Differential regulation of keratinocyte chemokinesis and chemotaxis through distinct nicotinic receptor subtypes

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Accepted 16 August 2004
Journal of Cell Science 117, 5665-5679 Published by The Company of Biologists 2004
doi:10.1242/jcs.01492

Summary

Nicotinergic agents can act as both chemokines and chemoattractants for cell migration. Epidermal keratinocytes both synthesize acetylcholine and use it as a paracrine and autocrine regulator of cell motility. To gain a mechanistic insight into nicotinergic control of keratinocyte motility, we determined types of nicotinic acetylcholine receptors and signaling pathways regulating keratinocyte chemokinesis and chemotaxis, using respective modifications of the agarose gel keratinocyte outgrowth assay. Random migration of keratinocytes was significantly ($P<0.05$) inhibited by hemicholinum-3, a metabolic inhibitor of acetylcholine synthesis, as well as by the α-conotoxins MII and AuIB, preferentially blocking α3-containing nicotinic acetylcholine receptors. The use of antisense oligonucleotides specific for nicotinic-acetylcholine-receptor subunits and knockout mice demonstrated pivotal role for the α3β2 channel in mediating acetylcholine-dependent chemokinesis. Signaling pathways downstream of α3β2 included activation of the protein-kinase-C isoform δ and RhoA-dependent events. The nicotinergic chemotaxis of keratinocytes was most pronounced towards the concentration gradient of choline, a potent agonist of α7 nicotinic acetylcholine receptor. The α7-preferring antagonist α-bungarotoxin significantly ($P<0.05$) diminished keratinocyte chemotaxis, further suggesting a central role for the α7 nicotinic acetylcholine receptor. This hypothesis was confirmed in experiments with anti-α7 antisense oligonucleotides and α7-knockout mice. The signaling pathway mediating α7-dependent keratinocyte chemotaxis included intracellular calcium, activation of calcium/calmodulin-dependent protein-kinase II, conventional isoforms of protein-kinase C, phosphatidylinositol-3-kinase and engagement of Rac/Cdc42. Redistribution of α7 immunoreactivity to the leading edge of keratinocytes upon exposure to a chemoattractant preceded crescent shape formation and directional migration. Application of high-resolution deconvolution microscopy demonstrated that, on the cell membrane of keratinocytes, the nicotinic acetylcholine receptor subunits localize with the integrin β_1. The obtained results demonstrate for the first time that α3 and α7 nicotinic acetylcholine receptors regulate keratinocyte chemokinesis and chemotaxis, respectively, and identify signaling pathways mediating these functions, which has clinical implications for wound healing and control of cancer metastases.

Key words: Keratinocytes, Chemokinesis, Chemotaxis, Acetylcholine, Nicotinic receptors α3 and α7, Knockout mice, Antisense oligonucleotides

Introduction

Lateral migration of eukaryotic cells is central to many important biological processes, such as embryogenesis, angiogenesis, metastasis, inflammation and wound healing (for reviews, see Firtel and Chung, 2000; Lauffenburger and Horwitz, 1996). Various kinds of chemical stimuli can modulate random cell migration (chemokinesis), some of which have also been shown to stimulate directional migration (chemotaxis) (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Many chemoattractants are ligands for G protein-coupled receptors (for a review, see Song and Zhong, 2000). Downstream pathways involve intracellular free Ca^{2+}, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) isoenzymes, phosphatidylinositol-3-kinase (PI3K) (Pettit and Fay, 1998; Siddiqui and English, 2000; Sotsios and Ward, 2000), as well as the small GTPases RhoA, Rac and Cdc42 (Fukata et al., 2003; Slater et al., 2001). Recently, it has become evident that many of the intracellular biochemical events mediating chemotaxis can occur in the absence of functional G-proteins, suggesting a role for G-protein-independent signaling mechanisms (Devreotes and Janetopoulos, 2003).

