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Epithelial sodium channel in human epidermal keratinocytes: expression of its subunits and relation to sodium transport and differentiation

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SUMMARY

The amiloride-sensitive epithelial sodium channel (ENaC) is a main determinant of sodium absorption in renal and colonic epithelial cells. Surprisingly, it is also expressed in non-transporting epithelia such as the epidermis. To gain insight into the putative role of ENaC in keratinocytes, we have evaluated its expression in human skin and in cultured human keratinocytes. Our results indicate that (1) ENaC is expressed in the epidermis and in cultured keratinocytes, at the mRNA and at the protein levels, (2) the ratio of expression of the different ENaC subunits is drastically modified at the protein level during cell growth and differentiation, with a selective upregulation of the beta subunit, (3) no transepithelial sodium transport function is apparent in cultured keratinocytes, but patch-clamp recordings indicate the existence of functional sodium channels with properties similar to those of the cloned ENaC and (4) ENaC inhibition does not alter keratinocyte proliferation, but it significantly decreases the frequency of dome formation in confluent keratinocyte cultures. These results document for the first time the characteristics of ENaC subunit expression in human keratinocytes, and suggest that ENaC may be important during differentiation.

Key words: ENaC, Benzamil, Amiloride, Epidermis, Hair follicle, Sodium transport

INTRODUCTION

The amiloride-sensitive epithelial sodium channel (ENaC) plays a key role in regulating transepithelial transport of sodium in electrically tight epithelia (Garty and Palmer, 1997). Its location in the apical membrane, facing the tubular fluid in the kidney collecting duct, drives sodium entry into the cell. Sodium is then extruded from the cell at the basolateral membrane by the Na^+-K^+-ATPase. Coordinate activities of apical sodium channels and the basolateral sodium pump allow efficient transcellular sodium transport without affecting cell sodium concentration. Three subunits (α, β and γ) of ENaC have been characterized at the molecular level. Each subunit is encoded by a different gene (Canessa et al., 1993, 1994; Lingueglia et al., 1993; McDonald et al., 1995; Voilley et al., 1994, 1995). Coexpression of α, β and γ ENaC subunits in Xenopus oocytes generates a sodium channel whose characteristics mimic those of the endogenous apical sodium channel of tight epithelia (Canessa et al., 1994; Garty and Palmer, 1997). ENaC subunits are found in a number of sodium-reabsorbing, electrically tight epithelia, such as the renal collecting duct, the surface epithelium of the distal colon and the ducts of salivary and sweat glands (Duc et al., 1994). Airway cells (nasal, tracheal epithelium and glands, type II pneumocytes) also express ENaC (Farman et al., 1997). The functional relevance of ENaC in regulating extracellular fluid volume and blood pressure is clearly illustrated by the identification of genetic defects of ENaC as the cause of certain human diseases such as Liddle syndrome (arterial hypertension due to ENaC mutations, leading to its permanent activation; Shimkets et al., 1994) or autosomal recessive type I pseudohypoaldosteronism (salt-wasting syndrome, due to mutations of ENaC that result in a loss of channel activity; Chang et al., 1996).

Surprisingly, ENaC transcripts have been identified in epidermis and hair follicles of the rat (Roudier-Pujol et al., 1996), and in the pluristratified epithelium of the esophagus (Farman et al., 1997). These tissues are not known to have a transepithelial transport function. In vivo, the skin (in human or rodents) is not exposed to external Na^+ and is not involved in ion or fluid absorption; in fact, the epidermis has a barrier function that prevents dessication and protects against pathogens (Eckert, 1989; Elias et al., 1988; Green, 1977; Green et al., 1982; Hardman et al., 1998; Holbrook and Wolf, 1987). The epidermis, the outermost layer of the skin, is a self-renewing pluristratified epithelium, which protects the body against environmental agressions. It is mainly consists of keratinocytes, which represent over 90% of the epidermal cells.

The basal keratinocytes can proliferate and generate cells that undergo terminal differentiation. This ultimately leads to the formation of the flattened anucleated and cornified cells constituting the stratum corneum, the outermost layer of the epidermis. This is the layer responsible for the barrier function of the epithelium. Terminal differentiation is precisely regulated both at the cellular and molecular levels. During keratinocyte terminal differentiation many genes, including keratins 1 and 10, transglutaminase type 1, filaggrin, involucrin and a number of other genes (Fuchs, 1995), are specifically expressed.

To gain insight into the role of ENaC in the skin, this study examines the expression and function of ENaC in cultured human keratinocytes. The inhibition of dome formation in confluent pluristratified keratinocyte cultures by benzamil, a specific ENaC inhibitor (Garty and Palmer, 1997), strongly suggests that this channel may have a function, althought net transepithelial transport could not be detected in cultured keratinocytes, which lack tight junctions. This is further confirmed by the presence of a benzamil-sensitive conductance in whole-cell patch-clamp experiments. Our results demonstrate that the α, β and γ ENaC subunits are expressed by human cultured keratinocytes; this expression varies in a pattern linked to terminal differentiation, including a change in the ratio of subunit expression at the protein level.

MATERIALS AND METHODS

Tissue samples

Human skin samples were obtained from patients undergoing reconstructive surgery; they were snap-frozen in liquid nitrogen and kept at –80°C until further processing. Frozen sections (10 μm) were obtained using a cryostat (Leica CM 3000), collected on Superfrost Plus slides and used for immunocytochemistry.

Cell culture

Normal human diploid epidermal keratinocytes, YF 29 isolated from the foreskin of a newborn, were cultured (Rheinwald and Green, 1975) and frozen stocks were made. Cells were thawed from the frozen stock and used between passages 4 and 6. They were cultured on a feeder layer of lethally irradiated (60 Gy) mouse embryonic 3T3 fibroblasts (Todaro and Green, 1963), in cFAD (a 3:1 mixture of the Dulbecco-Vogt modification of Eagle's medium and Ham's F12 medium; Gibco BRL) supplemented with 10% fetal calf serum (FBS, HyClone Laboratories Inc.) as described previously (Rheinwald and Green, 1975; Rochat et al., 1994).

To determine colony-forming efficiency, 100-1000 keratinocytes were cultured for 12 days. Cultures were fixed with 3.7% formaldehyde and stained with 1% B Rhodamine. Colonies were counted under a dissecting microscope (Barrandon and Green, 1987). Colony-forming efficiency is expressed as the number of colonies founded by 100 seeded cells. To determine the size of the keratinocytes, cells were suspended in a hemocytometer (Reichert-Jung) and photographed using an Axiovert 35 microscope (Zeiss). Cell size was measured on photographs using a micrometer (Zeiss). To determine dome formation, cultures were examined under a binocular microscope and the number of domes was counted.

