Receptor-Mediated Inhibition of Keratinocyte Migration by Nicotine Involves Modulations of Calcium Influx and Intracellular Concentration

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ABSTRACT

Early stages of wound healing rely on the ability of keratinocytes (KCs) to move over the denuded dermis to re-epithelialize the defect. The agarose gel keratinocyte outgrowth system (AGKOS) is an in vitro model of skin re-epithelialization designed to study the migratory function of KCs. Endogenously secreted acetylcholine controls crawling locomotion of KCs in AGKOS by binding to the cholinergic receptors of both the nicotinic and the muscarinic classes that are expressed by KCs. In this study, we used AGKOS to elucidate the nicotinic pathway of cholinergic control of keratinocyte migration. Activation of the nicotinic acetylcholine receptors decreased the migration distance of KC in a dose-dependent fashion without altering cell viability. Nicotine also increased in a dose-dependent manner transmembrane influx of 45Ca2+, and caused a transient rise in the concentration of [Ca2+]i. Perfect correlation between concentration responses found in the migration and 45Ca2+ influx assays suggested that nicotine-induced inhibition of crawling locomotion relies on modulation of Ca2+ metabolism in KCs. The effects of nicotine could be mediated by the a3- and the a7-containing nicotinic receptors visualized on KCs by immunostaining. Long-term incubation with nicotine up-regulated a7 and down-regulated a3 expression. Thus, nicotine exerts inhibitory effects on keratinocyte migration, and Ca2+ serves as a second messenger in the signaling pathway. These results help explain deleterious effects of nicotine on wound re-epithelialization, and suggest that smoking may delay wound healing via nicotinic receptor-mediated pathway.

Restoration of the epithelial barrier (i.e., re-epithelialization) is a major component of the natural process of wound healing. It is widely accepted that repair of epithelial wounds involves cellular migration (Woodley et al., 1993). Active proliferation of basal keratinocytes (KCs) at the wound edge, derived in part from the outer root sheath of hair follicles, may provide new generations of migratory KCs (Pang et al., 1978). Morphological studies of wounded epidermis have demonstrated that KCs migrate both as a cellular sheet and as individually crawling cells (for review, see Donaldson and Mahan, 1988). In the newly formed epidermal sheet, KCs undergo terminal differentiation to form a water-impermeable barrier, the stratum corneum. Thus, the process of skin re-epithelialization includes the following sequential steps of KC metamorphosis: detachment, migration, proliferation, attachment, and maturation. Failure to heal may stem from an error at any step.

To characterize the physiological control of the metamorphosis of KCs during wound healing, we developed an in vitro model of skin re-epithelialization termed agarose gel keratinocyte outgrowth system (AGKOS) (Grando et al., 1993b). Studies using the AGKOS demonstrated that the process of re-epithelialization is self-sustained and can be regulated by endogenously secreted mediators, such as acetylcholine (ACh) (Grando et al., 1993b). Epidermal KCs possess the enzymes for ACh synthesis and degradation, express nicotinic and muscarinic types of ACh receptors, respond to treatments with cholinergic drugs by dramatic changes in viability, and mediate all major processes of skin re-epithelialization (for review, see Grando, 1997). It was therefore proposed that a variety of cellular activities mediating KC metamorphoses during skin re-epithelialization could be controlled by a single “pace-maker” cytotransmitter: autocrine, juxtacrine, and paracrine ACh (Grando, 1997).

The nicotinic ACh receptors (nAChRs) are classical representatives of a large superfamily of ligand-gated ion channel proteins, or ionotropic receptors mediating the influx of Na+ and Ca2+ and efflux of K+. Undifferentiated KCs express ~5500 nAChRs/cell (Grando et al., 1995), which is very sim-
ilar to the number of nAChRs expressed by cultured bronchial epithelial cells (Maus et al., 1998). During maturation, KCs increase their nAChRs up to 35,400 receptors/cell (Grando et al., 1995). Patch-clamp studies have demonstrated that keratinocyte nAChRs are indeed functional ion channels and can mediate ion transport across the cell membrane (Grando et al., 1995). Polymerase chain reactions have amplified authentic human α3, α5, α7, β2, and β4 subunits from KCs, which indicates that KCs express both the heteromeric and homomeric nAChR channels (Grando et al., 1995, 1996). The nAChR subtypes expressed by KC also are called “neuronal” (ganglionic) nAChRs because they originally were thought to be expressed exclusively by neurons. The heteromeric nAChR channels on KC cell membrane can be composed of the α3, α5, β2, and β4 subunits, e.g., α3β2(β4)±α5, and the homomeric channels can be made from several α7 subunits (for review, see Conti-Tronconi et al., 1994).

In this study, we report that the nAChRs expressed by KCs are involved in cholinergic control of skin re-epithelialization. Nicotine binding to KC nAChRs inhibits KC migration and is accompanied by both transmembrane influx of $^{45}$Ca$^{2+}$ and an increase in the concentration of [Ca$^{2+}$]. These findings indicate that tobacco use can directly inhibit wound re-epithelialization.

