Defining the signaling mechanisms and effector proteins mediating phenotypic and mechanical plasticity of keratinocytes (KCs) during wound epithelialization is one of the major goals in epithelial cell biology. The acetylcholine (ACh)-gated ion channels, or nicotinic ACh receptors (nAChRs), mediate the nicotinergic signaling that controls crawling locomotion of KCs. To elucidate relative contributions of the ionic and protein kinase-mediated events elicited due to activation of α7 nAChRs, we quantitated expression of α2-integrin gene at the mRNA and protein levels and also measured Rho kinase activity in KCs stimulated with the α7 agonist AR-R17779 while blocking the Na+ or Ca2+ entry and/or inhibiting signaling kinases. The results demonstrated the existence of the two-component signaling systems coupling the ionic and protein events and signaling cascade downstream of α7 nAChR to simultaneous up-regulation of α2-integrin expression and activation of Rho kinase. The Raf/MEK1/ERK1/2 cascade up-regulating α2-integrin was activated due to both Ca2+-dependent recruitment of Ca2+/calmodulin-dependent protein kinase II and protein kinase C and Ca2+-independent activation of Ras. Likewise the phosphatidylinositol 3-kinase-mediated activation of Rho kinase was elicited due to both Ca2+ entry-dependent involvement of Ca2+/calmodulin-dependent protein kinase II and Ca2+-independent activation of Jak2. Thus, although the initial signals emanating from activated α7 nAChRs are different in nature the pathways intersect at common effector molecules providing for a common end point effect. This novel paradigm of nAChR-mediated coordination of the ionic and metabolic signaling events can allow an auto/paracrine ACh to simultaneously alter gene expression and induce reciprocal changes in the cytoskeleton and contractile system of KCs required to compete a particular step of wound epithelialization.

Defining the signaling mechanisms and effector proteins mediating phenotypic and mechanical plasticity of epidermal keratinocytes (KCs) during their lateral migration in a wound bed is one of the major goals in epithelial cell biology. The epithelial and some other types of non-neuronal cells synthesize, degrade, and respond to acetylcholine (ACh) that functions outside the nervous system as an auto/paracrine hormone or a cytotoxin (for a review, see Ref. 1). The non-neuronal ACh exhibits rapid and profound effects on gene expression due to activation of the muscarinic and nicotinic classes of cholinergic receptors coupling multiple signal transduction pathways. The muscarinic receptors are classic G protein-coupled transmembrane glycoproteins that mediate a metabolic response to ACh through the interactions of G proteins with signal transducing enzymes, leading to increases or decreases of second messengers, ion concentrations, and modulations of protein kinase activities. The nicotinic ACh receptors (nAChRs) are classic representatives of the superfamily of ligand-gated ion channel proteins, or ionotropic receptors, mediating the influx of Na+ and Ca2+ and efflux of K+ (2). In neurons, binding of ACh to nAChRs leads to cell membrane depolarization that allows influx of Ca2+ through voltage-sensitive calcium channels. Although a high resolution patch clamping technique recorded single channel currents from outside-out patches excised from cultured human epidermal KCs stimulated with ACh, the KCs grown in the medium containing 0.09 mM Ca2+ only rarely showed ACh-activated currents (3). This was surprising because under such low Ca2+ culture conditions, the nAChR ligands elicit a plethora of biologic effects on KCs (for reviews, see Refs. 4 and 5). The nAChRs regulate survival, proliferation, adhesion, and differentiation of KCs and a large variety of non-neuronal cells and, in particular, play a crucial role in coordinating cellular functions mediating epithelialization of skin (6–8) and lung (9) wounds. Hence elucidation of the signaling events elicited upon agonist binding to keratinocyte nAChRs is crucial for understanding the mechanisms of ACh signaling in non-neuronal cells, which has salient clinical implications.