Activation of ligand-gated ion channels has been shown to affect both chemokinesis and chemotaxis. The cholinergic agonist carbachol (CCh) enhances the chemotactic responsiveness of human monocytes to endotoxin-treated serum (Sandler et al., 1975). Nicotine, an agonist of acetylcholine (ACh)-gated ion channels or nicotinic ACh

receptors (nAChRs) is chemotactic for neutrophils (Nowak et al., 1990; Totti et al., 1984). Choline, a selective agonist of α7 nAChR (Alkondon et al., 1997; Papke et al., 1996), is a ligand for chemosensation and chemotaxis (Yassin et al., 2001). ACh and CCh induce chemotaxis of spermatozoa (Sliwa, 1995) and adult sensory-neuron growth cones (Kuffler, 1996; Tessier-Lavigne, 1994). The growth cone response to the activation of nAChRs requires the presence of extracellular Ca^{2+} and appears to be mediated by CaMKII (Zheng et al., 1994; Zheng et al., 1996).

Functional nAChRs are expressed in non-neuronal locations, where they regulate vital functions of various types of nonexcitable cells through Ca^{2+}-dependenet mechanisms (for a review, see Sharma and Vijayaraghavan, 2002). The homopentameric channels formed by α7 subunits and sensitive to α-bungarotoxin (αBTX) exhibit the highest measured Ca^{2+} permeability values, whereas heteropentameric non-αBTX-sensitive nAChRs containing α3 and one β (β2, β3 or β4) subunit have lower measured Ca^{2+} permeability (for a review, see Fucile, 2004). Thus, the value of Ca^{2+} permeability associated with a particular nAChR subtype is an important indication of its physiological role (Fucile, 2004).

There is an upward concentration gradient of free ACh within the multilayered epidermis, the uppermost division of the skin consisting of the stratified epithelial cells termed keratinocytes (KCs) (Nguyen et al., 2001). KCs synthesize and degrade ACh and use it as an autocrine and paracrine hormone regulating their motility and many other important functions (for a review, see Grando, 1997). The KCs that form the lower epidermal compartment constantly move upwards to renew the epidermis. They respond to ACh via two classes of cholinergic receptors: the muscarinic and the nicotinic receptors (Grando, 2001; Ndoye et al., 1998). On KC plasma membranes, the heteromeric nAChRs can be composed of various combinations of α3 with β2, β4 and α5 subunits, and α9 with α10 subunits, whereas the homopentameric channels are composed of α7 subunits. Cholinergic drugs acting at KC nAChRs have been shown to exhibit rapid, profound effects on cell shape and motility, which correlated with changes in the concentration of intracellular Ca^{2+} (Grando et al., 1995; Zia et al., 2000). An 'under' agarose gel KC outgrowth system (AGKOS) allows one to measure the chemokinetic and chemotactic effects of test compounds in a large cell population (10^4 per well), which renders this technique highly specific and sensitive (Grando et al., 1993a). Results of early studies using the AGKOS assay strongly suggested that endogenous ACh is required for KC outgrowth initiation. However, the individual roles of different nAChR types and receptor-dependent signaling pathways in the biological processes that initiate and maintain crawling locomotion of KCs remain to be elucidated.

This study was designed to gain a mechanistic insight into the nicotinergic control of KC chemokinesis and chemotaxis. We investigated effects of functional inhibition of different KC nAChR types by pharmacological antagonists, antisense oligonucleotides (AsOs) and null mutation of the nAChR subunit gene in receptor knockout (KO) mice on random and directional migration using the chemokinesis and chemotaxis modifications of the AGKOS assay, respectively. We demonstrated that endogenously produced and secreted ACh is essential for KC migration, and that individual types of nAChRs expressed in KCs produce distinct effects on crawling locomotion. The nicotinergic chemokinesis of KCs was predominantly mediated by the signaling events downstream of α3β2 and involved PKC-δ and the Rho/Rho-associated protein kinase (ROK) pathway. The α7-containing nAChR inhibited random migration but facilitated directional migration of KCs. The nicotinergic chemotaxis of human KCs was predominantly regulated via the Ca^{2+}-dependent pathway involving CaMKII, PI3K and conventional isoforms of PKC, as well as Rac and Cdc42. The obtained results have clinical implications for wound healing and control of cancer metastases.

