The Ras/Raf-1/MEK1/ERK Signaling Pathway Coupled to Integrin Expression Mediates Cholinergic Regulation of Keratinocyte Directional Migration*

Alexander I. Chernyavsky‡, Juan Arredondo§, Evert Karlsson¶, Ignaz Wessler‡, and Sergei A. Grando

From the 6Department of Dermatology, University of California, School of Medicine, Davis, California 95616, the 6Section of Experimental Geriatrics, NEUROTEC, Karolinska Institute, 141 86 Huddinge, Sweden, and the 6Institute of Pathology, University Hospital, Johannes Gutenberg University of Mainz, D-55101 Mainz, Germany

The physiologic mechanisms that determine directionality of lateral migration are a subject of intense research. Galvanotropism in a direct current (DC) electric field represents a natural model of cell re-orientation toward the direction of future migration. Keratinocyte migration is regulated through both the nicotinic and muscarinic classes of acetylcholine (ACh) receptors. We sought to identify the signaling pathway mediating the cholinergic regulation of chemotaxis and galvanotropism. The pharmacologic and molecular modifiers of the Ras/Raf-1/ERK signaling pathway altered both chemotaxis toward choline and galvanotropism toward the cathode in a similar way, indicating that the same signaling steps were involved. The galvanotropism was abrogated due to inhibition of ACh production by hemicholinium-3 and restored by exogenously added carbachol. The concentration gradients of ACh and choline toward the cathode in a DC field were established by high-performance liquid chromatographic measurements. This suggested that keratinocyte galvanotaxis is, in effect, chemotaxis toward the concentration gradient of ACh, which it creates in a DC field due to its highly positive charge. A time-course immunofluorescence study of the membrane redistribution of ACh receptors in keratinocytes exposed to a DC field revealed rapid relocation to and clustering at the leading edge of α7 nicotinic and M1 muscarinic receptors. Their inactivation with selective antagonists or small interfering RNAs inhibited galvanotropism, which could be prevented by transfecting the cells with constitutively active MEK1. The end-point effect of the cooperative signaling downstream from α7 and M1 through the MEK1/ERK was an up-regulated expression of α2 and α5 integrins, as judged from the results of real-time PCR and quantitative immunoblotting. Thus, α7 works together with M1 to orient a keratinocyte toward direction of its future migration. Both α7 and M1 apparently engage the Ras/Raf/MEK/ERK pathway to up-regulate expression of the “sedentary” integrins required for stabilization of the lamellipodium at the keratinocyte leading edge.

Human epidermal keratinocytes (KCs)2 can synthesize and secrete acetylcholine (ACh) and use it as a local hormone for an autocrine and paracrine control of their vital functions, including motility (1). ACh and its congeners are chemoattractant for KCs, neurons, and other cell types (2–8). To characterize the physiologic control of keratinocyte migration, we developed an in vitro model of skin epithelialization, termed the agarose gel keratinocyte outgrowth system (AGKOS) (9). Using AGKOS, we have demonstrated that activation of the keratinocyte nicotinic ACh receptors (nAChRs) comprised by α7 subunits exhibit reciprocal effects on cell motility by stimulating directional (chemotaxis) and inhibiting random (chemokinesis) migration and that ACh-gated ion channels containing α3 subunit stimulate chemokinesis of KCs (8). More recently, α7 nAChR has been shown to mediate chemotaxis of vascular smooth muscle cells toward nicotine (10). The muscarinic ACh receptor (mAChR) subtypes M3 and M4 also exhibit reciprocal regulation of the keratinocyte migratory function by inhibiting or stimulating it via the signaling pathways coupled to preferential expression of either sedentary (α2 and α5) or migratory (β2 and β3) integrins, respectively (11). We next sought to gain a mechanistic insight into the cholinergic regulation of the directionality of keratinocyte migration.