In non-neuronal cells, nAChRs regulate the expression of many genes. For instance, 118 genes are up-regulated and 97 are down-regulated in the human macrophage-like cell line U937 (10). In KCs, activation of nAChRs alters expression of the genes encoding cell receptor, signal transduction, cell cycle regulation, apoptosis, and cell-cell and cell-substrate

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2 The abbreviations used are: KC, keratinocyte; ACh, acetylcholine; nAChR, nicotinic ACh receptor; ROK, Rho kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; CaMKII, Ca2+ /calmodulin-dependent protein kinase II; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MLA, methyllycaconitine; DN-MEK, dominant negative MEK1 mutant; CA-MEK, constitutively active MEK1; siRNA, small interfering RNA; siRNA-NC, negative control siRNA; qPCR, quantitative PCR.
adhesion proteins (for reviews, see Refs. 4 and 5). On the keratinocyte plasma membrane, the nicotinergic signals can be elicited due to activation of several classic nAChR subtypes. The homomeric nAChRs expressed in KCs can comprise α7 or α9 subunits, whereas the heteromeric nAChRs can comprise the α3, α5, α9, α10, β1, β2, and β4 subunits, e.g. α3(β2/β4)±α5 and α9α10 (3, 11–15). We have documented that downstream of keratinocyte nAChRs the signaling pathways can involve elevation of intracellular Ca2+, activation of the protein kinase C (PKC) isoforms, Ca2+/calmodulin-dependent protein kinase II (CaMKII), Jak2, phosphotyrosinositol 3-kinase (PI3K), Akt, p38 mitogen-activated protein kinase (MAPK), phospholipase C, Src, epidermal growth factor receptor kinase, Rac, and Rho as well as the Ras/Raf-1/MEK/ERK pathway (6–8, 16, 17). There are many potential targets of these signaling cascades, including nuclear processes, metabolic pathways, and structural components of the cytoskeleton, but the receptor-mediated mechanisms of signal induction remain to be identified.

It has been demonstrated that keratinocyte α7 nAChR directs chemotaxis of KCs, which is a critical early step in wound epithelialization, and that the downstream signaling from this receptor in KCs involves intracellular free Ca2++, CaMKII, PKC, PI3K, Jak2, and the Ras/Raf-1/MEK/ERK cascade and leads to up-regulation of α2-integrin gene expression (7, 8, 18). Noteworthy is that PI3K plays a central role in coordinating Rho-mediated signaling events during cell migration (for a review, see Ref. 19). It is well known that α7 subunits form a homomeric receptor/channel that has a high relative permeability to Ca2+ (20, 21). Indeed Ca2+ ions that enter KCs through α7-containing ACh-gated ion channels can raise the concentration of intracellular free Ca2+ in KCs (11, 22), indicating that ionic events contribute to the nicotinergic effects on KCs. However, experiments with several types of non-neuronal cells demonstrated that the nicotinergic effects can be elicited in the absence of Na+ or Ca2+ entry (23–26). Therefore, the downstream signaling from α7 nAChR expressed in KCs may also proceed via parallel, ionic, and protein kinase signaling pathways.

In this study, we tested a hypothesis that the coordinated regulation of α2-integrin expression and reciprocal alterations in the cytoskeleton that mediate ACh control of keratinocyte lateral migration through α7 nAChR involves both ionic events and protein kinase signaling cascades. The results demonstrated the existence of the two-component signaling systems allowing simultaneous up-regulation of α2-integrin expression and ROK activation. The Raf/MEK1/ERK1/2 cascade up-regulating α2-integrin was activated due to both the Ca2+-dependent recruitment of CaMKII and PKC and the Ca2+-independent activation of Ras. Likewise the PI3K-mediated activation of ROK was elicited due to both Ca2+ entry-dependent involvement of CaMKII and Ca2+-independent activation of Jak2. Thus, although the initial signals emanating from activated α7 nAChR are different in nature the pathways intersect at common effector molecules providing for a common end point effect.