For electrophysiological studies, keratinocytes were grown on Snapwell filters (Costar) as follows: keratinocytes were seeded on top of the filter and 3T3 fibroblasts on the bottom of the well; cells were bathed on each side with 8 ml of minimum medium as previously described (Blot-Chabaud et al., 1996; Djelidi et al., 1997).

Elutriation

Human keratinocytes were cultured in flasks, trypsinized 2 days after the average confluency. Cells were suspended at 2×10^7 cells/ml in DMEM containing 5% bovine serum (HyClone Laboratories Inc.) and DNase (0.04 mg/ml). Cells (5.2×10^8 cells) were separated in function of their size (Tseng and Green, 1994), using a Beckman J2-21 M centrifuge equipped with a JE-5.0 rotor and a Standard Chamber of elutriation (Beckman, Gagny, France). The speed of the rotor was 140 ***g*** (elutriation boundary) at 15°C. The elutriation buffer was 138 mM NaCl, 5 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 5 mM $NaH_2PO_4.H_2O$, 10 mM glucose and Red phenol (0.01 g/l), pH 7.2. The initial flow rate (4 ml/minute) permitted the admission of 5×10^8 cells into the Standard Chamber. The flow rate was then increased by steps of 100 ml at 4.5, 8, 12, 16 and 21 ml/minute. Five fractions of cells were collected, centrifuged at 4°C and resuspended in cFAD. Samples of each fraction were used to determine cell number, cell size and colony-forming efficiency. The remaining cells were lysed for RNA extraction.

Ribonuclease protection assay

Total RNA was extracted from cultured cells lysed in guanidinium thiocyanate (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 0.1 M β-mercaptoethanol) for RNAse protection assay as described previously (Escoubet et al., 1997). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal loading standard. Antisense cRNA probes were synthesized using [γ-^{32}P]dUTP (15 TBq/mmol; Amersham Corp.) according to the manufacturer's protocol (Promega). The GAPDH probe was synthesized using a cDNA inserted in Bluescript (Escoubet et al., 1997). The cDNAs for α, β and γ hENaC (subcloned into pCRII), for human involucrin (in Bluescript) and for human basonuclin (in pSPORT1), were used to synthesize antisense cRNA probes. The lengths of probes were: 354 nt for α hENaC subunit with a protected fragment of 223 nt (nt 875-1098), 274 nt for β hENaC subunit with a protected fragment of 190 nt (nt 777-967), and 204 nt for γ hENaC subunit with a protected fragment of 107 nt (nt 1481-1588); 192 nt for human involucrin with a protected fragment of 118 nt (nt 547-665), 374 nt for human basonuclin with a protected fragment of 306 nt (nt 4305-4611) and 183 nt for GAPDH with a protected fragment of 164 nt (nt 707-871). Radioactivity corresponding to the protected fragments was quantified with an Instant Imager (Packard).

Antibodies

Rabbit polyclonal anti-rENaC (i.e. rat-specific) α, β and γ subunit antibodies (against a fusion protein between GST and portions of the rat α, β or γ subunit) have been previously described (Djelidi et al., 1997). Rabbit polyclonal anti-hENaC α and γ subunit antibodies (i.e. human-specific) were raised against synthetic peptides coupled to keyhole limpet haemocyanin (Neosystem; Strasbourg, France). The peptide sequence was PGLMKGNKREEQGLG for α subunit (amino acids 17-32, located in the cytoplasmic N terminal part of α hENaC), and SLYGFPESRKRREAES for γ subunit (amino acids 126-142 in the extracellular loop of γ hENaC). These antibodies were purified on HITrap affinity columns (Pharmacia Biotech) according to the manufacturer's recommendations. To assess the specificity of the antibodies (see Fig. 4), competition experiments were performed in the presence of the corresponding fusion proteins (100 μg/ml) for anti-rENaC antibodies or in the presence of peptides (200 μg/ml) for anti-hENaC antibodies.

Immunocytochemistry

Frozen sections (10 μm) of human skin were fixed for 10 minutes at –20°C in acetone and rinsed in PBS. Endogenous peroxidase activity was inhibited with 5% H_2O_2 in PBS. The sections were then rinsed in PBS, incubated in FCS for 20 minutes, and overnight at 4°C in a 1/4 dilution of the purified antibody (rabbit polyclonal anti-hENaC α and γ subunit antibodies). Sections were then rinsed in PBS, incubated

for 30 minutes at room temperature in a 1/40 dilution of goat anti-rabbit immunoglobulins (Dako), rinsed in PBS and incubated for 60 minutes with 1/100 diluted Peroxidase Anti-Peroxidase (PAP) rabbit immunoglobulins (Dako). They were then rinsed in PBS, incubated for 10 minutes at room temperature with diamidinobenzidine (Sigma Fast) and rinsed again before they were dehydrated and mounted in Eukitt.

Immunoprecipitation

Cultured keratinocytes were labeled with [^{35}S]methionine (37.5 Bq/mmol, Amersham) for 1.5 hour, and immunoprecipitation was performed as previously detailed (Djelidi et al., 1997). Briefly, cells were extracted in ice-cold lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM PMSF). [^{14}C]ovalbumin (Amersham, 0.5 mCi) was added to the cell lysates prior to the assay as a standard and coprecipitated with a polyclonal anti-ovalbumin antibody (Nordic). Antibodies against ovalbumin and rENaC antisera (5 μl) or hENaC antisera (30 μl) were incubated overnight at 4°C with cell lysates (100 μg protein per assay). Immunoprecipitates were incubated with Protein A-Sepharose CL4B beads (Pharmacia LKB) and samples of eluted immunoprecipitates were submitted to PAGE (7.5%). The signal was quantified with an Instant Imager (Packard). The results were normalized to the signal obtained with ovalbumin.

Electron microscopy

Keratinocytes grown on filters were fixed with 2% glutaraldehyde in phosphate buffer, pH 7.4 for 30 minutes at room temperature and handled as previously described (Blot-Chabaud et al., 1996). Ultrathin sections were examined with a Philips EM 410 electron microscope.

Functional studies

Keratinocytes were grown on a plastic surface and fluid under the domes (about 5 μl) was collected using a thin glass pipette (outer diameter 2 μm). Samples (less than 1 nl) were analyzed for sodium and potassium concentration by emission flame microspectrophotometry (ACTA, Ouistream, France).