In wound healing, the direction of migration is determined when a keratinocyte extends a flattened cytoplasmic protrusion from the free basolateral side into the wound. This cytoplasmic protrusion, or leading lamella (pseudopodium), is free from organelles; it has the optically dense leading edge, or lamellipodium, and long, straight, stiff cylindrical rods, or filopodia, extending outward. Lamellipodium of a crawling cell attaches tightly to the substratum. Consistent with the idea that ion channels are involved at the leading lamella in directional migration of KCs are the results of experiments involving cathodal cell migration (galvanotaxis) and directed lamellipodium extension (galvanotropism) in direct current (DC) electric field (12). The exposed KCs orient the axis of direction of their migration parallel to the field lines and migrate toward the cathode. Because a membrane potential gradient asymmetrically alters transmembrane ion fluxes across the cell, the accumulation of ion channels leading to membrane depolarization at the leading lamellae have been proposed to explain provocative activity and thereby direct cell locomotion (13). An example of such field-induced redistribution of ion channels is accumulation of nAChRs at the cathode-facing cell pole, which continues to aggregate after the field has been terminated (14–17). Turning of neuronal cells toward ACh gradients required the presence of extracellular Ca2+ and involved calcium/calmodulin-dependent protein kinase II (18, 19). Accumulation of nAChRs on the cathodal side of a cell allows a focal ion flux and local receptor; DC, direct current; MEX, mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase; TX, α-bun- garotoxin; HC-3, hemicholinium-3; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CA-MEK, constitutively active-MEK; DN-MEK, dominant-negative-MEK; siRNA, small interference RNA; siRNA-NC, negative control siRNA; KGM, keratinocyte growth medium; MT7, M1-toxin 1.
osmotic swelling required for leading lamella and lamellipodium formation (18). This phenomenon mimics known accumulation of muscle nACHRs at sites of nerve input where ACh is released (reviewed in Ref. 20). Due to its highly positive charge (21), ACh secreted by KCs should migrate toward the cathode, thus creating its own concentration gradient in a DC field. Hence, migration toward the cathode may represent migration toward the concentration gradient of ACh.

Redistribution of α7 immunoreactivity to the leading edge of KCs upon exposure to a cholinergic chemotaxtract precedes crescent shape formation and directional migration (8), suggesting that accumulation of α7 nACHRs is required for lamellipodium formation. The signaling downstream from α7 nACHR can proceed via several pathways. The Ca2+ ions that enter KCs through α7-made ACh-gated channels can raise the concentration of intracellular free Ca2+ (22, 23). The pathway mediating α7-dependent keratinocyte chemotaxis includes intracellular free Ca2+, activation of calcium/calmodulin-dependent protein kinase II, conventional isoforms of protein kinase C, phosphatidylinositol 3-kinase, and recruited Rac/Cdc42 (8). In various types of epithelial cells, α7 nACHR has been shown to utilize the Ras/Raf/MEK/ERK signaling pathway (24–26). This pathway has been implicated in the physiologic regulation of cell migration and chemotaxis (27, 28). Furthermore, ERK signaling pathways are engaged in healing of lens epithelial monolayer wounds and in the DC field-directed migration of the wound edge (29). The putative mechanism of signaling along this pathway involves up-regulated expression of the sedentary integrin α5 (30). We therefore hypothesized that α7-mediated signaling can contribute to galvanotropism of KCs in a DC field, and that the Ras/Raf/MEK/ERK pathway subserves the function of α7 and, possibly, some other subtypes of keratinocyte ACh receptors regulating directional migration of KCs.

In this study, we measured the effects of pharmacologic and molecular modifiers of the specific steps in the Ras/Raf-1/MEK1/ERK signaling pathway in KCs crawling toward the concentration gradient of the α7 agonist choline, and in KCs turning toward the cathode in a DC electric field. We found that α7 works together with M1 to orient a keratinocyte toward direction of its future migration, and that both α7 and M1 receptors can employ the Ras/Raf-1/MEK1/ERK pathway to up-regulate expression of the α2 and α3 integrins required for stabilization of the lamellipodium.

MATERIALS AND METHODS

*Cholinergic Regulation of Directional Migration—*In accordance to published protocol of the chemotaxis assay (8), the KCs seeded in the chemotaxis AGKOS plate were exposed to the concentration gradient of the α7 agonist choline for 10 days with daily changes of KGM and refreshing the chemotaxtract solution. The cells were fed with a choline-free KGM custom prepared by Cascade Biologics (Portland, OR). After migration was terminated, a blueprint of the outgrowth was obtained and used to compute the directional migration distance. Some KCs were first transfected with siRNA and/or MEK1 mutants and then used in the chemotaxis assay.
Cholinergic Regulation of Directional Migration