### MATERIALS AND METHODS

**Chemicals and Protein Kinase Assay Kits**—The preferential α7 nAChR agonist AR-177779 (27) was a gift from AstraZeneca Pharmaceuticals (Wilmington, DE). The α7-prefering antagonist methlycholactonite (MLA) (28) was purchased from Sigma-Aldrich. The noncompetitive inhibitor of the Ras acceptor protein manumycin A (29), the cRaf-1 kinase inhibitorGW5074 (5-ido-3-[3,5-dibromo-4-hydroxyphenyl]-methylene)-2-indoline) (30), the Jak2 inhibitor AG-490, and the cell-permeable, potent, and selective inhibitor of MEK “MEK inhibitor I” (31) were from Calbiochem-Novabiochem. The selective inhibitor of CaMKII KN-62, the selective and cell-permeable inhibitor of all PKC isoforms chelerythrine, and the highly specific PI3K inhibitor Ly-294002 were purchased from Axxora, LLC (San Diego, CA). The plasmins encoding the constitutively active MEK1 (CA-MEK) with two point mutations (S218E and S222E) and a deletion of amino acid residues 31–52 and the dominant negative MEK1 mutant (DN-MEK) that contains three point mutations (K97R, S218A, and S222A) and thus could neither be phosphorylated by its activators nor phospho-rylate its downstream effector ERKs were purchased from Biomyx Technology (San Diego, CA). The PKC activity assay kit was purchased from Stressgen Bioreagents (Ann Arbor, MI), the phospho-ERK1/2 (Thr-185/Tyr-187)-linked enzyme-linked immunosorbent assay kit was from Millipore (Billerica, MA), the Rho kinase assay kit was from CycLex Co. (Nagano, Japan), and the Jak2 kinase assay kit was purchased from Cell Signaling Technology, Inc. (Boston, MA). All assay kits were used to determine the activity of a corresponding kinase in experimental and control KCs following the protocols provided by the manufacturers.

**Keratinocyte Exposure Experiments**—Human keratinocyte cultures were started from normal neonatal foreskins (32). The cells were grown in 75-cm² flasks (Corning Glass Works, Corning, NY) in serum-free keratinocyte growth medium containing 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract (Invitrogen) at 37 °C in a humid, 5% CO2 incubator at 0.09 mm calcium. The keratinocyte cultures used in experiments were between passages 2 and 4 (~80% confluent). The cells were washed thrice with oxygenated Krebs-Ringer HEPES buffer, pH 7.4, containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 2.5 mM HEPES, 5.6 mM glucose, and 0.5% bovine serum albumin with or without 2.6 mM CaCl2++ and incubated for 1 h at 37 °C with test compound dissolved in Krebs-Ringer HEPES buffer. Then the monolayers were washed with prewarmed keratinocyte growth medium and incubated in this medium for 1 h to recover. After that, the 3-h exposure to test compounds was repeated. Control cells were exposed to Krebs-Ringer HEPES buffer without test compounds. Some experimental cells were incubated in Na+-free sucrose buffer in which NaCl was replaced with an isotonic concentration of sucrose (270 mM) and Tris-HCl buffer, pH 7.4, was used instead of HEPES.

**Transfection Experiments**—The presdesigned and tested small interfering RNA (siRNA) targeting human CHRNA7 (GenBank™ accession number NM_000746) mRNA sequence 5’-GGACAGAUCACUAAUUCACA-3’ and negative control siRNA (siRNA-NC) targeting luciferase gene with
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Figure 1. Alterations in α2-integrin gene expression. The qPCR (A) and in-cell Western (B) analyses of the effects of test conditions on the α7 nAChR-dependent activation of α2-integrin gene expression in human epidermal KCs at the mRNA and protein levels, respectively, were performed as described under “Materials and Methods.” To standardize the analysis, the gene expression ratios in the control cells, i.e. intact KCs, were taken as 1. The following experimental treatments were used: 50 μM AR-R17779, 100 nM MLA, 1 μM MEK inhibitor I (MEK Inh), 10 μM AG-490, 10 μM Ly-294002, 1 μM chelerythrine, 0.3 mM Cd²⁺, 0.1 mM Zn²⁺, and Ca²⁺- or Na⁺-free media. Some cells were transfected with siRNA-α7 versus siRNA-NC and DN-MEK versus CA-MEK. C shows the dose-dependent up-regulation of α2-integrin in normal (○) and Ca²⁺-free medium (●). Triplicate experiments were performed with KCs from each of the three cell donors used in this study (n = 3). Asterisks indicate significant (p < 0.05) differences from control KCs. The pound signs indicate significant (p < 0.05) differences from KCs exposed to AR-R17779 alone.

