Central role of α7 nicotinic receptor in differentiation of the stratified squamous epithelium

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Several ganglionic nicotinic acetylcholine receptor (nAChR) types are abundantly expressed in nonneuronal locations, but their functions remain unknown. We found that keratinocyte α7 nAChR controls homeostasis and terminal differentiation of epidermal keratinocytes required for formation of the skin barrier. The effects of functional inactivation of α7 nAChR on keratinocyte cell cycle progression, differentiation, and apoptosis were studied in cell monolayers treated with α-bungarotoxin or antisense oligonucleotides and in the skin of Acra7 homozygous mice lacking α7 nAChR channels. Elimination of the α7 signaling pathway blocked nicotine-induced influx of 45Ca2+ and also inhibited terminal differentiation of these cells at the transcriptional and/or translational level. On the other hand, inhibition of the α7 nAChR pathway favored cell cycle progression. In the epidermis of α7−/− mice, the abnormalities in keratinocyte gene expression were associated with phenotypic changes characteristic of delayed epidermal turnover. The lack of α7 was associated with up-regulated expression of the α3 containing nAChR channels that lack α5 subunit, and both homomeric α9- and heteromeric α9α10-made nAChRs. Thus, this study demonstrates that ACh signaling through α7 nAChR channels controls late stages of keratinocyte development in the epidermis by regulating expression of the cell cycle progression, apoptosis, and terminal differentiation genes and that these effects are mediated, at least in part, by alterations in transmembrane Ca2+ influx.

Introduction

Recent progress in the identification of genes encoding new members of the neuronal nicotinic acetylcholine receptor (nAChR)* subunit gene superfamily and developing in vivo models for specific subunit gene deletions has revealed that the expression of the acetylcholine (ACh)-gated ion channels is not limited to neurons (Grando, 1997; Lindstrom, 1997). On the other hand, recent results with receptor subunit knockout (KO) mice indicated that certain ganglionic nAChR subtypes are not essential for normal neurological function (Orr-Urtreger et al., 1997; Paylor et al., 1998). These unexpected findings suggested that one of the major biological functions of neuronal-type nAChRs is to subserve trophic, hormone-like effects of ACh in both neuronal and nonneuronal locations. Elucidation of the nonneuronal function of nAChRs, therefore, may lead to better understanding of a complex signaling mechanism mediating interactions of the peripheral nervous system with the surrounding tissues, wherein different cell types use ACh as a common messenger, or a pacemaker.

ACh is a ubiquitous chemical in life that, although is best known for its role in neurotransmission, is produced by practically all types of live cells and is remarkably abundant in the epidermis and other types of the surface epithelium (Grando et al., 1993b; Wessler et al., 1999). It has become evident that ACh can regulate tissue homeostasis in an autocrine and paracrine fashions by exhibiting a plethora of biological effects on different cell types (Wessler et al., 1998).

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The level of free tissue ACh is controlled by the cholinergic enzymes choline acetyltransferase and acetylcholinesterase that are present in nonneuronal cells lining the cutaneous, respiratory and alimentary tracts, and blood vessels. In these nonneuronal locations, ACh signaling can be mediated by muscarinic and nicotinic receptors. Binding of ACh to the cell membrane receptors elicits several diverse and simultaneous biochemical events, the "biological sum" of which, together with cumulative effects of other hormonal and environmental stimuli, determines a distinct change in the cell cycle.

The nAChRs are classic representatives of the Cys loop superfamily of ligand-gated ion channel proteins or ionotropic receptors, mediating the influx of Na\(^+\) and Ca\(^{2+}\) and efflux of K\(^+\) (Steinbach, 1990). The differences in subunit composition of nAChRs determine the functional and pharmacological characteristics of the ion channels formed. 12 nAChR subunit genes encoding a pentameric protein have been identified and designated \(\alpha2-\alpha10\) and three \(\beta2-\beta4\), and each subunit has four putative transmembrane-spanning domains (M1–M4) and a similar topological structure. Each of \(\alpha7\), \(\alpha8\), and \(\alpha9\) subunits is capable of forming functional homomeric nAChR channels, which are \(\alpha\)-bungarotoxin (\(\alpha\)-BTX) sensitive. RT-PCR has amplified \(\alpha3\), \(\alpha5\), \(\alpha7\), \(\alpha9\), \(\alpha10\), \(\beta2\), and \(\beta4\) subunits from human keratinocytes (Grando et al., 1995, 1996; Nguyen et al., 2000a, 2001; Sgard et al., 2002), indicating that keratinocytes express both heteromeric and homomeric nAChR channels on their cell membrane. The differences in subunit composition of nAChRs determine the functional and pharmacological characteristics of the ion channels formed.

Current research results indicate that biological effects of ACh in the skin are finely tuned to regulation of each phase of the cell cycle via the intracellular signaling pathways coupled by each particular type of nAChRs (Grando, 1997, 2001). In keratinocytes, nAChRs control cell viability, proliferation, differentiation, adhesion, and motility, and constant stimulation of keratinocyte nAChRs with endogenously secreted ACh produced by these cells is essential for cell survival. We have demonstrated recently that programmed cell death of keratinocytes culminates in apoptotic secretion of a humectant upon secretagogue action of ACh and that activation of ACh signaling through the \(\alpha7\) nAChR, which is predominantly expressed by mature keratinocytes, is essential for a sustained turnover of the epidermis in humans (Nguyen et al., 2001).