Transport properties of keratinocytes grown on filters were evaluated by the short circuit current technique as previously described (Blot Chabaud et al., 1996; Djelidi et al., 1997) after the cultures had reached confluency and become pluristratified. The filters were mounted onto a voltage clamp system (Costar Corp., World Precision Instruments) to measure transepithelial voltage and resistance. Short-circuit current I_{SC} (μA/cm^2) was measured by clamping transepithelial potential to 0 mV for 1 second.

Patch-clamp experiments were conducted on keratinocytes grown on a plastic surface. Keratinocytes with the largest size were aspirated individually with a Pasteur pipette (Barrandon and Green, 1985), seeded and cultured with benzamil (10^{-7} M) for 6 days (to prevent an eventual sodium influx into the cell, possibly responsible for cell damage or death). The patch-clamp experiments were conducted after benzamil withdrawal. Recordings were performed in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). All experiments were performed at room temperature (18°C-23°C). Patch pipettes of 4-8 MΩ were filled with a solution containing 120 mM CsF, 10 mM CsCl, 10 mM EGTA and 10 mM Hepes. CsOH was added to adjust the pH to 7.2. The osmolarity was 265 mOsm/kg. The standard external solution contained 130 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes. NaOH was added to adjust the pH to 7.3. Benzamil was applied to the cell by means of a multibarrel fast perfusion system. Solutions flowed continuously by gravity from all barrels. Currents were recorded with a List EPC-7 amplifier (Darmstadt, Germany). The voltage-clamp current was filtered (8-pole Bessel) with a corner frequency of 100 Hertz. Sampling frequency was 400 Hertz. Data were acquired and analyzed by Strathclyde Electrophysiology Software (a gift from Dr J. Dempster) and pClamp6 (Axon Instruments, Foster City, CA, USA).

RESULTS

Human keratinocytes, when cultured, may form a pluristratified layer that has many characteristics of the epidermis. Electron microscopic examination of confluent cultured keratinocytes showed that cells grew as a multilayer (Fig. 1A), with distinct types of cell junctions. Gap junctions were present (Fig. 1B), and numerous desmosomes (Fig. 1C) were observed. Hemidesmosomes (Fig. 1D) also developed on the basal keratinocytes when cultured onto a filter. No tight junctions were found, in particular in the domes. Intracellular glycogen stores were often present. The upper layer cells did not show extensive microvilli or organized cornified layers.

ENaC expression in cultured human keratinocytes

It has been previously reported that ENaC subunit mRNAs are

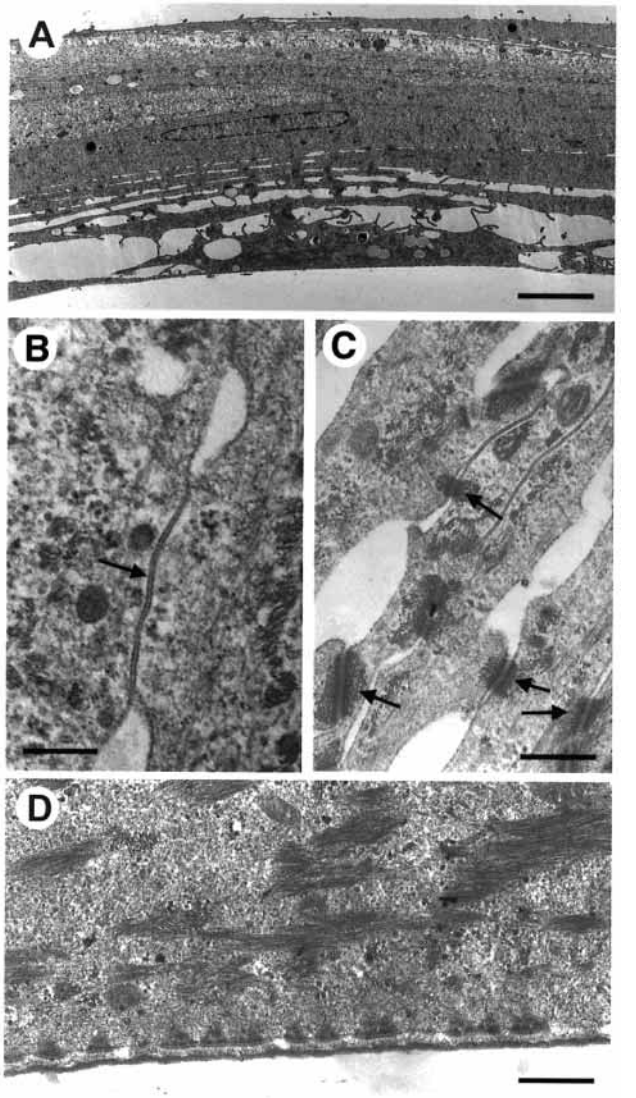

Fig. 1. Ultrastructure of human keratinocyte cultures. Keratinocytes were grown on a plastic surface (A,B) and on filters (C,D). (A) Low magnification view of the multilayer. (B-D) Higher magnifications illustrating the observed junctions. (B) Gap junctions (arrow); (C) desmosomes (arrows); (D) hemidesmosomes in basal layer of cells in contact with the filter (lower part of the picture). No tight junctions were detected. Bars, 2.2 μm (A); 0.26 μm (B); 0.42 μm (C,D).

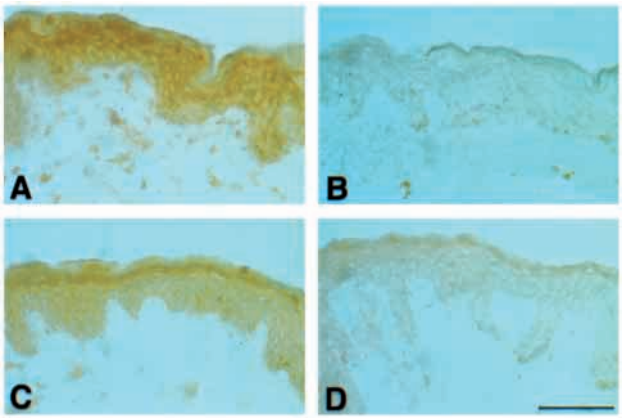

Fig. 2. Immunolocalization of α and γ ENaC subunits in human skin. Skin sections were immunostained with antipeptide antibodies against human α subunit (A,B) or γ subunit (C,D). The staining was largely attenuated by coincubation with the respective immunizing peptides (B,D). Bar, 83 μm.

expressed in rat epidermis and hair follicles (Roudier-Pujol et al., 1996). However, little is known about expression of ENaC in the human skin. The presence of α and γ subunits is now demonstrated by immunocytochemistry in human epidermis (Fig. 2). Anti-α (Fig. 2A) and anti-γ (Fig. 2C) antisera gave a clear signal in epidermal keratinocytes, while the dermis (lower part of each photograph) was negative. The specificity of the immunostaining was provided by the extinction of the signal when each of the antisera was incubated in the presence of the corresponding immunizing peptide (Fig. 2B for α and Fig. 2D for γ). In addition, no signal was detectable when the primary antibody was omitted (not shown).