The target sequence 5’-CGTACGCGGAATACTTCGA-3’ were purchased from Dharmacon (Lafayette, CO). For transfection with siRNAs, we followed the standard protocol described in detail elsewhere (8). Briefly KCs were treated with increasing concentrations of siRNA in the transfection solution with the DharmaFECT™ 1 siRNA Transfection Reagent (Dharmacon) for 16 h at 37 °C in a humid, 5% CO₂ incubator. On the next day, the transfection medium was replaced by keratinocyte growth medium, and the cells were incubated for 72 h to determine at which time point maximum inhibition of the receptor protein expression occurs. The siRNA transfection efficiency was also assayed using fluorescein isothiocyanate-labeled luciferase GL2 duplex (Dharmacon). It was experimentally established that maximal, i.e. >90%, inhibition of expression occurred 48 h after transfection (data not shown) at which point the cells were used in exposure experiments. The same generic protocol of keratinocyte transfection was used to express MEK1 kinase mutants.

Real Time Quantitative PCR (qPCR) Experiments—Total RNA was extracted from KCs at the end of exposure experiments with the RNeasy Mini kit (Qiagen, Valencia, CA) and used in the qPCR assay of α2-integrin gene expression as detailed elsewhere (17). The qPCR primers 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-Demand provided by Applied Biosystems. The qPCRs were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance with the manufacturer’s protocol as described by us in detail elsewhere (33). To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data from triplicate samples were analyzed with sequence detector software (Applied Biosystems) and expressed as mean ± S.D. of α2-integrin mRNA relative to that of control.
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AR-R17779-dependent up-regulation of \( \alpha_7 \)-integrin in Ca\(^{2+}\)-free medium was inhibited by >50% at three different AR-R17779 concentrations, i.e. 25, 50, and 100 \( \mu \text{M} \) (Fig. 1C), suggesting that \( \alpha_7 \) nAChR-mediated Ca\(^{2+}\) influx, rather than cell membrane depolarization, contributes to the signaling cascade leading to up-regulation of the \( \alpha_7 \)-integrin gene expression. This conjecture was supported by a partial decrease of the AR-R17779-dependent up-regulation of \( \alpha_7 \)-integrin expression due to inhibition of Ca\(^{2+}\) influx through nAChR by Cd\(^{2+}\) or Zn\(^{2+}\) (35, 36).

Both in the absence of extracellular Ca\(^{2+}\) and in the presence of Cd\(^{2+}\) or Zn\(^{2+}\), the AR-R17779-dependent elevation of \( \alpha_7 \)-integrin mRNA and protein was completely blocked by inhibiting MEK1 activity (Fig. 1), indicating that MEK signaling down-

\[ \text{AR-R17779} + \text{MLA} + \text{siRNA-\( \alpha_7 \)}} \]
\[ \text{Ca}^2\text{-free medium} + \text{Manumycin A} \]
\[ \text{GW5074} + \text{MEK Inh} \]
\[ \text{DN-MEK} + \text{CA-MEK} \]
\[ \text{Ca}^2\text{\textsuperscript{2+}} \]
\[ \text{Zn}^2\text{\textsuperscript{2+}} \]
\[ \text{AG-490} + \text{Ly-29402} \]
\[ \text{Chelerythrine} \]
\[ \text{Na}\textsuperscript{+}-\text{free medium} \]