Galvanotropism Assay—KC cells were seeded onto culture treated Thermorax® coverslips (Nalge Nunc International, Rochester, NY) at a cell density of $2.5 \times 10^4$, incubated overnight, mounted on the galvanotaxis chamber in a drop of KGM containing 0.2 mM Ca$^{2+}$ (49), and exposed for 1 h at 37 °C to a DC electric field with a constant voltage of 100 mV/mm and a current of $\sim 0.5$ mA, which is equivalent to the physiological strength of the DC field detectable near the edge of skin wound (50). Compliant with the standard design (51), the galvanotaxis chamber was assembled on a glass slide using as spacers the number 1 glass coverslips (thickness 130–170 μm) onto which the Thermorax® coverslip with KCs was waxed, forming a 10-mm wide, ∼150-μm high, and 22-mm long trough bordered by 2% agar bridge electrodes at each end of the trough. After exposure, the KCs were fixed, and the number of cells oriented toward the cathode versus anode was counted at the magnification 10× in at least two randomly selected fields with $\sim 50–70$ individual KCs from three different donors in each, and compared with corresponding control cells. Fig. 1 shows the high power view of human KCs prior to and after exposures that allows observation of cell morphology. The experiments were performed with KCs from three different donors, and the results are expressed as percentage of appropriate control.

To visualize the ACh receptors and $\alpha_2$ and $\alpha_5$ integrins on the cell surface of KCs exposed to the DC field, the cells were fixed for 3 min in 3% freshly depolymerized paraformaldehyde that contained 7% sucrose, thus avoiding cell permeabilization, washed, and incubated overnight at 4 °C with a primary antibody, and then for 1 h at room temperature with secondary, fluorescein isothiocyanate-conjugated antibody (Sigma-Al
drich, Inc.). The fluorescence was examined with an Axiovert 135 fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). The specificity of antibody binding was demonstrated by omitting the primary antibody or by replacing it with an irrelevant antibody of the same isotype and species as the primary antibody, as detailed elsewhere (45).

To determine the effect of the DC field on distribution of ACh molecules in a solution, a constant voltage of 100 mV/mm and a current of $\sim 0.5$ mA were applied to a 1 ml solution of ACh in Tris-buffered saline, phosphate-buffered saline, or distilled water, and aliquots of the solution were pipetted out 3 min after beginning of the exposure at the anodal and the cathodal borders and subjected to ACh measurement. Measurement of ACh and Choline—ACh and choline were measured by cationic exchange high-pressure liquid chromatography combined with bioreactors and electrochemical detection, as described in detail elsewhere (52). The BAS 481 microbore system was used (Bioanalytical Systems Inc., West Lafayette, IN). ACh and choline were separated on an analytical SepStik column (1 $\times$ 530 mm; BAS, Axel Semrau GmbH, Sprockhövel, Germany) using a mobile phase of 45 mM phosphate buffer and 0.3 mM EDTA (adjusted to pH 8.5). The analytical column was followed by an immobilized enzyme reactor (SepStik IMER.2/pkg; BAS) containing acetylcholinesterase to hydrolyze ACh and choline oxidase to produce H$_2$O$_2$. H$_2$O$_2$ flowing across a platinum electrode (reference electrode Ag/AgCl, set at 0.5 V) is oxidized, producing a current that is proportional to the amount of ACh in the sample. The 20-μl samples were injected by an automatic injector (Bio-Rad AS100). The amounts of ACh and choline were calculated by comparison with external standard containing 1 pmol/20 μl of both ACh and choline.

ERK1/2 Activity Assay—To measure the effects of a DC field on the activities of ERK1 and ERK2 in KCs, we employed the phospho-ERK1/2 TiterZyme® enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI), and followed the protocol provided by the manufacturer. Briefly, $\sim 1 \times 10^7$ KCs were seeded on a standard glass coverslip, incubated overnight to allow cell-substrate adherence, washed, and exposed for 30 min to the DC field described above. The cells were lysed with the lysis buffer, and the lysate was transferred to a microtiter plate with immobilized ERK monoclonal antibody. After a short incubation, the excess sample was washed out and a rabbit polyclonal antibody to phosphoERK was added. The amount of bound anti-phosphoERK antibody to phosphoERK was measured at 450 nm, and the results are expressed as optical density (A) values.

