The ENaC Channel is Required for Normal Epidermal Differentiation

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Ionic fluxes are important for critical aspects of keratinocyte differentiation, including synthesis of differentiation-specific proteins, enzymatic catalysis of protein cross-linking, post-transcriptional processing of profilaggrin, and lipid secretion. The epithelial sodium channel is expressed in epidermis and the expression of its α and β subunits is enhanced as keratinocytes differentiate. In order to ascertain the role of the epithelial sodium channel in epidermal differentiation, we examined skin of mice in which the epithelial sodium channel α subunit had been deleted. Newborn −/− mice, in which the α subunit had been completely inactivated, demonstrated epithelial hyperplasia, abnormal nuclei, premature secretion of lipids, and abnormal keratohyaline granules. In addition, immunohistochemistry demonstrated that expression of the differentiation markers K1, K6, and involucrin were abnormal. These data suggest that the epithelial sodium channel modulates ionic signaling for specific aspects of epidermal differentiation, such as synthesis or processing of differentiation-specific proteins, and lipid secretion. Key words: epithelial sodium channel/Scnna sodium transport/skin/gene targeting. J Invest Dermatol 118:589–594, 2002

The amiloride sensitive epithelial Na⁺ channel (ENaC) is an important modulator of Na⁺ homeostasis, and thereby plays a critical role in regulating blood pressure (reviewed by Hummler and Horisberger, 1999), renal function (Shimkets et al., 1994; Snyder et al., 1995; Chang et al., 1996; Grünender et al., 1997), and fluid balance in the lung (Stutts et al., 1995; Hummler et al., 1996). ENaC distribution is widespread (reviewed in Garty and Palmer, 1997; Lin et al., 1999; Mirshahi et al., 1999) and has been found in a variety of epithelia, including epidermis, and in sweat glands (Roudier-Pujol et al., 1996; Brouard et al., 1999; Oda et al., 1999). Expression of ENaC subunits is increased in more differentiated keratinocytes, and is found only in the later stages of fetal epidermal development (Oda et al., 1999), suggesting that expression of this channel is linked to keratinocyte differentiation.

Mammalian epidermis generally is not considered as a transporting epithelium, because transeutaneous movement of water and ions is thought to be regulated passively by an external permeability barrier composed of corneocytes embedded in a lipid-enriched intercellular matrix (Elias and Friend, 1975). Yet, crucial aspects of keratinocyte differentiation, including the synthesis of cornified envelope and other differentiation-specific proteins (Hennings et al., 1983; Mauro et al., 1993; 1997), conversion of profilaggrin to filaggrin (Resing et al., 1993), and secretion of stratum corneum lipid precursors (Lee et al., 1994), are controlled by transmembrane ionic fluxes, particularly Ca²⁺. Moreover, Na⁺ influx also modulates Ca²⁺-induced keratinocyte differentiation (Hennings et al., 1983), presumably by modulations in membrane potential (Mauro et al., 1993; Lee et al., 1994). Further, application of amiloride, a known ENaC inhibitor, blocks Ca²⁺-induced differentiation in keratinocytes, although this result could be due to amiloride blockade of a nonspecific cation channel (Mauro et al., 1995).

In order to determine the importance of ENaC for keratinocyte and epidermal differentiation, we studied keratinocytes and epidermis from mice in whom the ENaC α subunit had been deleted (Hummler et al., 1996). These mice display a distinct phenotype, in which keratohyalin granule assembly or processing and lipid secretion appear to be altered. Epidermal differentiation also is retarded in these mice, and epidermal hyperplasia is present. These findings suggest that ENaC processes might modulate specific milestones in epidermal differentiation.

MATERIALS AND METHODS

Animals Genotyping of mice was performed using polymerase chain reaction (PCR) on DNA isolated from tail tips and subjected to PCR-based analysis using primers as described previously (Hummler et al., 1997). Eight wild-type (+/+), 16 heterozygous mutant (+/−), and 12 homozygous mutant (−/−) newborn mice, and five −/+ (−/−Tg) and four +/+ animals in which the α-ENaC subunit was selectively restored to lung tissue, enabling the mice to live beyond the perinatal period, were prepared as described previously (Hummler et al., 1997). Animal experiments followed approved institutional protocols at the University of Lausanne.

Histology Epidermal thickness was measured from the base of the stratum basale to the apex of the stratum granulosum in histologic sections of newborn α-ENaC +/+ , +/−, and −/− mice. Sections were measured with the observer blinded to the genotype of the animals. Statistical significance was calculated using the ANOVA test.