Materials and Methods

Chemicals and Tissue Culture Reagents. The nicotinic agonist nicotine and the antagonist of neuronal-type nAChRs mecamylamine were purchased from Sigma Chemical Co. (St. Louis, MO). KC growth medium (KGM) containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract was purchased from Gibco-BRL (Cambridge, MA). Fura-2 and calcium calibration kit were obtained from Molecular Probes (Eugene, OR) and ionomycin from Calbiochem (San Diego, CA). Agarose type HSA was from Accurate Scientific (Westbury, NY). Heat-inactivated newborn calf serum, 0.05% trypsin, trypsin blue dye solution, and Wright’s stain were from Sigma Chemical Co.

Cell Culture. KCs were isolated from human epidermis by treating newborn human foreskin with a trypsin solution overnight. The individual cells were suspended in serum-free KGM, plated in standard tissue culture flasks, and cultured at 37°C in a humidified atmosphere of 5% CO$_2$ according to the procedure detailed in Grando et al. (1993a). The cultures were expanded until they reached 70 to 80% confluency, at which time the cells were harvested and used in the experiments.

AGKOS Assay. Six-well tissue culture-agarose gel plates were prepared as described previously (Grando et al., 1993b), except the agarose gel was always prepared on the day of experiment. First prepared as described previously (Grando et al., 1993b), except the AGKOS were converted into the percentage of control. The control value for KCs from each particular donor was determined by measuring the baseline migration distance (in millimeters), and taken as 100%.

Generation of Anti-nAChR Antibodies. The antisera specific for the α3 and α7 nAChR subunits were generated in rabbits as reported previously (Ndoye et al., 1998). Briefly, a synthetic peptide analog of the carboxyl terminus of α3, CPLMAREDADA (residue numbers 496–503), and α7, CFVEAVSDKFDA (residue numbers 493–502), were conjugated onto bovine thyroglobulin specifically through the Cys residue that was incorporated into the peptide structure, and the conjugate was purified before being used to immunize rabbits. The antisera produced by the rabbits were tested by enzyme-linked immunosorbent assay for their ability to recognize specifically the α3 and α7 receptor subunits. Positive antisera were selected, and then tested in Western blots of KC homogonate (data not shown). Only antisera that recognized the α3 and α7 subunits in enzyme-linked immunosorbent assay and yielded on Western blots the protein bands that could be abolished by preincubating the antisera with the specific peptide used for immunization were used in the experiments reported herein. The α3 antisera produced three bands on the Western blots at apparent molecular masses of 46, 51, and 58 kDa, respectively, which are similar to those reported for the α3 subunit isoforms present in chick ciliary ganglia (Halvorsen and Berg, 1990). The α7 antisera produced a single band at an apparent molecular mass of 63 kDa, which is similar to the previously reported molecular mass for this nAChR subunit (Chen et al., 1998).

Immunofluorescence (IIF) Assays. The indirect IIF experiments were performed as detailed previously (Zia et al., 1997) with freshly frozen normal human skin specimens or cultured KC monolayers as a substrate. Briefly, first passage human foreskin KCs were seeded onto glass coverslips, and incubated for 2 days in KGM to form monolayers. The monolayers were then fixed for 3 min with 3% freshly depolymerized paraformaldehyde that contained 7% sucrose, washed, and incubated overnight at 4°C with the rabbit anti-receptor antibody. The rabbit anti-α3 and -α7 antisera were diluted with PBS that contained 1.0 mg/ml BSA. Binding of primary antibody was visualized by incubating the skin section or a coverslip with cultured KCs at room temperature for 1 h with fluorescein isothiocyanate-labeled donkey anti-rabbit IgG secondary antibody (dilution 1:100) purchased from Pierce (Rockford, IL). The specificity of antibody binding in the IIF experiments was demonstrated by abolishing the staining by omitting the primary antibody and by preincubating the rabbit antisemur with the specific peptide used for immunization. The specimens were examined with an Axiovert 135 fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). To calculate relative amounts of receptor subunits expressed by KCs preincubated with nicotine, the acquired immunofluorescent images were analyzed by a semiquantitative IIF assay (Ndoye et al., 1998). At least three different randomly selected segments of each microscopic fields were analyzed. Each segment included at least three different cells.