Real-time PCR Assay—The assay was performed as we previously described (48). Briefly, total RNA was extracted from cultured KCs at the end of experiments using the RNeasy® Mini Kit (Qiagen, Valencia, CA) following the protocol provided by the manufacturer. Primers for the genes encoding human integrins $\alpha_2$, $\alpha_5$, $\alpha_6$, and $\alpha_7$ were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA), and the service Assays-on-Design provided by Applied Biosystems. Obtained gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase to correct even minor variations in mRNA extraction and reverse transcription.
Western Blotting Assay—As detailed elsewhere (48), proteins were isolated by adding 1.5 ml of isopropanol alcohol per 1 ml of TRIzol reagent (Invitrogen) to the phenol-ethanol supernatant of homogenates of KCs, washed, dissolved in a sample buffer, separated via 4–15% SDS-PAGE, and electroblotted onto a 0.2-μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were developed using the ECL + Plus chemiluminescence detection system (Amershams Biosciences) and scanned with Storm 860/FluorImager (Molecular Dynamics, Mountain View, CA). The relative density of scanned bands was determined by area integration using ImageQuant software (Molecular Dynamics), and the results are expressed as integrated intensity of pixels of the spot excluding the background. The final results were expressed as ratios of the densitometry value of each integrin to that of glyceraldehyde-3-phosphate dehydrogenase in the same lane, compared with the values obtained in control samples. The protein content ratio in each control sample was always set equal to 1.

Statistics—The results of quantitative experiments were expressed as means ± standard deviation (S.D.). Significance was determined using Student’s t test.

RESULTS

Signaling through the Ras/Raf-1/MEK1/ERK Pathway Mediates the α7-dependent Chemotaxis of KCs—The chemotaxis of KCs toward concentration gradient of the α7 agonist choline was significantly (p < 0.05) inhibited by the Ras inhibitor manumycin A, 3 μM, and the cRaf-1 inhibitor GW5074, 0.1 μM (Fig. 2A). Neither inhibitor could decrease the directional migration distance of KCs transfected with CA-MEK (p > 0.05). An evidence of the involvement of MEK1/ERK in the signaling pathway mediating the chemotactic response of KCs to choline was obtained in experiments with 1 μM MEK Inhibitor I that significantly (p < 0.05) decreased directional migration. Similar results were obtained using 10 μM U0126 (Fig. 2A). To ultimately establish the role for the MEK1/ERK-mediated signaling, we transfected KCs with DNM-EIK. The dominant negative, but not control, mutant significantly (p < 0.05) inhibited directional migration of KCs (Fig. 2A).

To functionally inactivate the α7 nAChR, some cells were transfected with siRNA-α7 prior to exposures. The uptake of siRNA by KCs was determined in immunofluorescence experiments using fluorescein isothiocyanate-conjugated siRNA (data not shown). The efficacy of α7 gene knock-down was measured in Western blot assays wherein the relative amount of α7 nAChR subunit protein was estimated using α7-specific antibody characterized in the past (44). Transfection with anti-α7 siRNA decreased the relative amount of subunit protein by ~90% (Fig. 2B). Transfection with siRNA-NC did not alter receptor protein level (data not shown).

Functional inactivation of signaling from α7 nAChR by transfecting the cells with siRNA-α7, but not siRNA-NC, or treating KCs with 1 μM αBtx in both cases abolished their chemotactic response to choline (p < 0.05). This inhibitory effect could not be prevented by overexpressing CA-MEK (Fig. 2A). The pan-muscarinic antagonist atropine, 50 μM, did not have any effect on the chemotaxis of KCs toward choline. These results indicated that α7 nAChR uniquely mediated directional migration of KCs toward the concentration gradient of choline and that the signaling proceeded through the Ras/Raf-1/MEK1/ERK steps.

Role for ACh in Galvanotropism of KCs in a DC Electric Field—Galvanotropism in a DC field represents a natural model for studying the intrinsic mechanisms mediating re-orientation of KCs prior to the onset of crawling locomotion toward a chemotactant. A concentration gradient of ACh in a DC field (current strength: 0.5 mA; duration of stimulation: 30 s) was found by comparing its concentration at the anodal (0.67 ± 0.06 mM) and the cathodal (0.79 ± 0.16 mM; n = 4; p > 0.05) sides of the chamber filled with a Tris-buffered saline containing nominally 1 mM ACh. Likewise a small but statistically significant concentration gradient of choline in a DC field was found: 0.31 ± 0.01 mM at the anode versus 0.39 ± 0.03 at the cathode (n = 4; p < 0.05). Respective differences were reproduced in experiments in which ACh and choline were dissolved in either phosphate-buffered saline or distilled water (data not shown).

Keratinocyte re-orientation toward the cathode could be abolished, due to inhibition of ACh production by 20 μM HC-3, and restored by an exogenously added agonist carbachol, 1 mM (Fig. 2C). This observation suggested a supposition that in a DC electric field, the KCs migrating toward the cathode actually move toward the concentration gradient of ACh, because carbachol, just like ACh, migrates toward the cathode, due to its highly positive charge, and creates its own concentration gradient within the DC electric field.