Electron microscopy Full-thickness skin samples were obtained from euthanized animals, minced to < 0.5 mm³, and fixed in modified

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Karnovsky's fixative overnight. All samples were then divided and postfixed in the dark in either 1% ruthenium tetroxide or 1% aqueous osmium tetroxide containing 1.5% potassium ferrocyanide. Thin sections were examined, with or without further contrasting with lead citrate, in a Zeiss 10 A electron microscope operated at 60 kV.

Reverse transcription PCR (RT-PCR) analysis Keratinocytes from α-ENaC +/+, +/−, and −/− mice were isolated and cultured as described previously (Hennings et al., 1980; Hennings, 1994). Total RNA (500 μg) was extracted from α-ENaC −/− and control keratinocytes, reverse transcribed, and amplified by PCR using the Titan One Tube RT-PCR System (Roche Laboratories). Amplified fragments were separated on a 2% agarose gel and visualized by staining with ethidium bromide. Primers were used as described previously (Hummler et al., 1996).

Immunohistochemistry: K1, K6, and K14 Skin samples from newborn mice were frozen in OCT compound. Frozen skin sections were fixed 10 min in paraformaldehyde 4%. For immunohistochemistry of differentiation markers, monospecific rabbit antisera (dilutions according to BAbCO Laboratories, Berkeley, CA) produced against mouse keratin 1, 6, and 14 (BAbCO Laboratories) were incubated 1 h at room temperature with frozen skin sections, followed by incubation for 45 min at room temperature with antirabbit IgY CY3 secondary antibody diluted at 1:100 (Jackson Immunoresearch Laboratories).

Immunohistochemistry: involucrin Skin samples from newborn +/+, +/−, and −/− mice were fixed in 4% paraformaldehyde. Sections of skin were exposed to involucrin antibody (Covance, Richmond, CA) at a dilution of 1:500, followed by a biotinylated goat antirabbit secondary antibody and ABC peroxidase (both from Vector Laboratories, Burlingame, CA) to precipitate diaminobenzidine. Sections were counterstained with methylgreen.

RESULTS

Biopsies were obtained from newborn −/− mice soon after birth, before respiratory distress had developed or hyperplasia might develop in response to a deficient barrier. Although pathologic skin changes were not apparent upon gross examination of newborn skin, light microscopy showed thickening of all suprabasal levels (Fig 1a) attributable to an increase in the average number of cell layers from three to four, six, seven. In addition, −/− epidermis demonstrated focal abnormalities in epidermal maturation, includ-
ing a failure of suprabasal cells to flatten progressively, as well as nuclear atypia, and decreased amounts of keratohyalin. The epidermis of −/− mice was significantly thicker than that of +/+ and +/+ mice, seen both in hematoxylin and eosin sections and confirmed by measurements of epidermal thickness (Fig 1b). Epidermal thickness of +/+ and +/+ mice was not significantly different (Fig 1b), nor were defects in differentiation seen in +/− mice, suggesting that almost complete absence of the ENaC is required to produce defects in epidermal differentiation.

Electron microscopy revealed additional defects. Keratohyaline granules were decreased in number (Fig 2a), and premature lipid secretion was noted in the mid-stratum granulosum in −/− mouse epidermis (Fig 2a). Mice in which transgenic expression of an α-ENaC cDNA rescues the perinatal lethality of α knockout mice (Hummler et al., 1997) were also examined. These animals retain approximately 15% of residual activity as estimated in lung explants (Hummler et al., 1996). Skin samples from these −/− Tg mice retain the premature lipid secretion (Fig 3b), but demonstrate normal nuclei and keratohyaline granules and lack epidermal hyperplasia, suggesting that almost complete ablation of activity is necessary for disruption of keratinocyte differentiation (although not for control of lipid secretion) (Table I). Post-secretory processing of the lipids appeared to be normal, as assessed by ruthenium tetroxide staining (data not shown). Neither the β nor the γ subunits were upregulated in keratinocytes from α-ENaC −/− mice (Fig 4).

Immunohistochemistry of murine epidermis demonstrated delayed expression of involucrin in the −/− newborn mice (Fig 5). Similarly, the expression of K1 was increased in −/− suprabasal layers compared to the +/+ mice (Fig 5). Expression of the basal marker K14 was similar in the interfollicular epidermis. The epidermis from α-ENaC knockout mice reacted positively with a mouse-specific K6 antibody, a marker for both the inner layer of the outer root sheath and nonspecific epidermal hyperplasia (Sundberg et al., 1997) (Fig 6), confirming the epidermal hyperplasia observed with hematoxylin and eosin studies. The pathologic findings of normal versus knockout mice are summarized in Table I.