Measurement of $^{45}$Ca$^{2+}$ Influx. The assay of transmembrane influx of $^{45}$Ca$^{2+}$ was performed essentially as described in Grando et al. (1996). Briefly, KCs were resuspended in KGM and loaded in Eppendorf tubes at a final concentration of $1 \times 10^6$ cells/50 μl/tube. The cell aliquots then received 300 μl of KGM containing $^{45}$Ca$^{2+}$ (1.2 mM $^{45}$Ca$^{2+}$ at a final specific activity of 11.6 μCi/mmole; NEN, Boston, MA), incubated for 1 min at 37°C in the absence (baseline) or presence of test nicotinic drugs (experiment). After washing three
times by centrifugation at 250g for 1 min in a Beckman microcentrifuge in ice-cold, radioactive calcium-free KGM, the cells were solubilized in 100 μl of Triton X-100 (Sigma Chemical Co.), transferred into scintillation vials containing 5.0 ml of the scintillation cocktail Ecolume (ICN Pharmaceuticals, Costa Mesa, CA), and 45Ca2+ taken up by KCs was measured in an LKB liquid scintillation counter. Nicotinic drug-induced changes in 45Ca2+ influx were expressed as a percentage of basal influx, taken as 100%.

**Measurement of [Ca2+]i.** The concentration of [Ca2+]i in KCs was measured with calcium-sensitive Fura-2 acetoxymethyl ester by the fluorescence ratiometric method detailed in Zia et al. (1997). Briefly, KCs were plated onto two-well glass coverslip chambers (Nunc Nalgene, Naperville, IL) at a density of 1.5 × 105 cells/cm2 and cultured in KGM in a humid CO2 incubator at 37°C. On the day of an experiment, the cells were washed with HEPES/Hanks’ buffer consisting of 20 mM HEPES, 132 mM NaCl, 5.4 mM KCl, 0.44 mM KH2PO4, 0.34 mM Na2HPO4, 0.41 mM MgSO4, 0.49 mM MgCl2, 0.03 mM CaCl2, 5.5 mM glucose, and 0.05% BSA (pH 7.4); loaded for 60 min at room temperature with 5 μM Fura-2 in the HEPES/Hanks’ buffer; washed twice with the same buffer; and allowed to recover for 15 min by incubating in a CO2 incubator at 37°C. The chamber slide was then mounted on the preheated stage at 37°C of an inverted Axiovert 135 microscope equipped with an incubator chamber and an automated filter wheel that was controlled by the computer software IP Lab Ratio Imaging (Signal Analytical, Vienna, VA). The Fura-2 fluorochrome was excited sequentially at wavelengths of 340 and 380 nm and its emission at 510 nm was detected. The [Ca2+]i was continuously monitored by capturing and digitalizing images of 20 cells for each experiment and by subtracting background. At the end of each experiment, 340:380-nm fluorescence ratios were calibrated by measuring minimum and maximum fluorescence (Rmin and Rmax). The Rmax was achieved by adding 5 μM ionomycin to 1.2 mM CaCl2 containing HEPES/Hanks’ buffer, and the Rmin was obtained by adding 4 mM ethyleneglycol-bis(B-aminoethyl ether)-N,N,N’,N’-tetraacetic acid to the culture medium. The concentrations of [Ca2+]i, were calculated from 340:380-nm fluorescence ratios. The Kd value of 216 nM was determined with the calcium calibration kit from Molecular Probes with the protocol provided by the manufacturer.

**Statistics.** The results of the quantitative assays were expressed as mean ± S.D. Significance was determined with Student’s t test. The relationship between nicotine effects on migration and transmembrane 45Ca2+ influx was determined with correlation analysis.

**Results**

**Migration of KCs In Vitro.** The AGKOS has been designed to study the migratory function of KCs in prolonged experiments. It measures the response of a large cell population (>104 cells) to an experimental treatment (Grando et al., 1993b). Approximately 2 h after KCs were loaded into a 3-mm-diameter well in the agarose gel, they formed a round megacolony of tightly packed cells corresponding to the size of the well. The cells at the periphery of the colony flattened and advanced under the agarose gel outward from the colony. The drugs that were tested were always added on the 24th h of incubation, and the crawling locomotion of the KCs was observed through the transparent agarose gel with an inverted phase-contrast microscope. KCs moved as individual cells as well as a cellular sheet that constituted the leading front of the colony. Measurements of the KC outgrowth, the migration distance, were always made from the edge of the 3-mm well that outlined the circumference of the original colony to the front of the outgrowth. The magnitude of outgrowth was partially donor dependent, and the migration distances of untreated KCs over the 10-day period of observation ranged between 2.5 and 5.0 mm.

**Activation of KC nAChRs Inhibits Cell Migration.** To determine nicotine effects on KC migration, KCs were incubated in AGKOS plates for 10 days in the presence of the nicotinic agonist nicotine given alone or in combination with mecamylamine, a specific antagonist of the neuronal-type nAChRs expressed by KCs (Grando et al., 1995). As shown in Fig. 1A, activation of KC nAChRs with 100 μM nicotine significantly (P < .05) reduced the migration distance. The inhibitory effect of nicotine was mediated by activation of KC nAChRs because it could be abolished by mecamylamine (Fig. 1A). When given alone, mecamylamine did not significantly alter KC migration (P > .05). The cytotoxicity was not responsible for the inhibitory effect observed with nicotine. Cell viability assays, based on the trypan blue dye exclusion, revealed an average of 8.3% dead cells after 10 days of incubation of KCs from different cell donors at 100 μM nicotine. This is not statistically different (P > .05) when compared either with the 12.8% dead cells found in the cultures incubated in KGM containing 100 μM mecamylamine alone or with the 10.3% dead cells present in the control, untreated cultures of KCs from the same cell donors.