Materials and Methods

Chemicals and tissue culture reagents

The pan-nicotinic agonist nicotine, the potent agonist of α3-containing nAChRs epibatidine (Wang et al., 1996a), the α7-selective agonist choline (Alkondon et al., 1997; Papke et al., 1996), the α3-nAChR-preferring antagonist mecamylamine (which has relatively small effects on α7 nAChR) (Frazier et al., 1998; Papke et al., 2001), tubocurarine (which can efficiently block the α3, α7 and α9 nAChRs) (Chavez-Noriega et al., 1997; Verbitsky et al., 2000), the α7 antagonist αBTX (Quik et al., 1996), the α9 antagonist strychnine (Rothlin et al., 1999), the metabolic inhibitor of ACh synthesis hemicholinium-3 (HC-3) (Guyenet et al., 1973; Veldsema-Currie et al., 1984), the muscarinic agonists muscarine and oxotremorine-M and the PI3K inhibitor wortmannin were purchased from Sigma-Aldrich (St Louis, MO). The preferential blockers of α3β2 and α3β4 nAChRs, α-conotoxins MII (αCtxMII) and AuIB (αCtxAuIB), respectively, were synthesized by Advanced ChemTech, Louisville, KY. αCtxMII and αCtxAuIB block their respective target receptors with an IC_{50} of 0.5 nM and 0.75 μM, and block other nAChR subunit combinations with IC_{50} values 2-4 orders of magnitude lower (Cartier et al., 1996; Luo et al., 1998; Nicke et al., 2003). The cell-permeable chelator of intracellular free Ca^{2+} 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA/AM), the selective inhibitors of CaMKII KN-62 and KN-93, the selective and cell-permeable inhibitor of all PKC isoforms chelerythrine, the highly specific PI3K inhibitor Ly-294002, the functional inhibitor of Rho proteins C3 exoenzyme (C3), and the cell-permeable and selective inhibitor of ROK Y-27632 were purchased from Axxora (San Diego, CA). The *Clostridium difficile* toxin B (TxB), a high-molecular-weight glucosyltransferase that inhibits Rho, Rac and Cdc42 by glycosylation of a threonine residue (Just et al., 1996), the PKC inhibitor Gö-6976 (which selectively inhibits PKC-α and -β isozymes without affecting the -δ, -ε and -ζ isoforms) and rottlerin (a PKC inhibitor that exhibits greater selectivity for the δ isoform) were from Calbiochem-Novabiochem (La Jolla, CA). The serum-free keratinocyte growth medium (KGM) containing 5 ng ml^{-1} epidermal growth factor and 50 μg ml^{-1} bovine pituitary extract were purchased from Gibco-BRL (Cambridge, MA). Agarose type HSA was from Accurate Chemical & Scientific Corporation (Westbury, NY). Heat-inactivated newborn calf serum, 0.05% trypsin, trypan-blue dye solution and Wright's stain were from Sigma-Aldrich. Rabbit anti-α9 antibody was developed and characterized by us previously (Nguyen et al., 2000b). Rabbit antibodies against α3, α5, β2 and α7 nAChR subunits, which were also characterized by us previously (Nguyen et al., 2000a; Zia et al., 2000), are commercially available from Research and Diagnostic Antibodies (Benicia, CA). The fluorescein-isothiocyanate (FITC)-labeled polyclonal antibody against cytokeratin 5 was from BAbCO (Richmond, CA) and that against filaggrin and loricrin from Covance Research Products (Berkeley, CA). The monoclonal antibody against Ki-67 was purchased from DAKO (Carpinteria, CA), that against PCNA from Santa Cruz Biotechnology (Santa Cruz, CA), that against cytokeratin 10 from Novocastra Laboratories (Newcastle upon Tyne, UK) and that against β1 integrin from Oncogene Research Products (San Diego, CA). Anti-β-actin

primary antibody and all secondary, FITC-labeled antibodies were purchased from Sigma-Aldrich.

Cultures of human KCs and AsOs transfection

Human KC cultures were started from normal neonatal foreskins, as we described in detail elsewhere (Grando et al., 1993b). The KC cultures used in AsOs assays were between passages 2 and 4. The phosphorothioated and FITC-tagged AsOs and the phosphorothioated, equally sized control (sense) oligonucleotides were commercially synthesized by Operon (Alameda, CA). The oligonucleotide sequences used in this study are shown in Table 1. The uniqueness of the sequences targeted by each of the AsOs was determined by comparing the targeted sequence against sequences found in GenBank and other data bases using BLAST (Altschul et al., 1990). AsOs were mixed with LipofectAMINE PLUS™ reagent (Gibco BRL) and transfected into human KCs grown to approximately 50% confluence, as detailed elsewhere (Arredondo et al., 2002; Nguyen et al., 2004). Each experimental culture received 20 nM AsOs and the control cultures received the same dose of control (sense) oligonucleotide diluted in KGM. The AsOs uptake by KC was monitored using FITC-conjugated AsOs and the efficacy of inhibition of nAChR subunit expression was determined by western blotting (Arredondo et al., 2001).