**FIGURE 2. Measurement of ERK1/2 activity**. A, the effects of test conditions on ERK1/2 activity in human epidermal KCs were determined as described under "Materials and Methods" following the protocol for ERK1/2 activity measurement provided by the manufacturer of the assay kit. The ERK1/2 activity in intact KCs was taken as 1. The following experimental treatments were used: 50 \( \mu \text{M} \) AR-R17779, 100 \( \mu \text{M} \) MLA, 3 \( \mu \text{M} \) manumycin A, 0.1 \( \mu \text{M} \) GW5074, 1 \( \mu \text{M} \) MEK inhibitor I (MEK Inh), 10 \( \mu \text{M} \) AG-490, 10 \( \mu \text{M} \) Ly-29402, 1 \( \mu \text{M} \) chelerythrine, 0.3 \( \text{mM} \) Cd\(^{2+}\), 0.1 \( \text{mM} \) Zn\(^{2+}\), and \text{Ca}\(^{2+}\) or Na\(^{+}\)-free media. Some cells were transfected with siRNA-\( \alpha_7 \) versus siRNA-NC and DN-MEK versus CA-MEK. B, concentration-dependent effects of AR-R17779 on ERK1/2 activity in normal (○) and Ca\(^{2+}\)-free medium (●). Triplicate experiments were performed with KCs from each of the three cell donors used in this study (\( n = 3 \)). Asterisks indicate significant (\( p < 0.05 \)) differences from control KCs. The pound signs indicate significant (\( p < 0.05 \)) differences from KCs exposed to AR-R17779 alone.

**In-cell Western Assay**—The in-cell Western assay was performed as described by us in detail elsewhere (34) using the reagents and equipment from LI-COR Biotechnology (Lincoln, NE). After exposure of 3 \( \times \) 10\(^4\) KCs/well of a 96-well plate to test agents, the experimental and control cells were fixed in situ, washed, permeabilized with Triton solution, incubated with the LI-COR OdysseyBlocking Buffer for 1.5 h, and then treated overnight at 4 °C with a primary rabbit polyclonal antibody to \( \alpha_2 \)-integrin (Chemicon International, Inc., Temecula, CA). After that, the cells were washed and stained for 1 h at room temperature with a secondary LI-CORIRDye 800CW goat anti-rabbit or IRDye 800CW antibody diluted 1:800. Sapphire700 (1:1000) was used to normalize for cell number/well. The protein expression was quantitated using the LI-COR Odyssey Imaging System.

**Statistical Analysis**—All experiments were performed in triplicates, and the results were expressed as mean ± S.D. Statistical significance was determined using Student’s \( t \) test. Differences were deemed significant if the calculated \( p \) value was <0.05.
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stream from α7 nAChR can be elicited independently from ion influx. The α7 nAChR pathway coupled to up-regulation of α7-integrin also involved PKC whose inhibition with chelerythrine produced a reciprocal decrease of the AR-R17779-induced up-regulation of the α7-integrin gene expression. This putative signaling pathway apparently did not involve Jak2 and PI3K because their inhibition with AG-490 and Ly-294002, respectively, did not alter the AR-R17779-dependent elevation of α7-integrin mRNA and protein (Fig. 1). These results demonstrated the existence of the two-component signaling system mediating up-regulation of the α7-integrin gene expression upon stimulation of α7 nAChR with an agonist.

Identification of the Signaling Elements Comprising the Pathways Up-regulating the α7-Integrin Gene Expression Downstream of α7 nAChR—AR-R17779 produced a dose-dependent increase of ERK1/2 activity, which could be inhibited by MLA and siRNA-α7 (Fig. 2), thus substantiating the notion that downstream signaling from α7 nAChR that up-regulates the α7-integrin gene expression involves the MEK1/ERK1/2 step.