To determine whether the nicotine effects on KC migration are dose-dependent, we incubated KCs in AGKOS plates for 10 days in the presence of nicotine concentrations ranging from 10−18 to 10−9 M. Measurements of the migration distances at the end of incubation demonstrated that nicotine inhibited crawling locomotion of KCs in a dose-dependent manner (Fig. 1B). The decrease of the migration distance became significant (P < .05) at the nanomolar concentration of nicotine.

**Activation of KC nAChRs Elicits 45Ca2+ Influx.** The nAChR subunits found in KCs (for review, see Grando, 1997) can form ion channels that are permeable to Ca2+ (Attwell and Adams, 1991; Delbono et al., 1997; Quik et al., 1997), and agonist binding to KC nAChRs elicits Ca2+ influx (Grando et al., 1996). We previously also reported that incubation of KCs in the presence of nicotine results in rapid differentiation (i.e., senescence) of these cells (Grando et al., 1996). Because calcium plays a critical role in launching KC differentiation program (Martinez et al., 1999) and because differentiation of KCs slows their migration rate by approximately one-half (Obedencio et al., 1999), inhibition of KC migration by nicotine might be mediated by alterations of calcium-dependent regulation of the KC cell cycle. To test this hypothesis, we determined the relationship between the concentration response of KCs to nicotine determined in the migration assay and the concentration response measured in the 45Ca2+ influx assay. As expected, exposure of suspended KCs to increasing concentrations of nicotine elicited a dose-dependent increase of the amount of 45Ca2+ taken up by the cells (Fig. 1C). Because measurable changes in 45Ca2+ influx started at the nanomolar dose of nicotine, the concentration values of 10−9, 10−8, 10−7, 10−6, and 10−5 M were used to determine whether correlation exists. The correlation analysis of the concentration-response curves shown on Fig. 1, B and C, revealed a perfect inverse relationship between nicotine-induced decrease of migration distance and an increase of 45Ca2+ influx in KCs. The calculated Pearson’s correlation...
The migration distance was measured in millimeters as described in KGM (baseline migration distance) or in KGM containing nicotinic drugs. Human KCs were loaded into AGKOS plates, and incubated for 10 days in KGM containing increasing concentrations of nicotine with or without a 10-fold concentration of mecamylamine, and 45Ca2+ influx through KC nAChR channels. The ratiometric analysis of cells loaded with Fura-2 quantitates the total concentration of free cytoplasmic Ca2+, which can originate from the mobilization of intracellular stores and from the influx through Ca2+ channels in the plasma membrane (Grynkiewicz et al., 1985).

Exposure of the undifferentiated KCs that were cultured in KGM containing low, 0.09 mM Ca2+ to nanomolar doses of nicotine did not produce significant changes in [Ca2+]i, levels during the 90 to 120 min of observation that limits the standard ratio-imaging experiment. Higher concentration of nicotine was required to elicit an increase in the [Ca2+]i, in cultures of intact KCs. This agreed with previous findings that significant changes in [Ca2+]i, in response to activation of α7 nAChRs occur at a relatively high (10−4 M) concentration of nicotine (Delbono et al., 1997). Because in the past we found that a long-term (~10 days) preincubation of KCs with a low dose of nicotine increases sensitivity of the cells to nicotine-induced 45Ca2+ influx through KC nAChR channels (Grando et al., 1996), we hypothesized that preincubation of KCs with nicotine would yield cells that could respond to pharmacological doses of nicotine with measurable changes in [Ca2+]i. As expected, after 10 days of preincubation with 10 nM nicotine, the KCs responded to 1 mM nicotine with characteristic changes in the [Ca2+]i, (Fig. 2A). The [Ca2+]i concentration in these cells rose gradually, over a 4-min period, to 1.9 times the basal level, i.e., from 45 to 85 nM. This was followed by a gradual decrease to the level that was

**Materials and Methods.** The migration distance was partially donor-dependent, and ranged from ~2.5 to 5.0 mm/10 days in control, untreated cultures. The migration distances of experimental, nicotinic drug-treated cultures are expressed as percentage of the baseline migration of KCs from the same cell donor (control), which are assigned 100%. Duplicate or triplicate experiments were performed with KCs from each cell donor, and the combined results from at least three different cell donors were averaged. To measure nicotinic effects on transmembrane calcium influx, KCs were freshly isolated from human neonatal foreskins and resuspended in KGM containing increasing concentrations of nicotine with or without a 10-fold concentration of mecamylamine, and 45Ca2+ influx through KC nAChR channels. The permeability to Ca2+ of a neuronal-type nAChR channel comprised of α7 subunits is sufficient to elicit detectable increase in [Ca2+]i, (Delbono et al., 1997). However, the oscillations of [Ca2+]i are thought to mediate physiological regulation of vital cellular functions of KCs, including migration (Yuspa et al., 1988). Therefore, to elucidate the biochemical mechanisms of nicotine effects on KC migration, we examined the effects of nicotinic drugs on [Ca2+]i in cultured KCs with the calcium-sensitive dye Fura-2. The ratiometric analysis of cells loaded with Fura-2 quantitates the total concentration of free cytoplasmic Ca2+, which can originate from the mobilization of intracellular stores and from the influx through Ca2+ channels in the plasma membrane (Grynkiewicz et al., 1985).