nAChR mutant mice and murine KC cultures

We used previously described KO mice deficient in one of the nAChR subunits α3 (Xu et al., 1999a), α5 (Salas et al., 2003), α7 (Orr-Urtreger et al., 1997), α9 (Vetter et al., 1999) and β2 or β4 (Xu et al., 1999b). PCR primers used for genotyping are shown in Table 2. This study was approved by University of California Davis Committee on the Use of Animals in Research. Pure cultures of murine epidermal KCs were started from skin samples obtained from 2-3-day-old mice (Arredondo et al., 2002).

AGKOS assays

Second-passage human or murine KCs were suspended in KGM, counted in a hemocytometer, loaded at a high density (1×10^4 cells per 10 µl) into each 3-mm well in an agarose gel, as detailed elsewhere (Grando et al., 1993a; Zia et al., 2000). In the chemokinesis AGKOS assay, KCs were fed with KGM containing various concentrations of test compounds vs no treatment (control) and incubated for 10 days in a humid CO_2 incubator with daily changes of medium. The migration of KCs was stopped by fixing the cells in 0.25% glutaraldehyde and staining them with Wright's stain. To measure the effects of nicotinergic agents on the random migration distance (RMD) (i.e. the distance outward from the original 3-mm well to the leading edge), the image of each megacolony was projected to the screen and the blueprint obtained. To standardize measurements, three segments were drawn through the center of each megacolony at 60° intervals (Fig. 1A). The RMD was computed in µm using the following formula:

$$RMD = (B_1B_4 - A_1A_4) + (B_2B_5 - A_2A_5) + (B_3B_6 - A_3A_6) / 6 .$$

The distance of KC outgrowth was partially donor dependent, ranging from approximately 2 mm to 4.5 mm. To standardize results obtained in experiments using KCs from different donors, the mean RMD values were converted into the percentage of the control value. The control value for KCs from each particular donor was determined by measuring the baseline RMD (in µm) and taking it as 100%.