These data suggest that the ENaC α-subunit controls selective aspects of epidermal differentiation, including synthesis of markers of differentiation, keratohyalin granule formation or processing, and lipid secretion. Moreover, control of lipid secretion seems to require a greater degree of channel activity, whereas the contribution of the ENaC to epidermal differentiation can be maintained by relatively low levels of expression.

Table I. Summary of skin changes in α-ENaC −/− mice

<table>
<thead>
<tr>
<th>Feature</th>
<th>Knockout</th>
<th>Wild-type</th>
<th>Rescue</th>
</tr>
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<tbody>
<tr>
<td>Lipid secretion</td>
<td>Premature</td>
<td>Normal</td>
<td>Premature</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Present</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Maturation</td>
<td>Abnormal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Keratohyalin granules</td>
<td>Decreased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Abnormal</td>
<td>Normal</td>
<td>Not done</td>
</tr>
</tbody>
</table>

Figure 2. Electron microscopy of α-ENaC −/− versus +/+ mouse skin. (A) Decreased number of keratohyalin granules in the stratum granulosum (SG) and enlarged nuclei (N), in a homozygous knockout mouse skin biopsy. (B) Wild-type mice show abundant keratohyalin granules (KH). Scale bars: 0.5 μm.

Figure 3. α ENaC −/− and −/− Tg (transgenic rescue) mice display premature lipid secretion. (A) Mid-stratum granulosum of −/− mice shows focal secretion of lamellar body contents into intercellular domains (arrows). (B) Upper and mid-stratum granulosum of −/− Tg rescue mice. Lipid is secreted normally in the upper stratum granulosum, but also is secreted prematurely in the mid-stratum granulosum (arrows). Scale bars: 0.5 μm.
DISCUSSION

Our initial pharmacologic experiments, which demonstrated that an amiloride-sensitive conductance controls Ca\(^{2+}\)-induced keratinocyte differentiation (Mauro et al., 1995), led us next to probe keratinocytes and epidermis for the amiloride-sensitive ENaC. We and others demonstrated ENaC subunit expression in both keratinocytes and epidermis (Roudier-Pujol et al., 1996; Brouard et al., 1999; Oda et al., 1999). ENaC expression, particularly of the β subunit, increases in more differentiated keratinocytes (Oda et al., 1999). We now report that ENaC is important for specific processes of epidermal maturation and differentiation, based upon our observations in skin in which the α-ENaC subunit has been deleted.

The α-ENaC ±/± defects in keratohyalin formation/processing, premature lipid secretion, and abnormal expression of involucrin, K1, and K6 parallel the known increase in ENaC expression that occurs with differentiation (Oda et al., 1999). Differentiation in keratinocytes also appears to be modulated by other plasma membrane ion channels, including a nonselective cation channel (Mauro et al., 1995), nicotinic ACh channel (Grando et al., 1995), K\(^+\) channel (Mauro et al., 1997), and Cl\(^-\) channel (Mauro et al., 1993; Wohlrab and Markwardt, 1999). The mechanism(s) by which ENaC controls keratohyalin granule formation/dispersal and lipid secretion is (are) unknown, but probably relates to its control of Na\(^+\) and/or Ca\(^{2+}\) fluxes. Whereas the delayed expression of differentiation markers might simply reflect delayed skin maturation, α-ENaC ±/± mice also exhibit premature lipid secretion that is not seen with a simple delay in skin maturation. We have reproduced these lipid secretion abnormalities by short-term treatment of adult hairless mouse skin with 1 mM amiloride, a concentration that pharmacologically blocks the ENaC. Whereas amiloride also blocks the NHE1 antporter at this concentration, lipid secretion abnormalities were not seen in mice treated with a selective NHE inhibitor, HOE-694, nor were lipid secretory

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**Figure 4.** Detection of mRNA transcripts for α-, β-, and γ-ENaC in ENaC wild-type (α-ENaC +/+), heterozygous (+/-), and homozygous mutant (-/-) mouse keratinocytes. In α-ENaC -/- mice, no change in mRNA expression of the β- and γ-ENaC subunit was detected. The RT-PCR was controlled by detection of glyceraldehyde-3-phosphate dehydrogenase message. Positive control (+), α-ENaC cDNA; negative control (-), PCR reaction without RNA.