This study was designed to ultimately determine the role for \(\alpha7\) nAChR in mediating physiologic control of keratinocyte differentiation by endogenous ACh. Alterations in the nicotinergic regulation of keratinocyte cycle progression, differentiation, and apoptosis were investigated in three independent models of functionally inactivated \(\alpha7\) nAChR: in cultured human keratinocytes treated with \(\alpha\)-BTX or antisense oligonucleotides (AsOs) and epidermal keratinocytes grown from and residing in the skin of KO mice with homozygous-null mutation of the gene-encoding \(\alpha7\) nAChR subunit. We found that pharmacological blockade of \(\alpha7\) nAChR with \(\alpha\)-BTX inhibits nicotine (Nic)-induced influx of \(\text{Ca}^{2+}\) in human keratinocytes, which is associated with an inhibition of Nic-induced terminal differentiation of these cells. Functional inactivation of \(\alpha7\) nAChRs in cultured human keratinocytes with AsOs abolished high extracellular Ca\(^{2+}\)-induced up-regulated synthesis of the terminal differentiation proteins. Terminal differentiation gene expression was found to be down-regulated in the epidermis of \(\alpha7\) KO mice whose keratinocytes demonstrated profound alterations in the normal cell cycle progression and apoptosis when grown in culture. The \(\alpha7^{-/-}\) keratinocytes also demonstrated changes in the gene expression of \(\alpha3\), \(\alpha5\), \(\alpha9\), and \(\alpha10\) nAChR subunits, suggesting that ACh signaling in these cells is rerouted to alternative nicotinergic pathways.

Results

The \(\alpha\)-BTX–sensitive component of nicotinergic control of keratinocyte differentiation

Nicotinergic agents have been demonstrated to affect the rate of keratinocyte differentiation. It has been shown that Nic accelerates the rate of keratinocyte differentiation which can be abolished by mecamylamine (Grando et al., 1996), an antagonist of the “neuronal” types of the nAChRs expressed in epidermal keratinocytes (Grando et al., 1995). Since keratinocytes express both the heteromeric nAChRs containing \(\alpha3\) subunit, which are not sensitive to \(\alpha\)-BTX, and the homomeric \(\alpha7\) nAChR, which is highly sensitive to \(\alpha\)-BTX (Levandoski et al., 1999), we investigated effects of \(\alpha\)-BTX on the Nic-induced keratinocyte differentiation in confluent monolayers of the second passage human foreskin keratinocytes incubated in the medium containing 0.09 mM Ca\(^{2+}\), in which both test drugs were dissolved. As seen in Fig. 1, after 14 d of...
exposure to both Nic (10 μM) and α-BTX (1 μM) the number of keratinocytes that stained for the differentiation markers cytokeratin 10, transglutaminase, involucrin, or filaggrin, or spontaneously formed cornified envelopes was significantly (P < 0.01) diminished compared with that found in the positive control cultures exposed to 10 μM Nic alone and did not significantly differ from the control values (P > 0.05). After shorter incubation periods, i.e., 4, 8, or 10 d, the changes were less pronounced (unpublished data). The cellular staining patterns produced by antibodies to the differentiation markers were similar in experimental and control cultures. Thus, the ability of α-BTX to abolish Nic-induced differentiation of keratinocytes indicated that the differentiation-inducing effect of Nic is predominantly mediated by activation of α7 nAChR.

The α-BTX-sensitive component of the nicotinergic control of transmembrane 45Ca2+ influx in keratinocytes

Previously, we have shown that Nic increases 45Ca2+ influx into keratinocytes freshly dissociated from human epidermis, which could be abolished by mecamylamine (Grando et al., 1996). Using the same cell preparation, we tested the effects of α-BTX on 45Ca2+ influx. The presence of α-BTX in the solution diminished both basal (Fig. 2 A) and Nic (10 μM)-elicited (Fig. 2 B) 45Ca2+ influx. However, these effects did not reach significant levels at either of three (0.1, 1.0, and 10 μM) α-BTX concentrations tested (P > 0.05). Since incubation of keratinocytes in the presence of high extracellular Ca2+ up-regulates expression of the gene coding for α7 subunit (Zia et al., 2000), we hypothesized that preincubation of keratinocytes at high (1.2 mM) Ca2+ before the 45Ca2+ influx assay might increase the sensitivity of Nic-elicited 45Ca2+ influx to a blockage with α-BTX. As expected, in pretreated cells α-BTX inhibited 45Ca2+ influx in a dose-dependent manner (Fig. 2). To directly address the role of α7-made nAChR channels in mediating these α-BTX effects, we measured effects of short (15 min) and long (60 min) term preincubations at 1.2 mM Ca2+ on the relative amount of α-BTX binding to the cell membrane of keratinocytes and total amount of the α7 protein present in these cells, using ELISA with FITC-labeled α-BTX and Western blot with rabbit anti-α7 antibody, respectively. We found that a short term preincubation moderately and a long term preincubation significantly (P < 0.05) increased both cell surface expression of α7 nAChR and total amount of the receptor protein in the cells (Fig. 2, C and D). Together, these results indicated that α7-made nicotinic channels may be a major contributor to transmembrane influx of 45Ca2+ in epidermal keratinocytes at increased extracellular Ca2+ levels.

α7 AsOs alters terminal differentiation of cultured keratinocytes

Having found that pharmacological inactivation of α7 nAChR with α-BTX abolishes Nic-induced differentiation, we asked if elimination of α7-coupled pathway can block terminal differ-
entiation of keratinocytes. To inhibit α7 expression, we used phosphorothioated AsOs targeted to mRNA for the α7 nAChR subunit (Table I). In keratinocytes lacking α7 nAChR, terminal differentiation can be induced via a Nic-independent pathway mediated by other types of Ca²⁺-permeable ion channels due to increased concentration of extracellular Ca²⁺. Nuclear AsOs uptake of the FITC-tagged AsOs by keratinocytes was monitored using a fluorescence microscope (Fig. 3 A). Treatment protocol was optimized to allow maximal inhibition, i.e., >80%, as judged from the results of quantitative receptor protein analysis by Western blotting (Fig. 3 B). The specificity of antibody binding to the immunoblotting membranes was confirmed by (a) appearance of α7 protein band at the expected mol wt (Nguyen et al., 2000a) and (b) absence of this band in negative control experiments omitting primary antibody or replacing it with an irrelevant and species- and isotype-matching antibody (unpublished data).

