

Thanks to Friedrich Beermann for suggestions on the manuscript and to Hanspeter Gaeggeler for help with the photographic work. Thanks also to all members of the laboratory of E. Hummler was supported by grants from the Swiss National Science Foundation, the Novartis Foundation, and the Roche Foundation.

References