A Time-course Immunofluorescence Study of the Membrane Redistribution of ACh Receptors in KCs Exposed to a DC Electric Field—The membrane topology of the nAChR subunits α3, α5, α7, and α9 and the mAChR subtypes M1–M5 was observed using receptor-specific antibodies at 0, 15, 30, and 60 min after application of the DC field. The most rapid and consistent changes were seen with α7 nAChR and M1 mAChR (Fig. 3A). These two receptors relocated to and clustered at the cell pole-facing cathode within 15–30 min of field application. In contrast to other subtypes of mAChRs, e.g. M5 shown in Fig. 3B, that dispersed diffusely over the entire plasma membrane, the nAChR subunits α3 (Fig. 3B) and α5 (not shown) relocated toward the side of the cell facing the cathode without forming clusters at the leading edge, the lamellipodium. The number of KCs displaying the α7 and M1 clusters at the lamellipodium in the direction of the cathode 30 min after application of a DC field exceeded control levels by ~4-fold (Fig. 3C). Depriving KCs of endogenously produced ACh by HC-3 blocked completely the cathodal relocation of M1 and partially abolished that of α7 (Fig. 3, A and C). Addition of 1 mM carbachol to the HC-3-pretreated KCs restored the pattern of receptor relocation (Fig. 3C).

These results suggested that in addition to the electrophoretic forces (13), the concentration gradient of ACh causes accumulation of α7 at the keratinocyte leading edge in a DC field, and that relocation of M1 receptors is solely the function of ACh gradient.

Engagement of the Ras/Raf-1/MEK1/ERK Pathway in the Downstream Signaling from ACh Receptors during Keratinocyte Galvanotropism in a DC Electric Field—Manipulations with the nicotnergic signaling using pharmacologic and molecular modifiers of the Ras/Raf-1/MEK1/ERK pathway altered galvanotropism of KCs similarly to their chemotaxis toward choline, indicating that the same signaling steps were involved (Fig. 2C). In contrast to chemotaxis, however, transfection of KCs with CA-MEK abolished only partially, i.e. by ~50%, the inhibitory effects of siRNA-α7 and αBtx on keratinocyte galvanotropism. When these cells were also treated with the M1 inhibitor MT7, 30 nM, their ability to turn to the cathode was completely blocked (Fig. 2C). Given alone, MT7 decreased the number of KCs responding to the DC field by ~70%, and this inhibitory effect could be ameliorated if the cells were transfected with CA-MEK (Fig. 2C). Silencing the M1 gene expression with siRNA-M1, but not siRNA-NC, also blocked the galvanotropism by ~70%, and co-transfection with CA-MEK partially abolished this inhibitory effect. The ~90% efficacy of M1 silencing with siRNA-M1 was demonstrated by Western blotting of cellular proteins (Fig. 2B). The galvanotropism was completely blocked, however, if the CA-MEK-transfected KCs that were treated with MT7, or co-transfected with siRNA-M1, were also exposed to αBtx (Fig. 2C). Similar
Cholinergic Regulation of Directional Migration

![Graph]