To determine the effect of inhibited α7 nAChR expression on the unfolding of the keratinocyte differentiation program, preconfluent monolayers of human keratinocytes were fed with serum-free keratinocyte growth medium (KGM) containing a differentiation-inducing concentration of Ca²⁺, 1.2 mM, and incubated in a humid 5% CO₂ incubator at 37°C for 96 h in the presence (experiment) or absence (baseline) of a mixture of five phosphorothioated anti-α7 AsOs or the same concentration of sense oligonucleotide used as a negative control for AsOs (Table I). After incubation, relative amounts of differentiation marker proteins were measured in experimental and control cells and compared. As seen in Fig. 3 C, functional deletion of α7 nAChR

| ODN   | Sequence                  | Function          |
|-------|---------------------------|-------------------|
| α7.1  | 5’-(F)CCGTAAGACCAGCAGCAGACAACTTCAG-3’ | fluorescein ODN α7 antisense |
| α7.2  | 5’-GAGGACCATCAAGGACGGTGAT-3’          | phosphorothioated α7 antisense |
| α7.3  | 5’-GATCCAGAATCAAATCCCT-3’           | phosphorothioated α7 antisense |
| α7.4  | 5’-CAATGACTCCGAACCACCTCA-3’         | phosphorothioated α7 antisense |
| α7.5  | 5’-CATGGACGATGGAGAGAAGAA-3’         | phosphorothioated α7 antisense |
| α7.6  | 5’-ACGCCACATCCTCACTAA-3’            | phosphorothioated α7 antisense |
| α7.7  | 5’-ACGCGGCTGATGTACCTG-3’            | phosphorothioated α7 antisense |

aFluorescein.
bPhosphorothioate.

Figure 3. Anti-α7 AsOs prevents high extracellular Ca²⁺-induced terminal differentiation of human keratinocytes. (A) Intracellular accumulation of FITC-labeled α7 AsOs. FITC-labeled AsOs (Table I), 20 nM, was added to the second passage human keratinocytes. Localized FITC-labeled AsOs was viewed live via phase–contrast fluorescence microscopy after a 24-h incubation (×400). Note that anti-α7 AsOs is distributed into the nucleus and the cytoplasm. Control oligonucleotide was similarly distributed (unpublished data). (B) Effect of anti-α7 AsOs on the α7 nAChR subunit protein in human keratinocytes. The cells were seeded in 24-well plates at a density of 5 x 10⁴/well and incubated in a 5% CO₂ incubator for 72 h in KGM in the presence of Lipofectamine Plus™ alone (control), 20 nM of sense oligonucleotide, or 20 nM of each of five phosphorothioated AsOs (Table I). The anti-α7 AsOs dramatically reduced the intensity of the 60-kD receptor band in the immunoblot. Control (i.e., sense) oligonucleotide did not alter the total amount of α7 protein. (C) Alterations in the expression of differentiation markers in keratinocytes treated with anti-α7 AsOs. Relative amounts of filaggrin, loricrin, and cytokeratins (CK) 1 and 10 were analyzed by Western blotting of the total protein isolated from human keratinocytes transfected with anti-α7 AsOs or the control oligonucleotide described above, or intact keratinocytes after 96 h incubation of these cells in KGM containing 1.2 mM Ca²⁺, to induce terminal differentiation.
the basal layer, consists of several rows of suprabasal keratinocytes, including a superficially located layer of granular keratinocytes, and the uppermost stratum corneum, which is unusually loose and thick. Light microscopy of the hematoxylin and eosin stained 6-7 wk, whose epidermis usually consists of one to two rows of live nucleated keratinocytes and a compact horny layer comprised of dead comeocytes (Fig. 4 B), α7-/- mice featured thickened, multilayered epidermis (Fig. 4 C). In addition to the lowermost basal layer, the epidermis in α7-/- mice contained an additional two to three suprabasal rows of pale and enlarged keratinocytes and from one to three rows of granular keratinocytes located just below widened and loose horny layer. Thus, the phenotypic abnormalities in the epidermis of α7 KO mice were consistent with retention hyperkeratosis, which is a morphologic manifestation of delayed epidermal turnover.

To relate changes in the skin development of α7 KO mice to α7 nAChR-mediated control of keratinocyte cell cycle signaling pathways in vitro with the in vivo phenotype caused by the absence α7 nAChR channels in the epidermis, we studied pups delivered by α7-/- mice, followed by genotyping (Fig. 4 A). Compared with wild-type α7+/+ mice aged from 1 to 3 wk, whose epidermis usually consists of one to two rows of live nucleated keratinocytes and a compact horny layer comprised of dead comeocytes (Fig. 4 B), α7-/- mice featured thickened, multilayered epidermis (Fig. 4 C). In addition to the lowermost basal layer, the epidermis in α7-/- mice contained an additional two to three suprabasal rows of pale and enlarged keratinocytes and from one to three rows of granular keratinocytes located just below widened and loose horny layer. Thus, the phenotypic abnormalities in the epidermis of α7 KO mice were consistent with retention hyperkeratosis, which is a morphologic manifestation of delayed epidermal turnover.