**Activation of KC nAChRs Is Associated with an Increase of [Ca2+]i.** The permeability to Ca2+ of a neuronal-type nAChR channel comprised of α7 subunits is sufficient to elicit detectable increase in [Ca2+]i, (Delbono et al., 1997). However, the oscillations of [Ca2+]i are thought to mediate physiological regulation of vital cellular functions of KCs, including migration (Yuspa et al., 1988). Therefore, to elucidate the biochemical mechanisms of nicotine effects on KC migration, we examined the effects of nicotinic drugs on [Ca2+]i in cultured KCs with the calcium-sensitive dye Fura-2. The ratiometric analysis of cells loaded with Fura-2 quantitates the total concentration of free cytoplasmic Ca2+, which can originate from the mobilization of intracellular stores and from the influx through Ca2+ channels in the plasma membrane (Grynkiewicz et al., 1985).

**Fig. 1.** Correlation of nicotinic effects on KC migration and 45Ca2+ influx. To measure nicotinic effects on migration distance of KCs, first passage human KCs were loaded into AGKOS plates, and incubated for 10 days in KGM (baseline migration distance) or in KGM containing nicotinic drugs. The migration distance was measured in millimeters as described in

- **A**
  - Graph showing the migration distance as a percentage of control.
  - Conditions: Nic, 100 μM; Nic, 100 μM + Mec, 100 μM.

- **B**
  - Graph showing the migration distance as a percentage of control.
  - X-axis: Concentration of Nic (M).

- **C**
  - Graph showing the 45Ca2+ influx as a percentage of control.
  - X-axis: Concentration of Nic (M).
In marked contrast, the small basal cells attached to the epidermal basal membrane localized mainly to the cell membrane of undifferentiated, cating that the \( \text{Mec} \) on the \( \text{Ca}^{2+} \) nAChRs subunits (Grando et al., 1995, 1996). Because the other subunits form a homomeric channel, composed of several identical subunit proteins, and because the other subunits are assembled into a single heteromeric channel, such as \( \alpha_{3}\beta_{2}\beta_{4}\delta_{5}, \alpha_{3}\beta_{2}\delta_{5}, \alpha_{3}\beta_{4}\delta_{5}, \alpha_{3}\beta_{2}\beta_{4}, \alpha_{3}\beta_{2}, \) and \( \alpha_{3}\beta_{4} \), each of which contains an \( \alpha_{3} \) subunit, all of the various nAChRs expressed by KCs can be visualized with the use of just two antibodies, an anti-\( \alpha_{3} \) antibody and an anti-\( \alpha_{7} \) antibody. In vivo, the ACh-gated nicotinic channels composed of these receptor subunits showed a diametrically opposed distribution (Fig. 3, a and b). The \( \alpha_{3} \) immunoreactivity was localized mainly to the cell membrane of undifferentiated, small basal cells attached to the epidermal basal membrane. In marked contrast, the \( \alpha_{7} \) immunoreactivity was localized predominantly to terminally differentiated, large KCs comprising the stratum granulosum, the uppermost layer of viable epidermal cells.

In vitro, the IIF staining revealed that the \( \alpha_{3} \)-positive KCs are small cells that are evenly distributed throughout the colony (Fig. 3c). The immunostaining for \( \alpha_{3} \) was seen both in KC cultures incubated in KGM containing low and high concentrations of \( \text{Ca}^{2+} \), 0.09 and 1.8 mM, respectively, indicating that the \( \alpha_{3} \)-containing nAChRs are present at the earliest stages of KC maturation. At confluence, the \( \alpha_{3} \) subunits clustered in areas of the cell membrane associated with cell-to-cell contacts (data not shown). As seen in Fig. 3c, the IIF staining with the \( \alpha_{3} \)-specific antibody excluded the large, immotile KCs located between the small, actively moving \( \alpha_{3} \)-positive cells.

The \( \alpha_{7} \)-immunoreactive cells were large, immotile, terminally differentiated cells located predominantly in the more central parts of the colony (Fig. 3d). The \( \alpha_{7} \)-specific antibody did not stain the small, nondifferentiated KCs interspersed between the \( \alpha_{7} \)-positive large KCs. In contrast to the results obtained with the \( \alpha_{3} \)-specific antibody, preincubation of cultures in KGM containing a differentiation-inducing concentration of free \( \text{Ca}^{2+} \) (1.8 mM \( \text{Ca}^{2+} \)) resulted in an increase in the number of \( \alpha_{7} \)-immunoreactive KCs, which suggests that KC maturation and differentiation are associated with an increase in the number of homomeric \( \alpha_{7} \) nAChRs per cell.