Table 1. Oligodeoxynucleotides (ODN) used in this study

ODN	Sequence	Function
C1	5′-G*GCTGAGCACCGTCTATTTG*A-3′	Phosphorothioated sense control
C2	5′-T*GAAGTTTGGGTCCTGGTCTTAC*G-3′	Phosphorothioated sense control
C3	5′-A*TCTGGTTGCCTGACATC*G-3′	Phosphorothioated sense control
C4	5′-C*ATCTCCTTTTGCTGGATC*T-3′	Phosphorothioated sense control
α3.1	5′-(Fl)CCACCTGGAAATCCCCAACA-3′	Fluorescein α3 anti-sense
α3.2	5′-A*AATAGACGGTGCTCAGCCT*C-3′	Phosphorothioated α3 anti-sense
α3.3	5′-C*TCAAATAGACGGTGCTCAGC*C-3′	Phosphorothioated α3 anti-sense
α3.4	5′-T*TCGCCTTATCGTAGGACCAG*G-3′	Phosphorothioated α3 anti-sense
α3.5	5′-A*AGACGAGCACAGTGAGGAAG*G-3′	Phosphorothioated α3 anti-sense
α5.1	5′-(Fl)CAATAGAAGAAGGTTCAGAT-3′	Fluorescein α5 anti-sense
α5.2	5′-C*CATCATAAGTCCAAGAACC*A-3′	Phosphorothioated α5 anti-sense
α5.3	5′-T*GACATACGGATACCAGCAAC*A-3′	Phosphorothioated α5 anti-sense
α5.4	5′-C*AGGTGTTCCACAGGACGAA*C-3′	Phosphorothioated α5 anti-sense
α7.1	5′-(Fl)ATAGTAGAGCGTCCTGCG-3′	Fluorescein α7 anti-sense
α7.2	5′-A*AGGGATTGTAGTTCTTGAC*C-3′	Phosphorothioated α7 anti-sense
α7.3	5′-C*GTAAGACCAGGACCCAAACTTC*A-3′	Phosphorothioated α7 anti-sense
α7.4	5′-G*TGAGTGGTTGCGAGTCATTG*G-3′	Phosphorothioated α7 anti-sense
α7.5	5′-G*TTCTTCTCATCCACGTCCATG*A-3′	Phosphorothioated α7 anti-sense
α7.6	5′-C*GATGTAGCAGGAACTCTTGA*A-3′	Phosphorothioated α7 anti-sense
α9.1	5′-(Fl)AGATCCAGCAAAAGGAGATG-3′	Fluorescein α9 anti-sense
α9.2	5′-T*GAGCATATTTTCCATCTG*C-3′	Phosphorothioated α9 anti-sense
α9.3	5′-T*GGACGAAGAGCATTAGAA*T-3′	Phosphorothioated α9 anti-sense
α9.4	5′-C*TTAATCTGAGAGAGCGTA*A-3′	Phosphorothioated α9 anti-sense
α9.5	5′-T*GGCGGATCCACAAATAAG*C-3′	Phosphorothioated α9 anti-sense
β2.1	5′-(Fl)CTCACTCTTCAGCACCAAGTCG-3′	Fluorescein β2 anti-sense
β2.2	5′A*CACCATAAGCTGTACTGTCAC*C-3′	Phosphorothioated β2 anti-sense
β2.3	5′-C*GCTCCTCTGTATCCGTAC*C-3′	Phosphorothioated β2 anti-sense
β2.4	5′-A*GACATTGGTGGTCATGATCTG*C-3′	Phosphorothioated β2 anti-sense
β2.5	5′-T*GCCATCATAGGAGACCAC*G-3′	Phosphorothioated β2 anti-sense
β4.1	5′-(Fl)CGATGTCAGGCAACCAGAT-3′	Fluorescein β4 anti-sense
β4.2	5′-T*GCAGCTTGATGGAGATGAG*C-3′	Phosphorothioated β4 anti-sense
β4.3	5′-C*TCTCGCTCATTCACGCTGA*T-3′	Phosphorothioated β4 anti-sense
β4.4	5′-A*TCTCCGTGTGGTCATAGGT*C-3′	Phosphorothioated β4 anti-sense

Fl, fluorescein; asterisks indicate phosphorothioate.

Table 2. PCR primers used for genotyping

Primer	Sequence	Expected product size (bp) (WT/Mut)
α3 KO		500/200
WT/Mut forward	5′-GTGGATCCCTCCGGCCATCTTTAAGAG-3′	
WT reverse	5′-GACTGTGATGACAATGGACAAGGTGAC-3′	
Mut reverse	5′-GAGACTAGTGAGACGTGCTACTTCCATTTG-3′	
α5 KO		380/290
WT forward	5′-GTGAAAGAGAACGACGTCCGC-3′	
WT reverse	5′-GCCTCAGCCCCTGAATGGTAG-3′	
Mut forward	5′-CTTTTTGTCAAGACCGACCTGTCCG-3′	
Mut reverse	5′-CTCGATGCGATGTTTCGCTTGGTG-3′	
α7 KO		440/750
WT/Mut forward	5′-CCTGGTCCTGTGTGTTAAACTGCTTC-3′	
WT reverse	5′-CTGCTGGGAAATCCTAGGCACACTTGAG-3′	
Mut reverse	5′-GACAAGACCGGCTTCCATCCGAGTAC-3′	
α9 KO		383/480
WT forward	5′-CTGGACCTACAATGGAAACC-3′	
WT reverse	5′-GATCTCTGCCACCATTAGCT-3′	
Mut forward	5′-CTGTGCTCGACGTTGTCACT-3′	
Mut reverse	5′-CGAGCTCTAGAGAATTGATC-3′	
β2 KO		250/400
WT/Mut forward	5′-CTCTGACTGTAAAGGCAGTGGTTGCTATAG-3′	
WT reverse	5′-TAGCTATTGACGACGTCTTTAAGATCC-3′	
Mut reverse	5′-GAGACTAGTGAGACGTGCTACTTCCATTTG-3′	
β4 KO		300/150
WT/Mut forward	5′-TGTAGAGCGAGCATCCGAACA-3′	
WT reverse	5′-TCTCTACTTAGGCTGCCTGTCT-3′	
Mut reverse	5′-AGTACCTTCTGAGGCGGAAAGA-3′	

WT, wild-type; Mut, mutant.