Both elimination of the extracellular Ca2+ and inhibition of its nAChR permeation through nAChR by Cd2+ and Zn2+ partially decreased the AR-R17779-dependent ERK activation, whereas elimination of extracellular Na+ had no effect. Transfection of KCs with CA-MEK eliminated the inhibitory effects of Ca2+ deprivation on both ERK activity (Fig. 2) and α7-integrin gene expression (Fig. 1). Combining the deprivation of extracellular Ca2+ with inhibition of MEK in all cases totally blocked AR-R17779-dependent ERK activation. In Ca2+-free medium, Ras and Raf inhibitors also totally abolished the AR-R17779 effect, demonstrating that α7 signaling was initiated due to both Ca2+ influx and activation of Ras/Raf independently from Ca2+ influx.

To further identify effectors of the nAChR-coupled signaling pathways upstream of the MEK1/ERK1/2 step, we measured the effects of Jak2, PI3K, and PKC inhibitors on the AR-R17779-dependent ERK activation. Only the PKC inhibitor chelerythrine significantly (p < 0.05) decreased ERK activity (Fig. 2), indicating that PKC is involved upstream of the MEK1/ERK1/2 step.

Defining the Mechanism of PKC Involvement in the α7 Signaling Regulating α7-Integrin Expression—To distinguish between the ionic and protein kinase-mediated mechanisms of PKC engagement in the α7 nAChR signaling up-regulating the α7-integrin gene expression, we measured PKC activity in KCs deprived of extracellular Ca2+ or exposed to kinase inhibitors. The specificity of PKC activation due to agonistic stimulation of α7 nAChR was demonstrated by inhibiting the AR-R17779 effect by MLA and due to transfection with siRNA-α7 but not siRNA-NC (Fig. 3). The AR-R17779-dependent activation of PKC was completely inhibited due to elimination of Ca2+ from the culture medium, blocking the nAChR permeability to Ca2+ with Cd2+ or Zn2+, and treatment with the CaMII inhibitor KN-62 (Fig. 3). The Ras inhibitor manumycin A produced no significant effect (p > 0.05). These results demonstrated that PKC engagement downstream of α7 nAChR activation occurs predominantly due to Ca2+ influx and proceeds along the pathway involving CaMII.

The Signaling Mechanisms Regulating Rho Activity Downstream of α7 nAChR—Because ROK is believed to play a central role in coordinating the cholinergic control of keratinocyte migration through regulation of the integrin gene expression and cytoskeleton rearrangements (5), we next determined whether and how the downstream signaling from α7 nAChR engages ROK. Activation of α7 nAChR with AR-R17779 produced a concentration-dependent increase of the ROK activity that could be inhibited by MLA and transfection with siRNA-α7 (Fig. 4). The AR-R17779-induced ROK activation depended upon Ca2+ (Fig. 4B) but not Na+ entry. An inhibitor of PI3K completely decreased the AR-R17779-dependent ROK activation; the CaMII and Jak2 inhibitors decreased it only partially as it remained significantly elevated (p < 0.05), whereas a PKC inhibitor produced no significant effect (p > 0.05) (Fig. 4). Combining Jak2 inhibitor with a blockade of extracellular Ca2+ entry or inhibition of CaMII in all cases completely abolished the AR-R17779-induced activation of ROK. These results indicated that α7 nAChR-mediated activation of ROK may result due to Ca2+ influx-dependent engagement of the CaMII/PI3K step and also independently from Jak2 involvement.

Activation of Jak2 through α7 nAChR Does Not Depend on Na+ and Ca2+ Influx—We have demonstrated recently that activation of keratinocyte α7 nAChR leads to recruitment and phosphorylation of Jak2 (18). To determine whether the influx of extracellular Na+ and Ca2+ through α7 nAChR is necessary for Jak2 activation, we measured Jak2 activity in KCs incubated in a Ca2+- or Na+-free medium and found no significant changes in the AR-R19779-dependent Jak2 activation (p > 0.05) (Fig. 5). Inhibition of Ca2+ entry through α7 nAChR using Cd2+ or Zn2+ produced no significant effects either (p > 0.05).