Abnormal keratinocyte differentiation in the skin of α7 KO mice
To correlate changes in the cell cycle and differentiation gene expression resulting from inactivation of α7 nAChR-coupled signaling pathways in vitro with the in vivo phenotype caused by the absence α7 nAChR channels in the epidermis, we studied pups delivered by α7-/- mice, followed by genotyping (Fig. 4 A). Compared with wild-type α7+/+ mice aged from 1 to 3 wk, whose epidermis usually consists of one to two rows of live nucleated keratinocytes and a compact horny layer comprised of dead comeocytes (Fig. 4 B), α7-/- mice featured thickened, multilayered epidermis (Fig. 4 C). In addition to the lowermost basal layer, the epidermis in α7-/- mice contained an additional two to three suprabasal rows of pale and enlarged keratinocytes and from one to three rows of granular keratinocytes located just below widened and loose horny layer. Thus, the phenotypic abnormalities in the epidermis of α7 KO mice were consistent with retention hyperkeratosis, which is a morphologic manifestation of delayed epidermal turnover.

To relate changes in the skin development of α7 KO mice to α7 nAChR-mediated control of keratinocyte cell cycle
and differentiation, we performed quantitative analysis of mRNA and protein levels of the genes encoding keratinocyte differentiation markers in α7 KO compared with wild-type α7+/+ mice. Gene-specific primers for murine filaggrin and cytokeratins 1 and 10 (Table II) amplified products of the expected sizes (Fig. 4 D). The Acrα7 homozygous mutant mice showed decreased mRNA levels of all three terminal differentiation markers, ranging from 36 to 54%. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene amplification remained constant in each experiment (Fig. 4 D).

Findings of down-regulated expression of terminal differentiation genes in keratinocytes residing in the epidermis of α7−/− mice were corroborated by results of the Western blot assay (Fig. 4 E). The α7 deletion was associated with the decrease of the filaggrin and cytokeratin 1 and 10 proteins.

To ultimately determine changes in the differentiation proteins in epidermis of α7 KO mice, we measured the relative intensities of specific staining of keratinocytes produced by antibodies against the keratohyaline proteins filaggrin and loricin and the intermediate filament proteins cytokeratin 1 and 6, using semiquantitative immunofluorescence (IF) assay. We found that in the epidermis of α7−/− mice, the abundance of terminally differentiated keratinocytes expressing filaggrin, loricin, and cytokeratin 1 was significantly (P < 0.05) less than that in the epidermis of α7+/+ mice (Fig. 4 F). In marked contrast, the intensity of epidermal staining for cytokeratin 6, a marker of rapidly proliferating, immature keratinocytes (Foley et al., 1998; Gibbs et al., 2000), was significantly increased (P < 0.05), which is consistent with the appearance of the prolonged epidermal turnover phenotype in α7−/− mice.

### Abnormalities in cell cycle regulation of α7 KO keratinocytes

When cell cycle and apoptosis gene expression in α7−/− keratinocytes was analyzed by RT-PCR, we found that Ki-67, cyclin D1, and PCNA were increased by 52, 77, and 52%, respectively, compared with α7+/+ cells (Fig. 5 A). The mRNA level of p53 also increased by 54%. By immunoblotting, we found that the relative amount of Ki-67, cyclin D1, PCNA, and p53 were increased in α7−/− keratinocytes (Fig. 5 B). On the other hand, the mRNA and protein levels of caspase-3 decreased 24 and 57%, respectively, whereas those of Bcl-2 both increased (Fig. 5).

The results of the semiquantitative IF assay confirmed the above findings (Fig. 5 C). As expected from the results of RT-PCR and immunoblotting assays, we found significant (P < 0.05) increases of the relative amounts of keratinocyte Ki-67, PCNA, cyclin D1, and p53 and a decrease of caspase 3 in keratinocytes residing in the epidermis of α7 KO mice compared with the epidermis of wild-type mice. These results suggested that in the absence of α7 nAChR, the nicotinergic pathway of autocrine and paracrine control of keratinocyte growth and differentiation is predominantly mediated by other type(s) of ACh-gated ion channels that are coupled to maintenance of the immature cell phenotype.

### Altered expressions of nicotinic receptor subunits in α7 KO keratinocytes

To test a hypothesis that mutational deletion of Acrα7 in keratinocytes evokes changes in the relative amounts of different nAChR channels, we investigated expression of the genes coding for α3, α5, α9, and α10 subunits in α7−/− versus α7+/+ keratinocytes. By RT-PCR, we found that the expression of the gene coding for α3 in α7−/− keratinocytes was up-regulated by 56%, whereas that of α5 was apparently unchanged (Fig. 6 A). Results of the Western blotting assay showed an 86% increase of the relative amount of α3 protein in α7−/− keratinocytes (Fig. 6 B). The protein level of α5 was found to be unchanged. These results indicated that although the total number of α3 containing nAChRs increases in the epidermis of α7 KO mice, the proportion of the α3 nAChRs containing α5 subunit is actually less than in wild-type mice. The relative amounts of both mRNA and proteins of α9 and

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#### Table II. Murine genes studied by RT-PCR