In both tissue and cell culture specimens, the intensity of IIF staining observed with the \( \alpha_{7} \)-specific antibody was stronger than that observed with the \( \alpha_{3} \)-specific antibody (Fig. 3, a–d). Examination of the specimens at higher magnifications showed that the immunopositive clusters revealed by the anti-\( \alpha_{7} \) antibody on KC cell surfaces were larger than those revealed by the anti-\( \alpha_{3} \) antibody.

Taking into consideration the ability of nicotine to modulate the expression of its own receptors/channels (Peng et al., 1994; Zia et al., 1997), we asked how did a 10-day preincubation of KCs with nicotine, which both decreased the migration distance of KCs and rendered KCs with higher sensitivity to nicotine-induced increase of \( \text{Ca}^{2+} \), influence the repertoire of nAChR subunits expressed by these cells. The analysis of the relative amounts of \( \alpha_{3} \) and \( \alpha_{7} \) in exposed KCs revealed reciprocal changes in the relative amounts of these subunits (Fig. 3, e and f). On the cell surfaces of nicotine-
Fig. 3. Localization of KC nAChRs and semiquantitative analysis of long-term effects of nicotine on nAChR subunit composition. Rabbit polyclonal antibodies that were raised to unique protein sequences of the two main nAChR subunits, α3 (a and c) and α7 (b and d), were used to immunolocalize the receptors. Cryostat sections of freshly frozen specimens of normal human skins (a and b) and colonies of first passage human foreskin KCs grown for 2 days on coverslips (c and d) were fixed and immunostained with the receptor specific antibodies as detailed in Materials and Methods. a, immunostaining with the anti-α3 antibody revealed the characteristic membrane staining pattern of the immature basal cells at the bottom of epidermis. b, abundant immunostaining produced by the anti-α7 antibody in the granular layer of epidermis, which is comprised of terminally differentiated large KCs. c, anti-α3 antibody selectively stains cell surfaces of small KCs in cultures and does not immunostain the large, mature cells. d, immunostaining of cultured KCs with the anti-α7 antibody selectively highlights the large, mature KCs present in the more central part of this colony. Dilutions of primary antibodies in PBS: 1:50 (c), 1:250 (d), 1:300 (a), and 1:5000 (b). Scale bar, 50 μm. The IIF staining was eliminated when the primary rabbit anti-receptor antibody was omitted and when the rabbit antiserum was preincubated with the peptide used for immunization. No immunostaining was observed when KCs were treated with preimmune sera obtained from the same rabbits (data not shown). Relative amounts of α3 (e) and α7 (f) expressed by second passage normal human foreskin KCs after 10 days of incubation in KGM containing 10 nM or 100 μM nicotine were determined with a semiquantitative IIF assay described in Materials and Methods and compared with the baseline expression of these nAChR subunits in intact KCs (control), taken as 100%. Data are mean ± S.D. of relative amounts of subunit proteins. The α7 values were computed in five
treated KCs, the relative amount of α3 significantly ($P < .05$) decreased whereas the relative amount of α7 significantly ($P < .05$) increased compared with the baseline, taken as 100%. Both low (10 nM) and high (100 μM) doses of nicotine produced similar changes, except that up-regulation of the α7 expression was more pronounced at the nanomolar compared with the micromolar concentration of nicotine. These findings suggested that the effects of nicotine on both KC locomotion and calcium metabolism were mediated predominantly by KC α7 nAChRs.

**Discussion**

This study characterizes for the first time the nAChR-mediated pathway of the physiological autocrine, paracrine, and juxtacrine control of wound re-epithelialization by endogenously secreted ACh. Through this pathway, ACh may inhibit KC migratory function with $\text{Ca}^{2+}$ as a second messenger. The results provide a biochemical mechanism to explain the deleterious effects of nicotine on wound healing.

To characterize the cholinergic effects on crawling locomotion of KCs, we used an “under agarose” cell migration system, or AGKOS (Grando et al., 1993b). Immunostaining of AGKOS plates at the end of experiments revealed that KCs expressing the proliferation marker Ki-67 are localized between the leading front and more internal parts of the colony (Grando et al., 1993b). KCs comprising the leading front do not express the Ki-67 antigen, suggesting that migrating KCs do not divide. Using AGKOS, we previously demonstrated that ACh and its congeners exhibit a plethora of effects on KCs, including regulation of crawling locomotion, and that deprivation of endogenous ACh kills KCs (for review, see Grando, 1997; Grando and Horton, 1997). Hence, ACh may act as a cytokine for movement.