FIGURE 2. The Ras/Raf-1/MEK1/ERK pathway of nicotinic control of keratinocyte directional migration. A chemotaxis. The second passage human KCs were loaded into the chemotaxis AGKOS plates, incubated for 18 h to allow cells to adhere to the dish bottom after which 1 mM choline was added to the chemoattractant well, and the incubation was continued for 10 days with daily refreshment of the chemoattractant solution. The test agents were diluted in KGM and added directly to a well in AGKOS plate that contained KCs. Some cells were transfected with receptor-specific or control siRNA and/or MEK1 mutants, as detailed under "Materials and Methods." The following experimental treatments were used: 3 μM manumycin A (Mnmc); 3 μM manumycin A on KCs transfected with CA-MEK (CA-MEK + Mnmc); 0.1 μM GW5074; 0.1 μM GW5074 on the CA-MEK-transfected KCs (CA-MEK + GW5074); 1 μM MEK inhibitor I (MEK-inh); 10 μM U0126; transfection with DN-MEK; transfection with the control MEK1 mutant K97R (K97R); 1 μM Btx; 1 μM Btx on the CA-MEK-transfected KCs (CA-MEK + Btx); 1 μM atropine on the CA-MEK-transfected KCs (CA-MEK + Btx + Atr); transfection with siRNA-a7; co-transfection with siRNA-a7 and CA-MEK (CA-MEK + siRNA-a7); 10 μM atropine on KCs co-transfected with siRNA-a7 and CA-MEK (CA-MEK + siRNA-a7 + Atr); 10 μM atropine (Atr) and scrambled (normal control) siRNA (siRNA-NC). Triplicate experiments were performed with KCs from each of the three cell donors used in this study (n = 3). The results are expressed as means ± S.D.% of appropriate control. An asterisk denotes statistical significance, p < 0.05, compared with control. B, efficacy of siRNAs-a7 and siRNA-M, Representative results of Western blot analysis of the effects of siRNA-a7 and siRNA-M on α7 nAChR subunit and M1 subtype expression, respectively, in human KCs. The experimental cells were transfected as detailed under "Materials and Methods," and incubated for 72 h prior to harvesting the proteins and Western blot analysis of the relative amounts of α7 and M1 (experiment). The numbers underneath the bands are ratios of the densitometry value of each receptor protein to that of β-actin, compared with the values obtained in control, untreated KCs (taken as 1). An asterisk denotes statistical significance, p < 0.05, compared with the baseline determined in the intact KCs. C, galvanotropism. The cells were exposed for 1 h to a DC field in the galvanotaxis chamber as described under "Materials and Methods." In addition to the experimental conditions described in the panel A, the cells were treated with 20 μM HC-3; 20 μM HC-3 plus 1 μM carbachol (HC-3 + CCh); 30 nM MT7; 30 nM MT7 on KCs transfected with CA-MEK (CA-MEK + MT7); 30 nM MT7 on KCs co-transfected with siRNA-a7 and CA-MEK (CA-MEK + siRNA-a7 + MT7); 30 nM MT7 plus 1 μM Btx on KCs transfected with CA-MEK (CA-MEK + aBtx + MT7); 1 μM Btx on the KCs co-transfected with siRNA-M1 and CA-MEK (CA-MEK + siRNA-M1 + aBtx); or transfected with siRNA-M1 (siRNA-M1) or co-transfected with siRNA-M1 and CA-MEK (CA-MEK + siRNA-M1). Triplicate experiments were performed with KCs from each of the three cell donors used in this study (n = 3). The results are expressed as means ± S.D.% of appropriate control. An asterisk denotes statistical significance, p < 0.05, compared with control. D, ACh receptors mediate an increase of keratinocyte ERK1/2 activity by a DC field. The KCs grown on coverslips to a cell density of 10^5 were exposed for 30 min to the DC after which the monolayers were lysed, and the ERK1/2 activity was determined as detailed under "Materials and Methods." The experimental conditions used are shown in the graph. Manumycin was used at the concentration of 3 μM, GW5074 at 0.1 μM, PMA at 50 nM, and chemerythrine at 1 μM. These data are means ± S.D. of optical density measured at 450 nm in experiments using KCs from different donors (n = 3). All values are significantly, p < 0.05, different from the baseline value measured in the intact KCs and are shown as a horizontal dotted line. Bracket arrows denote statistical significance, p < 0.05, between specific conditions.

Results were obtained in experiments with the keratinocyte cultures in which endogenously produced and secreted ACh was substituted by exogenously added carbachol (data not shown).

In a series of confirmatory experiments, we tested a hypothesis that the DC field leads to activation of the Ras-Raf-ERK pathway via a pathway coupled by the α7, and M1 ACh receptors (Fig. 2D). Application of the DC field up-regulated the ERK1/2 activity in KCs by >2-fold (p < 0.05). This effect could be ameliorated to a various degree if the cells were pretreated with 3 μM manumycin or 0.1 μM GW5074, or transfected with siRNA-a7 or siRNA-M1, but not with siRNA-NC (Fig. 2D). Simultaneous silencing of the α7 and M1 genes produced a stronger inhibiting effect compared with silencing of each receptor gene alone. Because the Raf-ERK cascade in KCs exposed to a DC field could be hypothetically activated via the PKC-mediated pathway, we also studied the PMA-dependent activation of ERK1/2 (Fig. 2D). Neither ACh receptor gene silencing nor Ras or Raf inhibition produced any signifi-
cant changes in the PMA-induced ERK1/2 activity. The specificity of ERK1/2 activation to the engagement of PKC in this series of experiments was demonstrated by a pronounced inhibitory activity of the PKC inhibitor chelerythrine, 1 μM (Fig. 2D).