| Common name | Abbreviation | Gene name | Accession no.* | Primers |
|-------------|--------------|-----------|----------------|---------|
| Glyceraldehyde-3-phosphate dehydrogenase | Gapdh | Gapdh | M17701 | 214–234, 401–449 |
| nAChRs | | | | |
| Subunit α3 | α3 | Chrna3 | XO3440 | 434–455, 895–918 |
| Subunit α5 | α5 | Chrnα5 | AF204689 | 788–801, 1,238–1,257 |
| Subunit α7 | α7 | Chrnα7 | AF225980 | 555–575, 1,027–1,048 |
| Subunit α9 | α9 | Chrnα9 | AK010496 | 385–406, 821–842 |
| Subunit α10 | α10 | Chrnα10 | NM022639 | 340–358, 720–742 |
| Cell cycle markers | | | | |
| p53-dependent G2 arrest | p53 | Reprimo | AB043586 | 94–118, 463–482 |
| Proliferation-related Ki-67 antigen | Ki-67 | Mki67 | X82786 | 1,091–1,113, 1,570–1,589 |
| Proliferation cell nuclear antigen | PCNA | Pcna | X57800 | 131–150, 437–415 |
| Cyclin D1 | Cyl 1 | Ccnd1 | M64403 | 339–369, 797–820 |
| Cell differentiation markers | | | | |
| Cytokeratin 1 | CK1 | Krt1 | M27734 | 307–330, 744–767 |
| Cytokeratin 10 | CK10 | Krt10 | V00830 | 202–225, 665–642 |
| Filaggrin | Flg | | J03458 | 278–298, 588–609 |
| Cell apoptosis markers | | | | |
| Bcl-2, apoptosis inhibitor | Bcl-2 | Bcl2 | L31532 | 376–399, 730–751 |
| Caspase 3, apoptosis-related cysteine protease | CPP32 | Casp3 | U54801 | 225–246, 657–676 |

*Sequence data available from GenBank/EMBL/DDBJ.*
α10 subunits were elevated in α7−/− keratinocytes (Fig. 6). However, an increase of the protein level of α9 by 63% exceeded that of α10 subunit, indicating that both heteromeric α9α10 and the homomeric α9-made ACh-gated ion channels were up-regulated in α7−/− keratinocytes.

Discussion
This study provides several lines of evidence that the α7 nAChR-mediated signaling is critical for normal epidermal differentiation. We used three independent approaches to inhibit this nicotinergic pathway of ACh signaling in keratinocytes and consistently identified reciprocal changes in the expression of the cell cycle progression and differentiation regulators. The keratinocytes treated with α-BTX or a mixture of phosphorothioated AsOs targeted to mRNA for the α7 nAChR subunit, and the Acra7 homozygous mice lacking α7 nAChR channels, all demonstrated considerable down-regulation of terminal differentiation gene expression at the transcriptional and/or translational levels. On the other hand, inhibition of the α7 nAChR pathway favored expression of the cell cycle progression regulators stimulating cell growth and decreased expression of the proapoptotic caspase 3. The α7 nAChR-related changes in the cell cycle and differentiation caused a transient delay in skin development observed in α7 KO mice during the first 3 wk of their lives. These alterations in the genetically determined unfolding of the keratinocyte differentiation program in α7 KO mice and phenotypic abnormalities consistent with delayed epidermal turnover were associated with changes in the repertoire of keratinocyte nAChR subtypes, suggesting that the nicotinergic pathways dominating in the α7−/− keratinocytes are coupled to maintenance of the immature cell phenotype.

Findings of the new and important biological function of “neuronal” α7 nAChR in a nonneuronal location such as the physiologic control of homeostasis and terminal differentiation of the stratified squamous epithelium was anticipated based on the following reasons. First, despite multiple morphological, biochemical, and electrophysiological studies, the functions of neuronal α-BTX binding sites in the mammalian brain remain largely unknown. Furthermore, a mutation deleting the last three exons of the gene for the α7 nAChR subunit that completely eliminates its potential for participation in an ion channel does not alter normal general appearance, growth, survival, gait, anatomy, and baseline behavioral responses (Orr-Urtreger et al., 1997; Paylor et al., 1998).

Thus, although the Acra7 homozygous mutant mice demonstrated that the α7 subunit is not essential for normal devel-
opment or for apparently normal neurological function, they proved to have phenotypic abnormalities in the epidermis, thus providing a valuable tool for defining the functional role of the keratinocyte α7 nAChR channel in the epidermis.

Second, in addition to modulation of neurotransmitter release the α7 nAChR has been implicated in regulating neuronal growth and differentiation via a large variety of genomic and nongenomic effects, including the promotion of neuronal proliferation (Quik et al., 1994; Plummer et al., 2000), neuroprotection (Gueorguiev et al., 2000; Li et al., 2000; Garrido et al., 2001), and induction of apoptosis (Renshaw et al., 1993; Hory-Lee and Frank, 1995; Berger et al., 1998). Neuronal α7 nAChR acts through different intracellular transduction pathways to protect or kill cells (Li et al., 1999). It has been proposed that α7 nAChR helps regulate neuronal development by modulating intracellular Ca2+ levels and thus affecting neuronal differentiation and synaptogenesis (Broido and Leslie, 1999). The genomic effects downstream of α7 nAChR are represented by activation of tyrosine hydroxylase and dopamine β-hydroxylase gene expression in PC12 cells (Gueorguiev et al., 2000), whereas the nongenomic pathways involve regulation of protein phosphorylation (Schuller et al., 2000; Kihara et al., 2001).

Third, the α7 subunit is abundantly expressed in the epithelial cells lining skin, oral mucosa, esophagus, trachea, and bronchi, in which nicotinergic stimulation alters cellular metabolism of Ca2+ (Grando et al., 1996; Zia et al., 1997; Nguyen et al., 2000a), endothelial cells (Wang et al., 2001), and in cells surrounding large airways and blood vessels, alveolar type II cells, free alveolar macrophages, and pulmonary neuroendocrine cells (Sekhon et al., 1999). In the mammalian fetal lung, α7 nAChR may regulate neuropeptide release, collagen expression, and ultimately lung development (Sekhon et al., 1999). The expression of α7 nAChR channels on the cell membrane of nonneuronal cells is modulated by exposure to Nic (Zia et al., 1997; Arredondo et al., 2001), which may provide a mechanism for Nic-induced changes in gene expression (Arredondo et al., 2001; Zhang et al., 2001a,b), proliferation (Waggoner and Wang, 1994; Stone et al., 2001), apoptosis (LeSage et al., 1999; Heeschen et al., 2001), secretion (LeSage et al., 1999), and tumor growth (Heeschen et al., 2001) in nonneuronal locations.