In this study, we found that long-term exposure of KCs to nicotine decreases their migration distance. The inhibitory effect of nicotine was receptor mediated rather than toxic. This is illustrated by the following observations. First, the extent of migration inhibition correlated directly with the pharmacological dose of nicotine added to AGKOS plates. Second, mecamylamine, a specific antagonist of the neuronal-type nAChRs that are expressed by KCs, ablated the inhibitory effect of nicotine. And third, cytotoxicity was eliminated as a potential explanation by finding in the cultures treated with nicotine the number of dead cells that was similar to that found in untreated, control cell cultures originated from the same donors of KCs.

The response of KCs to chronic nicotine exposure appears to be directly opposite to that observed on short-term exposure. Short-term exposure to nicotine augments cytoplasmic motility and lateral migration of KCs and also aggravates other cell functions, such as proliferation and adhesion (Grando et al., 1995). Acute exposure of suspended KCs to nicotine results in attachment and spreading of the cells on the dish surface and development of intercellular contacts within 20 to 30 min, whereas untreated cells accomplish this only after 90 to 120 min (Grando et al., 1993b, 1995). The exposed cells flatten and extend cytoplasmic aprons toward neighboring cells, which is very similar to nAChR-mediated induction of actin-driven lamellar protrusions (Zheng et al., 1994). This response to short-term treatment with nicotine may partially explain why nicotine-treated rats with dry-ice blisters show more rapid wound healing compared with untreated controls (Westerman et al., 1993). Long-term exposure of KCs to nicotine produces opposite effects. In addition to inhibition of migration, found in this study, it has been demonstrated previously that chronic exposure to nicotine markedly increases the number of KCs forming cornified envelopes and staining with antibodies to the differentiation markers cytokeratins 10/11, filaggrin, involucrin, and transglutaminase type I (Theilig et al., 1994; Grando et al., 1996). In keeping with these findings is a recent report showing that differentiation decreases speed of moving KCs (Obedencio et al., 1999).

The inhibition of KC migration with nicotine in AGKOS assays was within the range of nicotine concentrations found in the blood of cigarette smokers and snuff users (Russell et al., 1981), indicating that the pathobiological effect of nicotine on re-epithelialization may play a role in delayed wound healing in tobacco users. Chronic exposure to nicotine-containing products, such as direct and second-hand tobacco smoke and chewing of smokeless tobacco, is associated with impaired wound healing (for review, see Smith and Fenske, 1996). Inhibition of wound re-epithelialization, a distinctly deleterious effect, has traditionally been ascribed to ischemia due to nicotine-induced vasoconstriction. However, KCs grow perfectly well in a serum-free environment (Gilchrest et al., 1982; Boyle and Ham, 1983), suggesting that other mechanisms may play significant roles. Among these, the direct effect of nicotine on KC migration described in this study. A combination of two effects of chronic nicotine exposure on KCs, namely, inhibited locomotion and increased terminal differentiation (Grando et al., 1996), helps explain impaired healing of cutaneous (Smith and Fenske, 1996) and intraoral (Jones and Tripelett, 1992) wounds in tobacco users. Furthermore, the mechanism underlying the negative effects of nicotine on KC motility may be similar to that proposed to explain the reduced ciliary beating of bronchial epithelial cells in smokers (Agius et al., 1995). Likewise, the expression of nAChRs on the surface of blood vessel endothelium (Macklin et al., 1998) and, most importantly, by vascular smooth muscles (S. A. Grando and A. Ndoye, unpublished observations), suggests that smoking-associated vasoconstriction is a direct effect of nicotine, rather than an indirect effect that is mediated by a release of catecholamine from the adrenal gland.

The drastic differences in effects observed between acute and chronic administration of nicotine to KCs may be related to the nicotine-induced alterations of KC nAChRs that are due to desensitization of overstimulated receptors and/or that are due to genomic effects of nicotine on the repertoire of cutaneous cholinergic enzymes and receptors regulating ACh metabolism and mediating ACh signaling through both the muscarinic and the nicotinic pathways. Both mechanisms have been described in the literature (Peng et al., 1994;
In this study, we found that nicotine elicits a switch in KC nACHRs subunit composition wherein the α7 subunit-containing nACHRs replace the α3 subunit-containing nACHRs on the cell membrane of KCs. Taking into consideration that rapid desensitization is a property of homomeric α7 subunit-containing nACHRs (Fenster et al., 1997), we expected to find a decreased amount of α7 in KCs preincubated with nicotine. However, we observed instead a significant (P < .05) increase in the relative amount α7 immunoreactivity in KCs incubated with either low or high dose of nicotine for 10 days, suggesting that during long-term exposures of KCs to nicotine a compensatory over-expression of α7 nACHRs occurs. Indeed, nicotine has been shown to stimulate the expression of α7 by the human neuroblastoma cell line SH-SY5Y (Peng et al., 1997). We have reported previously that smoking significantly (P < .05) increases the relative numbers of nACHRs in bronchial epithelial cells, and that this increase can be reproduced in cell cultures exposed to nicotine, including up-regulation of α7 (Zia et al., 1997). The results of this study also show that the pattern of nicotine-induced changes of the subunit composition of KC nACHRs matches that observed during normal differentiation of KCs in the epidermis, suggesting that nicotine accelerates the genetically determined program of KC differentiation leading to premature senescence, or terminal differentiation, of these cells.