We then investigated whether cultured keratinocytes express ENaC at the RNA and at the protein level. Results of RNAse protection assays are shown in Fig. 3. Probes specific for human α, β and γ ENaC subunit transcripts have been hybridized with RNAs obtained from keratinocytes and kidney, and with yeast tRNA. For each ENaC subunit, a protected band of appropriate size (221 nt for α, 190 nt for β and 107 nt for γ) was obtained in keratinocytes or kidney, while no signal was visible with yeast tRNA (Fig. 3). To document ENaC expression at the protein level, cultured keratinocytes were labeled with [^{35}S]methionine. Immunoprecipitation of the solubilized cell lysates was performed with antibodies generated against rat (Fig. 4A) and human (Fig. 4B) ENaC subunits. Results obtained with antibodies raised against a fusion protein between GST and portions of the α, β or γ subunit of the rat ENaC (Djelidi et al., 1997) are shown in Fig. 4A: for each subunit, a band was visible in the presence of antiserum, which was displaced when the antiserum was preincubated with the corresponding fusion protein. No immunoprecipitated materials were detected when preimmune serum was used. Thus, antibodies against rat ENaC sequences cross-react with human ENaC, a finding that is not surprising in view of the homologies between rat and human sequences. Other antibodies were generated against human peptide sequences. The material immunoprecipitated with anti-α or anti-γ antibodies yielded a band that was absent when the antisera were incubated with their corresponding immunizing peptides (Fig. 4B); preimmune sera were negative as well. These results demonstrate that human keratinocytes express ENaC in vitro and in vivo.

Evaluation of the role of ENaC in transepithelial sodium transport in cultured human keratinocytes

In epithelial cells of electrically tight epithelia, such as those of the renal collecting duct or the colonic surface cells, ENaC plays a major role in the control of transepithelial (apical to basolateral) sodium transport. Whether cultured keratinocytes can perform transepithelial sodium (and solute) transport was not known. We therefore evaluated the sodium transport properties of cultured keratinocytes.

In cultured cells of other epithelial origin (e.g. kidney, lung), domes are usually indicative of fluid transport, the fluid accumulating underneath the cells. When cultured on a plastic surface, keratinocytes reach confluency 11-14 days after seeding, depending on the initial density of the inoculum, and domes may form. Dome size varied from small domes (500 μm in diameter) as observed in renal cell lines, to very large ones (up to 1-3 mm in diameter) (Fig. 5). The composition of the fluid accumulated under the domes was then measured, in the presence and the absence of benzamil. This fluid contained 148.5±2.4 mM Na^+ and 5.1±0.1 mM K^+ (n=6) in the absence of benzamil pretreatment, and 151.2±1.7 mM Na^+ and 5.0±0.2 mM K^+ (n=5) on YF29 cells cultured in the presence of 10 nM

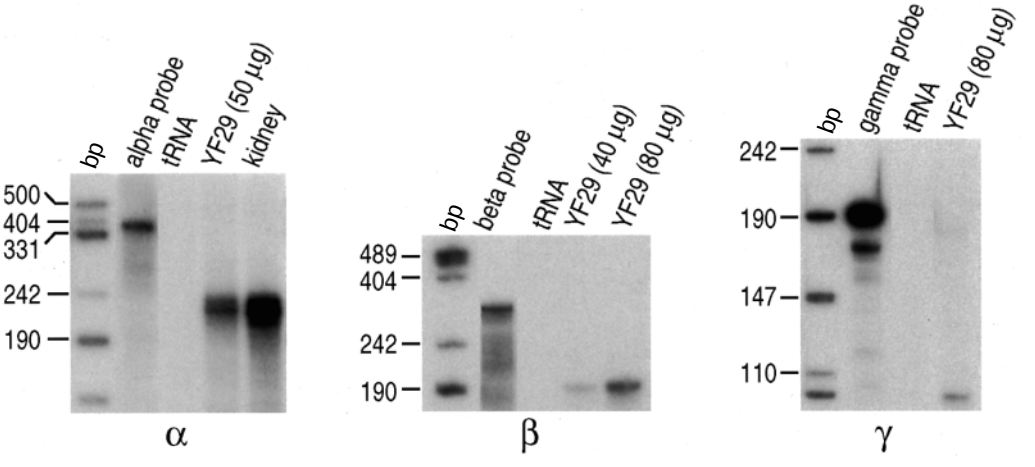

Fig. 3. Expression of ENaC transcripts in cultured human keratinocytes. RNase protection assays were performed with ^{32}P-labeled probes complementary to α, β and γ subunit ENaC mRNAs. Yeast tRNA (10 μg) was used as a negative control and human kidney RNA (20 μg) as positive control. The amount of keratinocyte (YF29 cells) mRNAs loaded onto the gels is indicated. Protected bands (see Materials and Methods) were observed in keratinocytes for each probe. The positions of polynucleotide markers are shown (lanes bp).