The contribution of different nAChR subunits to formation of ACh-gated nicotinic ion channels in the plasma membrane of keratinocytes changes with keratinocyte maturation (Zia et al., 2000). Antibody mapping studies in human epidermis have shown that the bulk of α7 immunoreactivity is localized to the cell membranes of mature keratinocytes comprising the granular layer (Nguyen et al., 2001). In keratinocyte cultures, the abundant expression of α7 was observed on the cell membrane of mature cells, which required precultivation of cultures in KGM containing a differentiation-inducing concentration of Ca2+ or Nic (Zia et al., 2000). In contrast, the α3-containing nAChRs are present at the earliest stages of keratinocyte development (Nguyen et al., 2000a; Zia et al., 2000). Extracellular Ca2+ has been shown to regulate responses of both α3- and α7-containing nAChRs on chick ciliary ganglion neurons (Liu and Berg, 1999). At

Figure 6. Alterations in the α3, α5, α9, and α10 nAChR subunit gene expression in α7 KO keratinocytes. (A) The levels of α3, α5, α9, and α10 nAChR subunit gene transcription in α7−/− keratinocytes. The detection of the nAChR subunit transcripts by RT-PCR was performed using gene specific primers for the murine α3, α5, α9, and α10 nAChR subunits (Table II) and cDNA from the second passage, ~75% confluent monolayers of neonatal α7 homozygous null (−/−) and wild-type (+/+) mice as template. Each pair of primers yielded a PCR product of the expected size: 485 bp for α3, 480 bp for α5, 458 bp for α9, and 463 bp for α10. (B) The levels of α3, α5, α9, and α10 nAChR subunit gene translation in α7−/− keratinocytes. The nAChR subunit proteins were visualized by Western blots of total proteins extracted from the same cells as in A. Results of a representative experiment showing protein bands recognized by rabbit polyclonal antibodies specific for α3, α5, α9, or α10 (Table III) resolved on 15% SDS-PAGE and immunoblotted as described in Materials and methods. The apparent mol wt of each receptor protein is shown in kD at the right side of the gel.
though both α3 and α7 subunits can contribute to the nAChRs that are permeable to Ca2+, the ACh-gated ion channels composed of the α7 subunits have the greatest Ca2+ permeability (Seguela et al., 1993). The results of this study demonstrated that the need to preincubate keratinocytes at differentiation-inducing concentrations of extracellular Ca2+ in order to increase the sensitivity of their response to Nic to a blockage with α-BTX in the α5Ca2+ influx assay is explained by up-regulated expression of α7 nAChRs.

Results of this study demonstrate that ACh signaling through α7 nAChR channels controls the maturation and the cornification stages of keratinocyte development in the epidermis. Downstream signaling from α7 nAChR regulates expression of cell cycle progression, apoptosis, and terminal differentiation regulators at the transcriptional and/or translational levels. These effects may be mediated, at least in part, by changes in Ca2+ metabolism. A "gain of function" mutation of the ACh-gated ion channels comprised by α7 subunits demonstrated that neurons expressing only mutant nAChRs are susceptible to abnormal apoptosis and degeneration, possibly due to increased Ca2+ influx (Treinin and Chaffie, 1995; Orr-Urtreger et al., 2000; Broide et al., 2001). We found that neither α-BTX could completely block Nic-induced differential of keratinocytes nor anti-α7 AsOs could completely abolish the process of cornification elicited by increasing the concentration of extracellular Ca2+ in KGM. Instead of using Nic to induce keratinocytes differentiation as in experiments with α-BTX, the differentiation of anti-α7 AsOs-treated keratinocytes was induced through alternative pathway(s) sensitive to high extracellular Ca2+, since in these cells the α7 nAChR pathway was inactivated due to treatment with anti-α7 AsOs. These finding suggest that the α7 nAChR-mediated pathway works together with other cholinergic and noncholinergic signaling pathways to sustain a constant advancement of a keratinocyte through its differentiation stages toward its programmed death. In acute experiments, such as treatment of cells with α-BTX or anti-α7 AsOs, the alternative pathway apparently could not get engaged fast enough to compensate for the missing function, which is illustrated by an approximately fivefold drop in the number of cells capable of spontaneous cornified envelope formation (Fig. 1). In marked contrast, in the epidermis of α7 KO mice the process of cornification, although delayed, proceeds via a normal path, surfacing skin of these mice with an impermeable barrier or the stratum corneum. Therefore, a lesser magnitude of changes of the gene expression in keratinocytes residing in the epidermis of α7 KO mice (Fig. 4 E) compared with keratinocytes treated with anti-α7 AsOs (Fig. 3 C), as judged from the results of the Western blotting assay, may be explained by putative physiologic backup mechanisms activated during the development of a KO mouse but lacking in the cells treated with AsOs in which the α7 AChRs are inactivated acutely at the posttranscriptional level.