**Figure 5.** Differentiation markers are abnormally expressed in α-ENaC -/- mice. Involucrin expression as determined by immunohistochemistry was restricted to the uppermost layers of the hyperplastic α-ENaC -/- mouse skin. K1 and K14 were detected immunohistochemically in newborn skin from α-ENaC-deficient and wild-type skin as described in Materials and Methods. Magnification: 400×.
proliferation; and in the ENaC modulation of differentiation found in undifferentiated (basal) versus differentiated (granular layer) keratinocytes. Although the Ca²⁺-permeable channels in the epidermis are crucial for normal lipid secretion, because different types of ion channels are required for normal lipid secretion. Decreased ENaC would hyperpolarize the membrane and decrease Ca²⁺ influx, thereby allowing unregulated lipid secretion. Decreased Ca²⁺ influx also inhibits the post-translational processing of prollagrin to filaggrin (Resing et al, 1993), which could explain the presence of abnormal keratohyalin granules in the ~+/~ granular cells (Presland et al, 1995).

In humans, mutations in all ENaC subunits have been reported, resulting in hypoactivity of the ENaC that results in pseudohypoaldosteronism, or PHA-1 (Gründler et al, 1997). No specific dermatologic findings are reported for this condition. All mutations tested so far retain significant rest activity of ENaC, however, which might explain the absence of severe pulmonary pathology in PHA-1 patients, whereas inactivation of the α-ENaC subunit is rapidly fatal due to respiratory failure (Hummler et al, 1996; Bonny et al, 1999). The knockouts for the β- and γ-ENaC subunit are available, but were not analyzed for a potential skin phenotype (Barker et al, 1998; McDonald et al, 1999). Rest activity of the ENaC has been demonstrated in γ-ENaC ~~/~ mice (Barker et al, 1998). These findings are consistent with our experimental observations, reported above, that almost complete ablation of the α-ENaC subunit is required to produce defects in epidermal differentiation, although a higher level of ENaC expression is required for normal lipid secretion.

To a large extent, the histologic and ultrastructural findings in ENaC ~~/~ mice resemble features of psoriasis and other psoriasiform dermatoses. Although we have not examined these disease states for alterations in expression or function of the ENaC, the similar histologic presentation suggests that the ENaC channel could modulate, or even underlie, some of the defects in keratinocyte differentiation that are seen in these conditions. Graft experiments and skin-specific gene targeting of α-ENaC might help to further identify the role of this sodium channel in epidermis.

abnormalities seen in NHE1 ~~/~ mice, suggesting that lipid secretion is controlled by the ENaC. Finally, when skin from newborn α-ENaC ~~/~ mice was grafted onto athymic recipient mice, epidermal hyperplasia persisted for the duration of the grafts, i.e., up to 8 wk, whereas +/+ skin, grafted in parallel, appeared normal.

Currents through the ENaC could direct differentiation by signaling a number of different pathways. In fact, it is likely that the mechanism of action varies in spinous versus granular layer keratinocytes, and in the ENaC modulation of differentiation versus lipid secretion, because different types of ion channels are found in undifferentiated (basal) versus differentiated (granular layer) keratinocytes. Although the Ca²⁺-permeable channels in the granular layer keratinocytes have not yet been characterized by molecular or electrophysiologic methods, pharmacologic and ion-substitution experiments suggest that these keratinocytes express T- or L-type voltage-sensitive Ca²⁺ channels (Lee et al, 1992, 1994; Resing et al, 1993; Grando et al, 1996). Na⁺ influx through the ENaC could depolarize the plasma membrane, thus increasing Ca²⁺ influx through voltage-sensitive channels (Lee et al, 1992; Mauro et al, 1995). Moreover, raised intracellular Ca²⁺ blocks lipid secretion in a manner that is reversible by L-type channel blockers (Lee et al, 1992, 1994). Thus, abolishing Na⁺ influx through the ENaC would hyperpolarize the membrane and decrease Ca²⁺ influx, thereby allowing unregulated lipid secretion. Decreased Ca²⁺ influx also inhibits the post-translational processing of profilagrin to filaggrin (Resing et al, 1993), which could explain the presence of abnormal keratohyalin granules in the ~~/~ granular cells (Presland et al, 1995).

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Figure 6. K6 immunostaining confirms epidermal hyperplasia in skin from α-ENaC ~~/~ mice. Skin sections of newborn ~~/~ pups are positively stained for K6.