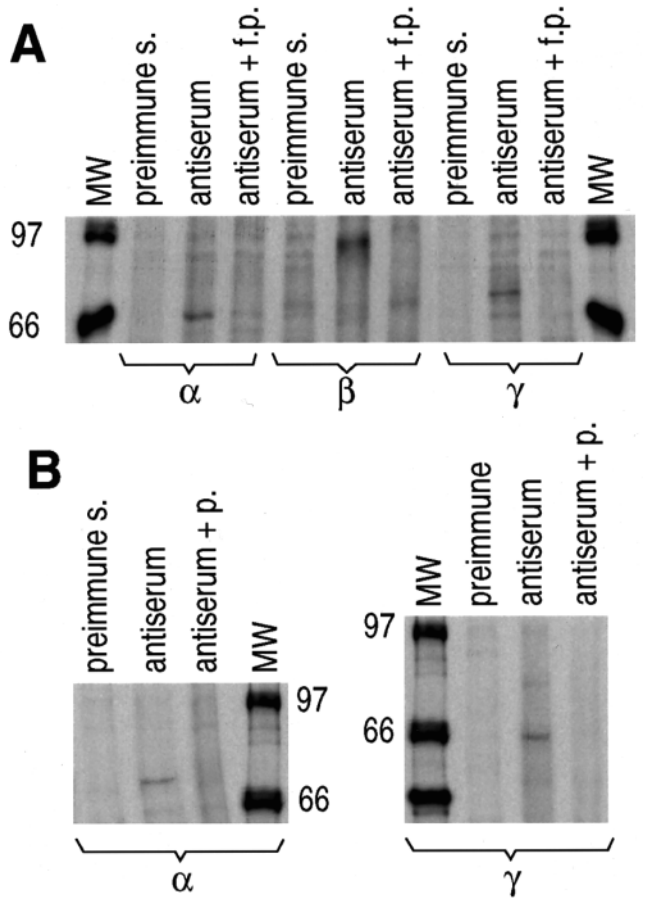

Fig. 4. Immunoprecipitation of ENaC subunits from cultured human keratinocytes. Cell extracts (100 μg) from YF29 cells (day 11 of culture for α and γ, and day 15 for β) labeled with [^{35}S]methionine were immunoprecipitated with antibodies against rat ENaC subunits (α, β and γ) (A) or with antibodies against human α and γ ENaC subunits (B). In each case, control immunoprecipitations were run using either the preimmune serum or the specific antibody plus the antigen (fusion protein (f.p.) for the antibodies against rat ENaC subunits, or peptide (p.) for the antibodies against human ENaC subunits). Specific bands with α, β and γ antisera were observed in keratinocytes, which were absent in the two control conditions. The positions of molecular mass markers (kDa) (lanes MW) are shown.

benzamil. Differences between these values, as well as differences between them and those of the culture medium bathing the cells, are not significant.

We then evaluated whether keratinocytes can transport sodium from the apical to the basolateral compartment, by analogy with the established role of ENaC in electrically tight epithelia. Keratinocytes were cultured to confluency on filters to form a continuous pluristratified epithelium. This allowed electrical measurements of transepithelial resistance and short circuit current. The mean electrical resistance of the preparations was 201±102 Ω/cm^2 (n=14), i.e. a very low value compared to that observed in differentiated renal collecting duct cells (for example the RCCD1 renal cell line has a resistance of 2000-4000 Ω/cm^2, as reported in Blot-Chabaud et al., 1996; Djelidi et al., 1997). These results are consistent with the absence of tight junctions. Transepithelial resistance of YF29 cells was not altered by the sodium channel inhibitor benzamil (10 nM) when added apically (188±59, n=7) or on the basal side (228±170, n=7). Amiloride (1-10 μM) was ineffective as well. Transepithelial potential difference (PD) and short-circuit current values (I_{sc}) were also very low (PD, –0.7±1.4 mV; I_{sc}, –2.4±9.4 μA/cm^2; both not different from zero), and insensitive to benzamil (not shown), indicating that there was no transfer of electrical charges from one side to the other of the multilayer epithelium.

Patch clamp analysis of ENaC in cultured keratinocytes

Since the in vivo function of ENaC in transporting epithelia is to promote sodium entry into the cell, the presence of functional ENaC at the surface of cultured keratinocytes was tested by patch-clamp recordings in the whole-cell configuration. Current flowing through ENaC was identified on the basis of a block by 100 nM benzamil. However, only a very small proportion of recorded cells (4/83) showed detectable currents. The current was large enough to allow accurate measurement of the on- and off-rate constants in only two of the four recorded cells. The rates were roughly the same in these two cells, and the data shown come from the cell showing the better signal-to-noise ratio. Channels showed a permeability ratio of sodium versus cesium (PNa/PCs) of >4.9, as calculated from reversal potential higher than +40 mV under biionic conditions (Na^+ out and Cs^+ in) (Fig. 6). Similar results were obtained either from voltage jump or from voltage ramp protocols. At –50 mV, the benzamil on-rate was 1.3 seconds

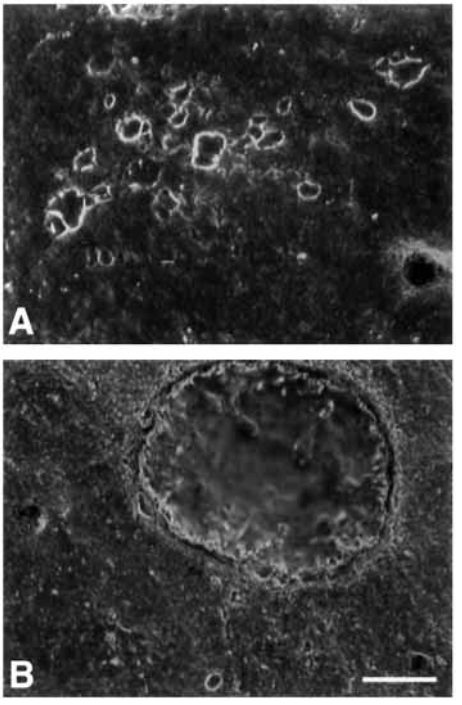

Fig. 5. Aspects of domes formed by confluent human keratinocytes. When cultured on plastic, YF29 cells form domes after reaching confluency. Some domes are relatively small (50-60 μm diameter) (A). Very large domes (as in B) of diameter 0.6-3 mm were frequently observed. The area of the dome shown in B is 0.1 mm^2. Bar, 120 μm.

Table 1. Lack of effect of sodium channel inhibitors on colony-forming efficiency and keratinocyte size

	Colony-forming efficiency (%) (*n*)	Cell diameter (μm) (*n*)
Control	19.7±2.5 (3)	16.3±2.0 (200)
Amiloride (10^{-6} M)	21.7±3.8 (3)	16.5±2.0 (165)
Control	25.3±2.1 (4)	17.5±3.7 (237)
Benzamil (10^{-8} M)	28.0±2.6 (4)	17.5±3.8 (227)

Table 2. Characteristics of the elutriated keratinocyte fractions

Fraction	Colony-forming efficiency (%)	Cell volume (μm^3)
1	33.6	1800
2	16.2	2800
3	6.6	3300
4	7.6	5300
5	6.8	5900

and off-rate was 3.12 seconds (four recordings), both values being much slower than our resolution limit. Onset and offset of benzamil block (Fig. 6 inset) yielded (assuming a bimolecular reaction) an apparent affinity of 42 nM at –50 mV and 19 nM at –70 mV; this is coherent with the described affinity of this compound for ENaC as calculated from the dose-response relationship (Canessa et al., 1994), as well as with the described mild voltage dependence of the amiloride block (Hamilton and Eaton, 1985). These characteristics clearly differ from the non-selective cation channel previously reported in keratinocytes (Mauro et al., 1995).