Taken together, these results indicated that the M1 mAChR cooperated with a7 in cell re-orientation toward a mixed nicotinic-and-muscarinic chemoattractant, such as ACh or carbachol, and that downstream signaling along both the nicotinic and muscarinic pathways proceeded through the common MEK1/ERK steps.

**Up-regulation of the Sedentary Integrin Gene Expression Is an Endpoint Effect of the Cholinergic Signaling through the Ras/Raf-1/MEK1/ERK Pathway**—The quantitative analysis of the effects of MEK1 kinase mutants on the ACh receptor-dependent expression of integrin genes at the mRNA and the protein levels brought consistent results. The KCs grown in 6-well plates to ~60% confluence were transfected with siRNA-a7 or siRNA-M1 (versus siRNA-NC) alone or in a combination with MEK1 kinase mutants, treated with 20 μM HC-3 to abolish ACh synthesis, and then stimulated with 1 mM carbachol for 24 h at 37 °C and 5% CO2. The mRNA and proteins were extracted and used in real-time PCR and Western blotting assays, respectively.

By real-time PCR, functional inactivation of signaling with siRNA decreased relative amounts of mRNA transcripts coding for α2 and α3 integrins by ~5- and 3-fold, respectively, and that of M1 by 4- and 6-fold, respectively, without altering the expression of the migratory integrins α5 and αv (Fig. 4A). In marked contrast, co-transfection of receptor-specific siRNAs with CA-MEK significantly up-regulated the

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**FIGURE 3. Membrane topology of ACh receptors in a DC field.** The second passage KCs seeded onto coverslips were exposed to a DC field for various periods of time (see “Results”), fixed to avoid cell membrane permeabilization, and stained with antibodies to the ACh receptors, as described under “Materials and Methods.” The vertical arrow indicates position of the cathode. A redistribution of the a7 and M1 receptors. Immunostaining of KCs with the a7- or M1-specific rabbit antibodies before (baseline) and 15 and 60 min after application of the DC field strength of 100 mV/mm. Note that the haphazard pattern of receptor distribution on the cell membrane of intact KCs changed after application of a DC field. Both receptors accumulated at the leading edge (lamellipodium), decorating the filopodia (anterior cytoplasmic spikes).
expression of $\alpha_2$ and $\alpha_3$ integrins approximately by 10 times (Fig. 4A). Similar up-regulation of $\alpha_2$ and $\alpha_3$ integrins was detected in KCs transfected with CA-MEK alone, but not with DN-MEK, which significantly ($p < 0.05$) decreased the integrin gene expression (Fig. 4A).

The protein levels of $\alpha_2$ and $\alpha_3$ integrins were significantly ($p < 0.05$) decreased due to $\alpha_7$, and M$_1$ gene silencing, and increased in KCs over-expressing CA-MEK (Fig. 4B). Transfection with siRNA-NC did not significantly alter the mRNA and protein levels of integrin expression in KCs ($p > 0.05$) (data not shown). The immunostaining of KCs moving toward the cathode in a DC field revealed accumulations of both $\alpha_2$ and $\alpha_3$ integrins at the leading edge, decorating anterior cytoplasmic protrusions, or filopodia (Fig. 3D), suggesting that up-regulated expression of the $\alpha_2$ and $\alpha_3$ integrins in this cell compartment is required for stabilization of the lamellipodium. Thus, the obtained results indicated that activation of either $\alpha_7$ or M$_1$ leads to up-regulation of the sedentary integrins $\alpha_2$ and $\alpha_3$ in KCs and that the signaling pathways downstream from both ACh receptor types include activation of the common effector step, MEK1/ERK, which alters integrin gene expression.

**DISCUSSION**

In this study we identified new steps in the signaling cascade that determine directionality of lateral migration of human KCs. We demonstrated that KCs can “sense” the chemoattractants such as ACh or carbachol via the cholinergic cell-surface receptors that activate signaling pathway altering integrin expression. KCs responded to the nicotinic chemoattractant choline through the $\alpha_7$ nAChR subtype that couples the Ras/Raf-1/MEK1/ERK signaling pathway. When KCs were exposed to the mixed nicotinic-and-muscarinic chemoattractant carbachol, $\alpha_7$ worked together with the M$_1$ receptor of the muscarinic family, which also used the MEK1/ERK step to execute its effect on the directionality of crawling locomotion. These results suggested that a physiological cooperation (synergism) between the $\alpha_7$-type of ACh-gated ion channels and the M$_1$-type of G protein-coupled transmembrane glycoproteins is required for keratinocyte re-orientation toward the concentration gradient of ACh.