Therefore, engagement of Jak2 in the α7 nAChR signaling coupled to activation of ROK does not require Ca\(^{2+}\) and Na\(^{+}\) influx.

**DISCUSSION**

The obtained results demonstrated for the first time the synergistic action of the ionic and protein kinase signal transduction events downstream of α7 nAChR in mediating the nicotinicergic up-regulation of α\(_2\)-integrin expression and ROK activity required for normal keratinocyte migration and wound epithelialization. The α\(_2\)-integrin gene expression was up-regulated through the Raf/MEK1/ERK1/2 cascade activated due to both the Ca\(^{2+}\)-dependent recruitment of CALMII and PKC and the Ca\(^{2+}\)-independent activation of Ras (Fig. 6). Likewise the PI3K-mediated activation of ROK was elicited due to both the Ca\(^{2+}\)-entry-dependent engagement of CALMII and the Ca\(^{2+}\)-independent activation of Jak2. Downstream of α7 nAChR, the distinct signals emanated from activated receptor intersected at the common signal transduction effectors, i.e. Raf in the cascade leading to α\(_2\)-integrin up-regulation and PI3K in the pathway activating ROK. This novel biologic mechanism may allow an auto/paracrine Ach to synchronize the ionic events and metabolic signaling cascades to simultaneously alter gene expression patterns and induce reciprocal changes in the cytoskeleton and contractile system that altogether drive crawling locomotion of KCs during wound epithelialization.

The signaling pathways downstream of nAChRs are involved in regulation of related cellular functions of KCs, such as proliferation, differentiation, apoptosis, adhesion, and migration (for reviews, see Refs. 4 and 5). The diverse nicotinic signals elicit biologic effects through divergent modifications of a large variety of effector molecules and structural proteins. We have demonstrated previously that activation of α\(_7\) nAChR helps orient a keratinocyte toward direction of its future migration via the Ras/Raf in the pathway leading edge (8). The α\(_2\)-integrin function can switch the Rho-dependent cell shape changes from a resting to a spreading morphology (37). RhoA induces the assembly of contractile actin-myosin filaments (stress fibers) and associated focal adhesion complexes (for a review, see Ref. 38). Thus, the biologic significance of the cooperative signaling from α\(_7\) nAChR that simultaneously up-regulates α\(_2\)- integrin and activates ROK stems from the fact that both of these effector systems are crucial for initiation of migration of resting KCs at the earliest stage of wound epithelialization.

The AR-R17779-dependent up-regulation of both α\(_2\)-integrin expression and ROK activity could be altered by blocking Ca\(^{2+}\) entry. This observation is in keeping with our earlier findings that the nAChR-dependent Ca\(^{2+}\) influx plays an important role in mediating Ach regulation of keratinocyte migration (22). It is well known that activation of nAChRs elicits Ca\(^{2+}\) influx that varies in magnitude between different receptor subtypes and that the Ach-gated ion channels comprising α7 subunits have the greatest Ca\(^{2+}\) permeability (20, 39). We have demonstrated previously that Ca\(^{2+}\) ions that enter KCs through α7 nAChRs can raise the concentration of intracellular free Ca\(^{2+}\) (11, 22). Therefore, it was not surprising that the
signaling downstream of α7 nAChR involved CaMKII that, in turn, relayed the downstream signaling to the two separate pathways, one leading to α2-integrin up-regulation through engagement of PKC and another one activating ROK through PI3K. Indeed it has been established that the Raf/MEK/ERK cascade can be activated independently from Ras in a PKC-dependent manner (40) and that PI3K is positively regulated by Ca2+ via CaMKII (41).