ENaC transcripts expression is modulated during terminal differentiation

In vivo expression of ENaC subunit mRNAs has been shown to vary according to the differentiation status of keratinocytes within the rat epidermis, with the highest levels of transcripts in the large differentiated suprabasal keratinocytes (Roudier-Pujol et al., 1996). When cultured, keratinocytes also increase their size during the process of differentiation (Watt and Green, 1982). Since the entry of sodium may increase cell volume, and since there is an inverse relationship between the size of a keratinocyte and its ability to form a colony (Barrandon and Green, 1985), we have examined the effects of ENaC inhibitors on growth and differentiation of keratinocytes. The ability of YF 29 cells to form colonies was not modified in the presence of amiloride (1 μM) or benzamil (10 nM) (Table 1). In addition, these drugs did not significantly modify cell volume (Table 1).

To evaluate the relationship between ENaC expression and cell differentiation, keratinocytes of different sizes were separated by elutriation, and the level of expression of ENaC transcripts was measured. ENaC expression was compared to that of basonuclin, which is expressed only in basal proliferative cells (Tseng and Green, 1994), and to that of involucrin, a marker of terminal differentiation (Rice and Green, 1979; Watt and Green, 1981). In addition, the colony-forming efficiency of each cell fraction was determined. Cell size varied between 2000 and 6000 μm^3 (Table 2). The colony-forming efficiency of the first fractions (containing the smallest cells) was high, and that of the later fractions (containing the largest cells) was low (Table 2). Low levels of expression of the α, β and γ ENaC mRNAs (Fig. 7) were observed in the first two fractions, while expression increased gradually with cell size. The pattern of expression of ENaC followed that of involucrin, and was inversely related to that of basonuclin. These results show that ENaC subunit mRNAs expression is linked to terminal differentiation.

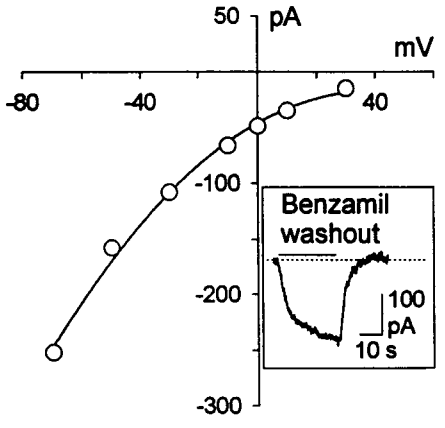

Fig. 6. Whole-cell patch-clamp recordings of cultured human keratinocytes. Benzamil-sensitive current-voltage relationship (Na^+ out / Cs^+ in) in YF29 cells: zero current was achieved upon 100 nM benzamil application. Current developed after 20 seconds of benzamil washout was measured at different holding potentials. Inset, current developed after benzamil washout at –50 mV. mV, millivolts; pA, picoamps; s, seconds.

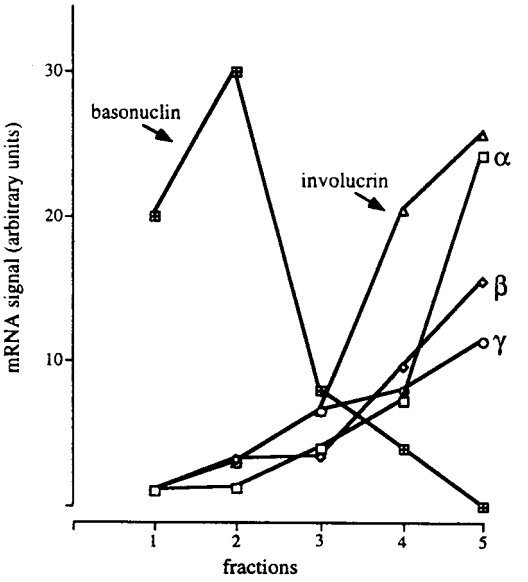

Fig. 7. Expression of ENaC, transcripts in cultured keratinocytes of different sizes. RNase protection assay was performed on elutriated keratinocytes (at day 10 of culture), corresponding to various differentiation states (from small undifferentiated cells to large differentiated ones (see Table 2). Signals (normalized to those of GAPDH) are given in arbitrary units per cell. The abundance of mRNAs encoding for ENaC subunits and for involucrin increases with cell size, while basonuclin, a marker of undifferentiated keratinocytes, predominates in the first two fractions.

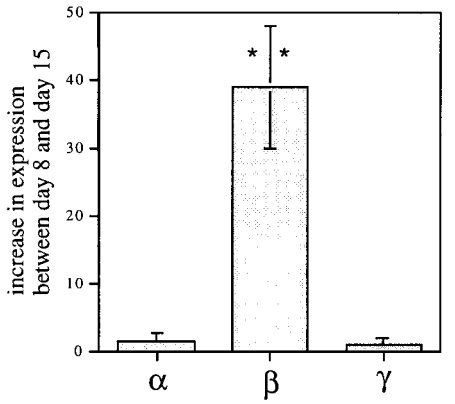

Fig. 8. Differential levels of ENaC subunits in cultured human keratinocytes. Immunoprecipitation of ENaC subunits after labelling with [^{35}S]methionine was compared in proliferating (day 8 of culture) and differentiated (day 15 of culture) keratinocytes. The immunoprecipitated bands were quantified, normalized to the ovalbumin signal and expressed as the ratio between values observed in differentiated keratinocytes (day 15) and in proliferating ones (day 8). Differentiation induces a selective rise in β ENaC subunit at the protein level. Each bar is the mean (± s.e.m.) of 3-4 experiments.

ENaC subunit ratio changes during keratinocyte differentiation

Changes in ENaC subunit expression during differentiation were also documented at the protein level. This was assessed by comparing YF 29 preconfluent cultures (day 8) rich in proliferative cells to very confluent cultures (day 15) rich in differentiated cells. Labeling of YF 29 cells with [^{35}S]methionine was followed by immunoprecipitation of each ENaC subunit with either rat-specific or human-specific antibodies. Each specific immunoprecipitated band was quantified and expressed in terms of the change between proliferative and differentiated keratinocytes (Fig. 8). No consistent change in the expression of either α or γ subunit protein was apparent between days 8 and 15. In contrast, the β subunit was strikingly up-regulated in differentiated keratinocytes as compared to proliferating ones. This demonstrates that the ratio of the sodium channel subunits changes when the human keratinocytes differentiate.