In the present work, we investigated involvement of the entire signaling pathway Ras/Raf-1/MEK1/ERK, culminating with ERK1/2 activation, in the cholinergic regulation of directional migration of KCs. However, the Raf/MEK/ERK cascade can be regulated independently from Ras in a PKC-dependent manner (53). Results of experiments with PMA stimulation of KCs convincingly demonstrated that the ERK1/2 activity sensitive to inhibition by manumycin and GW5074 did not result from activation of an alternative, PKC-mediated pathway. We did not attempt to discern individual contributions of Ras and/or cRaf-1 kinase in this study.

Redistribution of M$_1$ immunoreactivity to the leading edge of KCs preceded crescent shape formation required for directional migration, indicating that among the M$_1$–M$_5$ mAChR subtypes expressed in human KCs (45, 54), the M$_1$ was primarily involved. Both coupling of M$_1$ to the Ras/Raf/MAP kinase pathway and coupling of this pathway to up-regulation of integrin $\alpha_2$ have been demonstrated in other cell types (30, 55). In a previous study we demonstrated that activation of keratinocyte M$_2$ mAChR leads to an up-regulated expression of the sedentary integrin receptors $\alpha_3$ and $\alpha_6$ and arrested migration (11). It is well...
known that the odd-numbered mAChRs, M1, M3, and M5, all can couple the same intracellular signaling pathways (56). Hence, our findings indicate that M1, and possibly M3, share their control over the Ras/Raf-1/MEK1/ERK pathway with the α7-type of nAChRs. Because both M1 and α7 were implicated in the inhibition of lateral migration of KCs (8, 11), the common steps in the inhibitory mechanism responsible may be up-regulation of the expression of sedentary integrins in a crawling cell. At the beginning of migration up-regulated expression of αβ1 and/or αβ2 integrin receptors may be required to achieve a stable adhesion to the substrate of the lamellipodium extending in the direction of a chemotactrant. In a long run, however, overexpression of the sedentary integrins may slow down crawling locomotion.

The fact that the galvanotropism of KCs inhibited by a pharmacologic antagonist or siRNA against either α7 or M1 could be restored by CA-MEK only partially suggests that simultaneous activation of both of receptor is required for normal response. In addition to the MEK1/ERK pathway, each receptor type apparently activates other effector systems involved in the biochemical events mediating changes in cell polarity, such as Rac/Cdc42 coupled by α7 (8).

Relocation of α7 and M1 to the pole of the cell facing a chemotactrant, which preceded reorientation of the whole cell body, may provide for a compartmentalized activation of motor proteins via Rac/Cdc42, recruited by α7 (8), simultaneously with up-regulation of αβ1 and α5 integrins via the MEK1/ERK pathways, activated by α7 and M1. The relation of changes in the levels of these and other integrins to keratinocyte migration and skin wound re-epithelialization has been documented elsewhere (11). On the cell membrane of KCs, α7 nAChR co-localizes with β1 integrins (8). Because both αβ1 and αβ2 integrins accumulate at the lamellipodium, we believe that simultaneous activation of both the α7- and M1-coupled signaling pathways at the very beginning of migration may be required for an extension of the leading lamella (Rac/Cdc42) and its anchoring to the substrate (MEK1/ERK).

Our findings have direct implications to the mechanisms underlying both normal epidermal turnover and wound epithelialization. In the epidermis, KCs constantly move upward toward the concentration gradient of free ACh (57). The results obtained in this study indicate that keratinocyte galvanotaxis is, in effect, keratinocyte chemotaxis toward the gradient of ACh and/or choline in a DC field. Finding the differences between the concentration of ACh at the anode and cathode was not surprising, because of a well known utility of iontophoresis for administering ACh and its congeners, such as carbachol, into the skin under positively charged ACh is repelled away from the anode based on the general or not the DC field currents amplifies and synchronizes ACh synthesis and release by KCs.

Cholinergic Regulation of Directional Migration

Although the mechanisms by which a DC electric field stimulates epithelialization remain largely unknown, several protein kinase pathways, such as protein kinase C, cAMP-dependent protein kinase, and mitogen-activated protein kinase, are apparently involved (reviewed in Ref. 66). Results of this study substantiated involvement of the Ras/Raf-1/MEK1/ERK pathway in re-orientation of KCs required for both chemotaxis and galvanotaxis. In future studies, we will determine whether or not the DC field currents amplifies and synchronizes ACh synthesis and release by KCs.

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Cholinergic Regulation of Directional Migration

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