To test a hypothesis that mutational deletion of α7 brings about changes in the repertoire of nAChR channels, we determined the ratios of different α subunit gene expression in α7−/− keratinocytes. We found alterations in the expression of α3, α9, and α10 nAChRs subunits, indicating that the nicotinergic signaling in the skin of α7 KO mice is predominantly mediated via a nAChR complex containing α3 without α5 and both homomeric α9- and heteromeric α9α10-made nAChRs. This switch in subunit composition of the nAChR-gated ion channels may, in turn, bring about a corresponding switch in the ionic properties of the ion channels formed because of shifting of the nicotinergic signaling to the nAChRs that differ in subunit composition, pharmacology, conductance, and kinetics and in their permeability to and modulation by Ca2+. For instance, it has been shown that α5 subunit increases Ca2+ permeability of α3 nAChR so that the Ca2+ permeability of α3B2α5 nAChRs is comparable to that of α7 nAChRs (Gerzanich et al., 1998). Hence, a relative decrease of the proportion of α3 nAChRs containing α5 subunits, i.e., α3B2α5, in α7−/− keratinocytes can bring about corresponding changes in the ionic properties of the channel, leading to a complex changes in cell cycle regulation, including proliferation-inducing effects, DNA repair and replication anomalies, and antiapoptotic gene activation. On the other hand, up-regulated expression of α9-containing nAChRs that mediate proapoptotic action of ACh at the granular cell–cornocyte transition, which culminates in programmed cell death within the epidermis (Nguyen et al., 2001), may compensate for a lacking component of the nicotinergic control of terminal differentiation of α7−/− keratinocytes, allowing formation of the functional epidermal barrier in α7 KO mice. The α7 nAChR incorporating α9 subunits represents a novel ionotropic and metabotropic receptor/Ca2+ channel (Elgoyhen et al., 1994; Glowatzki et al., 1995, Wikstrom et al., 1998). Compared with homomeric α9 channels, the α9α10 nAChR channel displays faster and more extensive agonist-mediated desensitization, a distinct current-voltage relationship, and a biphasic response to changes in extracellular Ca2+ ions (Elgoyhen et al., 2001). Thus, although the use of KO mice is probably the most straightforward and rewarding approach to dissect biological function of each particular type of keratinocyte nAChRs, providing an unambiguous mechanistic insight into differential control of keratinocyte functions by ACh, the missing function may be partially compensated or obscured due to engagement of the alternative regulatory pathways.

In conclusion, the comprehensive analysis of the biological role of α7 nAChR in keratinocytes revealed its important role in sustaining normal unfolding of the genetically determined program of cell differentiation eventuating in cell death, or cornification, which is required for formation of the skin barrier. The ACh signaling through α7-made channels may evoke rapid and profound changes in the cellular metabolism of free Ca2+ due to modulation of its transmembrane flux. The downstream signaling apparently harbor both genomic and nongenomic effects, the biologic sum of which determines the rate of keratinocyte progression through the differential steps. In Acrα7 homozygous mutant mice, the missing regulatory pathway causes transient changes in skin phenotype characteristic of delayed epidermal turnover. The changes are partially compensated due to redirection of the nicotinergic signaling via the α3-type keratinocyte nAChRs that in the past were found to be associated with immature cell phenotype (Zia et al., 2000), and the α9-type keratinocyte nAChRs that are coupled to regulation of keratinocyte apoptotic secretion (Nguyen et al., 2001).
Materials and methods

Human keratinocyte culture experiments

Human keratinocyte cultures were started from normal neonatal foreskins as we described in detail earlier (Grando et al., 1993a). The cells were grown in 75-cm² flasks (Corning Glass Works) in KGM containing 0.09 mM Ca²⁺ (GIBCO BRL) at 37°C in a humidified 5% CO₂ incubator. To study nicotinic effects on cell differentiation, keratinocytes were seeded into 6-well tissue culture plates (Falcon 3046; Becton Dickinson) at a density of 10⁵/well, grown to ~75% confluence in 2 ml of KGM containing 0.09 mM Ca²⁺, after which the monolayers received KGM containing 10 µM Nic, 10 µM Nic plus 1 µM α-BTX (both from Sigma-Aldrich), or no additions (controls), and the incubation was continued for additional periods of time (as described in Results) with replacing KGM every other day. After incubation, the monolayers were washed with prewarmed Ca²⁺⁻ and Mg²⁺⁻free PBS (GIBCO BRL) and used either in immunocytochemical assay of differentiation marker expression (see below) or in a modification of the spontaneous cornified envelope formation assay (Rice and Green, 1979) as described in detail elsewhere (Grando et al., 1996).

α7 KO mice and murine keratinocyte cultures

The α7 KO mice used in experiments were Acra7-deficient (α7 null) mice generated as described previously (Orr-Urtreger et al., 1997). All control mice were α7−/− littermates of α7−/− animals. The mice were killed, and skin samples were collected. The samples destined for RNA and protein extraction were fresh-frozen in liquid nitrogen or freshly embedded in OCT Tissue Tek compound (Sakura) for use in IF experiments. All of the experiments were conducted by an experimenter that was blind to the genotype of the mice. The genotyping was performed by PCR and Southern analysis as detailed elsewhere (Orr-Urtreger et al., 1997). Cell cultures were grown at 37°C and 5% CO₂ in 25 cm² Falcon culture flasks using the cell culture techniques optimized for mouse keratinocytes (Li et al., 1995; Lee et al., 1997).

Immunocytochemical assay

Immunocytochemical analysis of nicotinic effects on the expression of differentiation markers was performed in situ in keratinocyte monolayers as described previously (Grando et al., 1996). Stained monolayers were examined microscopically and photographed. The numbers of cytokeratin 10-, transglutaminase-, involucrin-, and filaggrin-positive cells were counted in at least three different microscopic fields at the magnification ×200, and the results were expressed as a percentage of the total cells. At least 50 cell per each microscopic field were examined.