Influence of ENaC inhibition on the formation of domes

We evaluated whether sodium channel inhibitors can modify the ability of keratinocytes to form domes. Addition of increasing doses of amiloride for 24 hours to YF29 cells altered dome formation (Fig. 9A) by reducing the number of domes per well in a dose-dependent manner (half-inhibition was observed with 10 μM amiloride). This value is not equivalent to a K_i (documenting a direct inhibition of membrane ENaC) and likely reflects complex actions of the drug on a series of cellular events occuring during the 24 hours exposure of keratinocytes to amiloride. Because amiloride may interfere with several cell processes, including inhibiting the Na^+/H^+ exchanger, the effect of benzamil, a specific inhibitor of ENaC, was evaluated. A low dose of benzamil (10 nM) was chosen to treat keratinocytes for several days, to avoid as much as possible non-specific (or toxic) effects. When the cells were

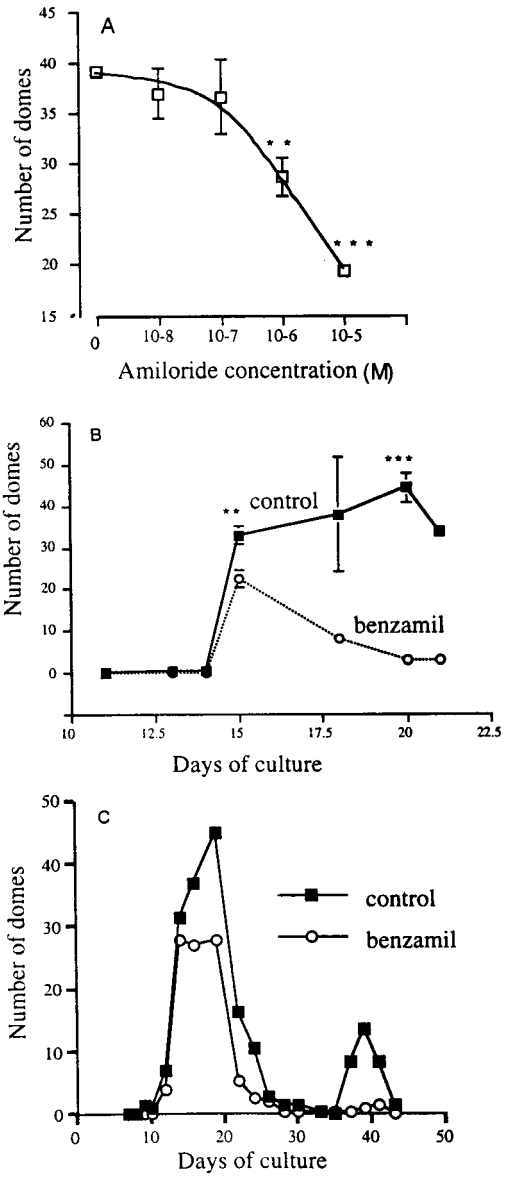

Fig. 9. Effect of sodium channel inhibitors on dome formation. Cultured keratinocytes (YF29) may form domes after they reach confluency (day 11-15). (A) Amiloride (10^{-8}-10^{-5} M), added to the cultures for 24 hours just after confluency, reduced the number of domes. (B) A low dose of benzamil (10^{-8} M), first added to the cultures at day 4 and continued throughout the experiment, also reduced the number of domes. Each point is the mean (± s.e.m.) of 4 experiments; some error bars are within the symbols. $^{**}P<0.01$, $^{***}P<0.001$. (C) Long-term follow-up of dome formation: in control conditions, cultured keratinocytes formed domes first between day 12-13 and day 24; then domes were no longer apparent for 10 days and formed again at day 36-40. When 10 nM benzamil was added to the cultured medium (day 4 and throughout), it affected (moderately) the number of domes during the first period of dome formation and inhibited completely the second period of dome formation (mean value of 2 experiments).

cultured in the presence of 10 nM benzamil, dome formation still occurred, but only transiently (Fig. 9B). In other experiments, the formation of the domes was monitored for 45

days (Fig. 9C): in control conditions, cultured keratinocytes formed domes between day 12-13 and day 24; then domes were no longer apparent for 10 days and formed again at day 36-40. When 10 nM benzamil was added to the cultured medium, it affected (moderately) the number of domes during the first period of dome formation and was able to inhibit completely the second period of dome formation. These results indicate that the chronic inhibition of ENaC activity drastically impairs the ability of keratinocytes to form sustained domes.

DISCUSSION

In electrically tight epithelia which reabsorb sodium, such as the renal collecting duct, the colonic epithelium or the ducts of salivary and sweat glands, ENaC subunits are expressed and amiloride-sensitive sodium transport has been detected (Duc et al., 1994; Garty and Palmer, 1997). These epithelia are polarized, with ENaC expression restricted to the apical membrane of the monolayer, thus favoring absorption of Na^+ from the renal, intestinal or duct fluid. At variance with these tissues, other epithelia (such as the epidermal keratinocytes) express ENaC transcripts (Roudier-Pujol et al., 1996) but their function, as a barrier, seems very distinct: indeed, the skin is at the interface of air and the extracellular medium and its function in vivo is to establish a barrier between these two compartments. Such a barrier is achieved by the watertight stratum corneum, i.e. the outermost layer of the pluristratified epithelium forming the epidermis. The surprising initial observation that the epithelial sodium channel ENaC is expressed in rat skin (Roudier-Pujol et al., 1996) led us to study its expression and putative role in human keratinocytes.

Our results provide evidence for the expression of ENaC at the mRNA and protein levels in human skin and in cultured human keratinocytes. Expression of the different ENaC subunit transcripts varies with the state of differentiation of cultured keratinocytes. Small proliferative basal cells expressed the three ENaC subunit transcripts at low levels. However, their expression is upregulated when the cells enlarge and differentiate. Members of the ENaC family form heteromultimers, with α β γ subunits forming the complete channel. The stoichiometry is considered to be $2\alpha/1\beta/1\gamma$ for some authors (Firsov et al., 1998) while others have suggested an association of $3\alpha/3\beta/3\gamma$ subunits (Snyder et al., 1998). However, it is noticeable that functional channel activity may also be obtained with the α subunit alone (Canessa et al., 1993; Lingueglia et al., 1993), or with a combination of two different subunits. The properties of channels formed of either $\alpha\beta$ or $\alpha\gamma$ ENaC have recently been characterized; they differed by their sensitivity to amiloride, their K_m values for external sodium and their channel conductances (McNicholas and Canessa, 1997). Interestingly, the stoichiometry of expression of ENaC subunit transcripts in vivo is not uniform among epithelial cells. While kidney collecting-duct cells and surface epithelium of the colon (Duc et al., 1994) express all three subunit mRNAs (at least when animals are challenged with aldosterone), epidermal keratinocytes (Roudier-Pujol et al., 1996) and several cell types of the respiratory tract (Farman et al., 1997) express essentially the α and the γ subunit transcripts, with little or no β mRNA. The present study provides evidence that ENaC expression, at the protein level, changes with keratinocyte differentiation; while proliferating undifferentiated keratinocytes express essentially α and γ subunits, differentiation is associated with a dramatic increase in synthesis of the β subunit of ENaC. These results are the first evidence in support of an apparent change in ENaC subunit stoichiometry as a result of differentiation, and they suggest a specific role for the β subunit of ENaC in the epidermis.