We found that the contribution of different nACHR subunits to formation of ACh-gated nicotinic ion channels in the plasma membrane of KCs changes with KC maturation. Both the heteromeric nACHRs, containing α3 subunits, and the homomeric nACHRs, comprised of α7 subunits, were found by immunostaining on the cell membrane of cultured KCs. Incubation of KCs at high extracellular concentration of Ca²⁺ launches a genetically determined cellular differentiation in KCs (Sharpe et al., 1993). In this study, it increased the immunostaining for the α7 subunit, which indicates that the expression of α7-containing nACHRs is differentiation dependent. Accordingly, both in vivo and in vitro the anti-α7 antibody stained more mature, immotile KCs. In contrast, the immunostaining for the α3 subunit did not require preincubation of KCs at high Ca²⁺, which indicates that the α3-containing nACHRs are present at the earliest stages of KC development. The anti-α3 antibody stained mainly small, motile, nondifferentiated KCs located in vitro at the leading front of the colony, and comprising in vivo the epidermal basal layer. Therefore, it can be postulated that the α3-containing nACHRs play a major role in mediating the effects of ACh and its nicotinic congeners at the earliest stages of KC-mediated re-epithelialization when α7-containing nACHRs are absent from or underexpressed on the cell membrane of crawling KCs. After the migrating KCs had stopped, attached to one another, and formed islands of epithelization, the α7-containing nACHRs may exert control over the nicotinic-mediated pathway of cholinergic regulation of re-epithelialization. Therefore, a switch in subunit composition of the nACHR-gated ion channels, which in turn, brings about a corresponding switch in the ionic properties of the ion channels on the KC cell membrane, is a more likely explanation of the profound differences observed in the effects between short- and long-term exposure to nicotine on KCs, rather than a mere desensitization of the α3-containing nACHRs.

The deleterious effect of nicotine on crawling locomotion of KCs correlated perfectly with stimulatory effect of nicotine on Ca²⁺ influx, suggesting that nicotine-induced alterations in KC calcium metabolism played a role. Furthermore, both a decreased migration distance and an increased sensitivity to nicotine-dependent rise in [Ca²⁺], were associated with up-regulation of the α7 subunit-containing nACHRs. Although 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). We found that nicotine induces elevation in cytosolic free Ca²⁺ similar to that described for bronchial epithelial cells, which have been shown to exhibit an increase in [Ca²⁺], on exposure to nicotine (Zia et al., 1997). The nicotine-induced increase in [Ca²⁺], levels in KCs was terminated by washing the cells to remove the nicotine. Mecamylamine blocked nicotine-induced [Ca²⁺] increase in nACHRs-mediated pathway. An increased of [Ca²⁺], could result from activation of the α7-containing nACHRs (Delbono et al., 1997; Quik et al., 1997). Interestingly, both in the studies with human embryonic kidney cells 293 with a stable expression of α7 nACHRs (Delbono et al., 1997) and in our studies with human neonatal KCs expressing native α7 nACHRs, the influx of Ca²⁺ could be observed starting at the nicotine dose that was much lower than that that required to elicit measurable changes in [Ca²⁺]. This phenomenon can be explained by the fact that an increase in the [Ca²⁺], measured in the Fura-2 assay is a summative effect of Ca²⁺ influx, mediated primarily via α7 nACHRs, and a set of intracellular events, such as Ca²⁺ extrusion, sequestration, and buffering, which may act to protect the cell from an unopposed raise in [Ca²⁺]. The need to preincubate KCs with nanomolar concentrations of nicotine to increase sensitivity of these cells to nicotine in the ratiometric assay of [Ca²⁺], may be explained by the above-discussed ability of nicotine to foster KC differentiation that is associated with up-regulated expression of its own receptors, particularly the α7-containing nACHRs.

In conclusion, activation of the nicotinic pathway in human KCs elicits a transmembrane influx of Ca²⁺, whereas activation of the muscarinic pathway inhibits Ca²⁺ influx and enhances Ca²⁺ efflux (Grando and Horton, 1997). Perhaps, the stimulatory effect of ACh on Ca²⁺ influx into the cells, mediated by its nicotinic class of cholinergic receptors, is balanced by an inhibitory effect, mediated by its muscarinic class of the cholinergic receptors, and simultaneous activation of both receptor classes may be required to produce a kind of a yin-yang autoregulatory balance. By selectively activating nACHRs, nicotine may imbalance this physiological equilibrium, and an imbalance between the nicotinic and muscarinic pathways of ACh regulation of Ca²⁺ metabolism may alter normal cellular performance. Therefore, changes in the [Ca²⁺], level may account for the inhibitory effect of nicotine on KC migration.

References
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