Table III. The primary antibodies used in IF assays

| Antibody specificity | Isotype | Host | Concentration | Epitope | Reactivity |
|---------------------|---------|------|---------------|---------|------------|
| nAChRα3 | IgG | Rabbit | 1 | CPLMAREDA | Human and rodent |
| nAChRα5 | IgG | Rabbit | 1 | CPVHIGNANK | Human and rodent |
| nAChRα7 | IgG | Rabbit | 1 | CFVEAVSKDFA | Human and rodent |
| nAChRα9 | IgG | Rabbit | 1 | CWHDAYLTWDRQYDRLD and CNKADDESEPVNTN | Human and rodent |
| nAChRα10 | IgG | Rabbit | 1 | RSHRAAQRHEDWKR | Human and rodent |
| p53 | IgG1 | Rabbit | 5 | RHSV | Human and rodent |
| Cyclin D1 | IgG2 | Rabbit | 1 | 1–295 | Human and rodent |
| PCNA | IgG2 | Rabbit | 2.5 | 1–261 | Human and rodent |
| Bel-2 | IgG | Rabbit | 0.5 | 20–30 aa | Human and rodent |
| Caspase 3 | IgG | Rabbit | 1 | whole protein | Human and rodent |
| CK 1 | IgG | Rabbit | 0.4 | SSVKFCSTTYSVGTCTC | Human and rodent |
| CK 10 | IgG | Rabbit | 1 | SGTGCGDQSQCGPNY | Human and rodent |
| Filaggrin | IgG | Rabbit | 0.5 | DSOVHSGYVQEGRRGH | Human and rodent |
| β-Actin | IgG1 | Mouse | 0.2 | PPAAVLVIPSGSGL | Human and rodent |
| Ki-67 | IgG1 | Rabbit | 1 | 2,597–2,896 | Human and rodent |

aResearch and Diagnostic Antibodies.

bRaised and characterized as detailed in Nguyen et al. (2000b).

The antiserum was raised against a 15 amino acid residue peptide (NH₂-RSHRAAQRHEDWKR-CONH₂) identical to aa 404–417 of the predicted α10 protein (Elgoyhen et al., 2001). Preimmune serum was isolated. After this, a rabbit received injections of the peptide at 14-d intervals (total of five injections; Pineda Antikörper-Service). Standard procedures were used to further characterize the antibody.

cThe antiserum was raised against a 15 amino acid residue peptide (NH₂-RSHRAAQRHEDWKR-CONH₂) identical to aa 404–417 of the predicted α10 protein (Elgoyhen et al., 2001). Preimmune serum was isolated. After this, a rabbit received injections of the peptide at 14-d intervals (total of five injections; Pineda Antikörper-Service). Standard procedures were used to further characterize the antibody.

dOncogene Research Products.

eBabCo.

fSanta Cruz Biotechnology, Inc.
tial amount of a7 protein was measured by Western blotting (as described below), and the membrane expression of this nACHR was evaluated using FITC-labeled a-BTX (Garcia-Borrón et al., 1990). Briefly, quadruplicate of experimental, i.e., 1.2 mM CaCl2-treated, and control, i.e., 0.09 mM CaCl2-treated, keratinocytes were resuspended in ice-cold PBS containing 10 μM FITC-labeled a-BTX (Molecular Probes, Inc.), incubated for 1 h at 4°C, washed at times with PBS, loaded in standard 96 well ELISA plates (Costar Corporation) at a concentration of 5 x 105/well, and the fluorescence intensity ratio (at 494 nm excitation and 518-nm emission wavelengths) was measured using the Perkin Elmer HTS 7000 instrument.

Western blot assay
Proteins were isolated from the phenol-ethanol supernatant of homogenized human or murine keratinocytes or skin samples of neonatal mice by adding 1.5 ml of isopropanol alcohol per 1 ml of Trizol Reagent (GIBCO BRL) and analyzed essentially as described in our protocol of a quantitative immunoblot assay (Arredondo et al., 2001). The specificities and working concentrations of primary antibodies used are listed in Table III. The membranes were developed using the ECL + Plus chemiluminescent detection system (Amer sham Biosciences). To visualize antibody binding, the membranes were scanned with Storm™/FluorImager (Molecular Dynamics), and band intensities were determined by area integration using ImageQuant software (Molecular Dynamics). To normalize for the protein content, the housekeeping protein actin was visualized in each sample with a mouse antiactin (C-20) antibody; GIBCO BRL) as described elsewhere (Chomczynski and Sacchi, 1987).

RT-PCR assay
Total RNA was extracted from cultured keratinocytes and murine skin using guanidinium thiocyanate phenol chloroform extraction procedure (Trizol Reagent; GIBCO BRL) as described elsewhere (Chomczynski and Sacchi, 1987). 1 μg of dried, DNase-treated RNA was reverse transcribed in 20 μl of RT-PCR mix (50 mM Tris, pH 8.3, 6 mM MgCl2, 40 mM KCl, 25 mM dNTPs, 1 μg Oligo-dT [GIBCO BRL], 1 mM DTT, 1 U RNase inhibitor [Boehringer] and 10 U SuperScript II [GIBCO BRL]) at 42°C for 2 h. The PCR was performed in a final volume of 50 μl containing 1 μl of the single strand cDNA product, 10 mM Tris-HCI (pH 9.0), 5 mM KCl, 5 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, and 2.5 U Taq DNA polymerase (Perkin Elmer) and 20 pmol of each forward (5′) and reverse (3′) primers. To allow a quantitative determination of relative gene expression levels (Arredondo et al., 2001), the cDNA content of the samples was normalized, and the linear range of amplification was determined for each primer set. For each experiment, the housekeeping gene GAPDH was amplified with 20–30 cycles to normalize the cDNA content of the samples. The amplification was performed at 94°C (1 min), 60°C (2 min), and 72°C (3 min) for 24–30 cycles. The specific primers used in this study are shown in Table II. The reported ratios derived from the combination of the three obtained in three independent experiments (n = 3). The images represent typical results from a series of three independent experiments. To standardize the analysis, the gene expression ratio in the control (or a7/+/+ sample) is always equal to 1.

Statistics
The results of the quantitative assays were expressed as mean ± SD. Significance was determined using Student’s t-test.

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