In the present study, we demonstrated that in addition to the Ca2+-dependent induction of protein kinases the two-component nicotinicergic signal transduction systems regulating α2-integrin gene expression and ROK activation in KCs also include the ion influx-independent events. To prevent Ca2+ entry through nAChRs we used Cd2+ and Zn2+, both of which have been shown to block ACh-evoked currents elicited due to activation of nAChRs (35, 36). Because on the one hand activation of nAChRs in KCs causes a limited depolarization of the cell membrane in the outside-out, but not the whole-cell, patches (3) and on the other hand the voltage-gated Ca2+ channels exist in KCs and play a role in skin barrier homeostasis (42), we tested the effect of Na+-free medium on AR-R17779 activity. As could be expected based on the fact that in contrast to neurons KCs are generally not considered to be excitable cells, changing the medium did not significantly alter the AR-R17779 effects (p > 0.05).

The functional cooperation of the ionic events and protein kinase signaling cascades was illustrated by the fact that inhibition of either Ca2+ entry or protein kinase activities produced only partial inhibition of the activity of AR-R17779, whereas a combined inhibition of both ionic and protein kinase pathways completely abolished its effects. Because others also reported that inhibition of Ca2+ influx using Ca2+ entry blockers or a Na+-free incubation solution only partially blocks the nicotinicergic effects (23, 25, 43–45), a similarly arranged two-component signaling system apparently operates in other cells downstream of nAChRs.

In both models of keratinocyte deprivation of the extracellular Ca2+ entry, i.e. due to treatment with Cd2+ or Zn2+ and using a Ca2+- or Na+-free medium, AR-R17779 activated Ras and Jak2. The exact nature of the non-excitatory events associated with AR-R17779 binding to α7 nAChR is not fully understood because nAChR subunits do not have intrinsic tyrosine kinase activity (46). Perhaps the AR-R17779 signaling observed by us in the absence of Ca2+ entry was elicited due to formation of functional multiprotein complexes of α7 subunit with the signaling proteins anchored to the cell membrane, such as Ras and Jak2 (47, 48). We have reported previously that activation of keratinocyte α7 nAChR leads to phosphorylation and activation of Jak2 (16, 18) and also initiates Ras-mediated signaling (8, 18, 49). Currently there is no published evidence that α7 can form complexes with Ras, but such a paradigm has been established for Jak2. It has been shown that agonist-induced complex formation between α7 and Jak2 leads to subsequent activation of PI3K (50) in agreement with the results of the present study. Our observations are also consistent with the notion that an important role of the Jak2/PI3K signaling cascade is the physiologic control of cell migration (51).
The demonstration of the functional coupling of nACHRs to signaling kinases further unveils a novel function of the subunit proteins that mediate nicotinic effects in non-excitable cells. As already mentioned, Jak2 physically associates with α7 upon stimulation with an agonist and becomes phosphorylated and activated (52). The α7 subunit can also physically and functionally associate with Src family kinases (53–56), whereas the subunits α3-α5 and β2 exhibit a positive interaction with the G protein subunits Gα16 and Gβγ (57). The nACHR proteins can associate with both protein kinase and protein-tyrosine phosphatase in large multimeric complexes (58). For instance, it was proposed that Src-associated phosphatase functions in early stimulation with an agonist and becomes phosphorylated and activated (52). The nAChR by autocrine/paracrine ACh controls Src activity and that interruption of ACh signaling releases Src activity, leading to catenin phosphorylation and weakening cell cohesion (59). Of particular interest is an observation that nicotinergic up-regulation of mitogenesis involves an α7-mediated recruitment of β-arrestin and thus facilitates Src activation (60). Therefore, the mode of interaction of nACHRs with the signal transduction molecules may vary depending on the type of receptor subunit involved, the kind of signaling pathway involved in mediating a particular nicotinergic function, and the cell type responding to an agonist. Thus, elucidation of the mechanisms of α7 nACHR signaling in non-excitable cells like KCs opens a door for a novel approach to pharmacologic regulation of the cellular functions regulated through the nACHRs expressed in non-neuronal cells.

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