The presence of a benzamil-dependent conductance in cultured keratinocytes as demonstrated by patch-clamp experiments indicates that ENaC may have a function. However, it should be emphasized that only a small proportion of the recorded cells showed functional channels. This was likely due to the fact that the experiments could only be performed on cells that had attached to the surface of the culture dish. Under these conditions, it was mostly small proliferative basal cells, which express low levels of ENaC, that were assayed, as opposed to the differentiated cells expressing high levels of ENaC, the latter having lost the capacity to adhere as a consequence of terminal differentiation. Alternatively, it is possible that sodium channels are not permanently inserted in the cell membrane of non-polarized epithelial cells such as the epidermal keratinocytes, in order to prevent a rapid increase of the intracellular sodium level which would lead to cell swelling and death. It is also possible that the ENaC is addressed to the cell membrane at a precise time during differentiation, leading to a transient rise in the intracellular sodium concentration. It has been shown that a rise in cell sodium (by means of ionophores) could activate the C-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, and the stress-activated protein kinase (SEK1). SEK1 can phosphorylate and activate JNK, which in turn phosphorylates C-Jun (Kuroki et al., 1997) leading to an increased transcriptional activity. Thus, alterations in cell sodium concentration could trigger a cascade of transduction signals ultimately interfering with keratinocyte-specific transcription factors.

Keratinocyte increase their size and lose their ability to form colonies in culture as they differentiate (Barrandon and Green, 1985), but the presence of benzamil or amiloride in the cultured medium does not seem to modify cell size and colony-forming ability, suggesting that ENaC is not involved in the control of the steady-state cell volume. Yet we cannot exclude that ENaC is involved in transient changes in cell volume.

The presence of domes in confluent cultures of non-stratified epithelial cells results from a transport of fluid (Lever, 1979). Dome formation in polarized epithelial monolayers with tight junctions, as documented for kidney cells or alveolar type II pneumocytes, depends on unidirectional ion and water transport through the epithelium (Jefferson et al., 1980; Lever, 1979; Sugahara et al., 1984). In these epithelia, domes are the site of an amiloride-sensitive transepithelial sodium transport; consistent with this transport function, the formation of domes is inhibited by amiloride and ouabain (a blocker of the Na^+-K^+-ATPase) (Lever, 1979). In fact the exact mechanisms of dome formation are still poorly understood. Sugahara et al. (1984) concluded that “domes in epithelial monolayers are not predominant special sites of active transport but more likely simple areas of weak attachment to the substratum”. For J. Lever, “induction of domes in MDCK cells may represent a

novel example of induction of differentiation in a cell culture system" (Lever, 1979). Interestingly, dome formation is also frequently observed after the keratinocytes have formed a confluent pluristratified epithelium in culture. The formation of a blister detaches the basal layer from the surface of the culture dish. In contrast to renal or pulmonary cells, we could not demonstrate that a transport of sodium actively contributed to this phenomenon. However, some transepithelial sodium transport may have occurred and evidence of it is compromised by a leak of sodium, due to the very low transepithelial resistance of cultured keratinocytes, from the fluid accumulated in the dome back to the cultured medium. This is consistent with the lack of tight junctions observed in our cells; however the presence (Kitajima, 1983) or absence (Chapman and Eadie, 1985) of such junctions in cultured keratinocytes remains controversial, possibly due to culture conditions. Nevertheless, the presence of benzamil or amiloride drastically reduces the formation or accumulation of domes in cultured keratinocytes, thus indicating that ENaC may be involved in this phenomenon by a mechanism that remains elusive. This effect may be reminiscent of an inhibition by amiloride of a low transepithelial Na^+ transport in keratinocytes, which was undetectable in our experiments. It should be pointed that, in vivo, the skin is a water-tight barrier between air and body compartments; human epidermal keratinocytes, in vivo, are not bathed with an external medium containing sodium, and form a barrier that is essential for terrestrial life (Hardman et al., 1998). This is at variance with amphibians, where the skin is an important site of sodium reabsorption (Crabbe, 1964). In mammals, sodium reabsorption by the skin is restricted to sweat glands (Quinton, 1987). As a matter of fact the mammalian skin forms an efficient barrier against water losses, that is developmentally regulated (Hardman et al., 1998). Initial stages of barrier formation are closely linked to the assembly of the cornifield envelope (Hardman et al., 1998). Amiloride or benzamil inhibition of dome formation in cultured keratinocytes which express ENaC may possibly be related to a non-classical function of this channel. It can be hypothesized that ENaC plays a role in the ability of keratinocytes to lose adherence to the culture dish when they differentiate. This feature is a priori unrelated to the classical function of ENaC and remains to be understood. Molecular dissection of the biochemical and molecular events accompanying dome formation should help to understand its links with differentiation

Interestingly, ENaC genes share some homologies with several *Caenorhabditis elegans* degenerin genes (Corey and Garcia-Anoveros, 1996; Garcia-Anoveros and Corey, 1997; Horisberger, 1998): *mec*-4, and *mec*-10 are involved in sensory touch transduction (Chalfie and Sulston, 1981; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994); unc8 is needed for locomotion (Tavernarakis et al., 1997), and unc105 (Liu et al., 1996) for stretch sensitivity. Another member of this gene family, the FMRFamide-activated channel (FaNaC) of *Helix aspersa*, is important for neurotransmission (Lingueglia et al., 1995). In mammals, several brain proton-gated channels have been characterized (Waldmann et al., 1996, 1997) and are thought to be required for the perception of pain. Altogether, some members of the degenerin/epithelial Na^+ channel superfamily encode several channels that are important for sensing mechanical or sensory stimuli, in addition to the sodium transport properties of the epithelial Na^+ channel.

We propose that ENaC may perform some functions – somewhat related to those of degenerins – in epidermal keratinocytes. Because benzamil and amiloride impair the normal phenotype (domes) of cultured keratinocytes, and considering the dramatic increase in the expression of ENaC β subunit after cells reach confluency, it is suggested that expression of ENaC may be important in these cells when they differentiate.

Examination of the skin of patients with mutations of ENaC, or of mice with targeted disruption of ENaC subunits, should help to evaluate the relevance of our findings in vivo.

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