In the chemotaxis AGKOS assay, KCs in KGM were loaded into a 3-mm well in agarose gel, as described above, incubated overnight (to allow cells to settle), after which a chemoattractant diluted in 10 μl PBS was inoculated in a 2-mm well cut on one side of the 3-mm well (Fig. 1B). The incubation was continued for 10 days with daily changes of KGM and chemoattractant. In control experiments, diffusion of the chemoattractant solution through the agarose gel was visualized using 1% methylene blue solution. Some human KCs were first transfected with AsOs and then exposed to a chemoattractant. After migration was terminated, a blueprint of the outgrowth was obtained and used to compute the directional migration distance (DMD). As shown in Fig. 1B, to standardize measurements, two segments in addition to the median segment B_1B_4 were drawn through the center of megacolony at the 30° intervals in the direction of the chemoattractant well. The DMD was computed in μm using the following formula:

$$DMD = (A_1B_1 + A_2B_2 + A_6B_6) - (A_3B_3 + A_4B_4 + A_5B_5) / 3 .$$

In the chemokinesis and the chemotaxis AGKOS assays of the effects of signal modifiers and the chemokinesis assays of AsOs-transfected human KCs and $nAChR^{-/-}$ murine KCs, we used the HC-3-pretreated cells whose migration was maintained by constant stimulation with CCh.

To control for possible changes in the rate of KC proliferation that could affect measurements of migration distances, we exposed some KCs in AGKOS plates to test compounds in the presence of the growth-arresting agent mitomycin C at 10 μg ml^{-1}. Although the cell numbers were significantly decreased in mitomycin-C-treated cultures, the migration distance did not differ from that in the control cultures that did not receive mitomycin C (P>0.05).

To visualize the nAChRs expressed on the cell surface of KCs situated in the chemotaxis AGKOS plates, the cells were fixed for 3 minutes in 3% freshly depolymerized paraformaldehyde that contained 7% sucrose, thus avoiding cell permeabilization, washed and incubated overnight at 4°C with an anti-receptor antibody and then for 1 hour at room temperature with the appropriate secondary, FITC-conjugated antibody. The fluorescence was examined with an Axiovert 135 fluorescence microscope (Carl Zeiss). Dual immunofluorescence labeling with an antibody to α3, or α7, nAChR and the integrin β_1 was analysed using the high-resolution deconvolution motorized system microscope Olympus Bx61 (Olympus America, Melville, NY) and the SlideBook 3.0.1 software (Intelligent Imaging Innovations, Santa Monica, CA). The specificity of antibody binding was demonstrated by omitting the primary antibody or by replacing primary antibody with an irrelevant antibody of the same isotype and species as the primary antibody, as detailed elsewhere (Grando et al., 1993b; Ndoye et al., 1998).

The phenotype of KCs moving under agarose was characterized immunohistochemically by counting in the outgrowth the number of cells specifically stained with antibodies to certain cell state and differentiation markers. In both untreated (control) and cholinergic-agent-treated (experiment) AGKOS plates, approximately 4-9% of KCs were positively stained for Ki-67, 8-14% for PCNA, 3-5% for filaggrin, 5-7% for loricrin and 91-99% and 4-8% for the cytokeratins 5 and 10, respectively, indicating that migrating KCs that compose the outgrowth are predominantly undifferentiated basal cells that ceased to divide, as would be expected because KC locomotion and division are independent (Woodley et al., 1993).

The effects of the 10-day exposures to test compounds on KC viability were investigated by the trypan-blue dye-exclusion assays. Except for the HC-3-treated cultures in which endogenous ACh was substituted with nicotinic or muscarinic agonists, none of the experimental conditions used affected cell viability. The number of the trypan-blue dye-positive KCs in experimental and control cultures ranged from approximately 7% to 11%.

Statistics

The results of quantitative experiments were expressed as means±s.d. Significance was determined using Student's *t* test.

Results

Studies of cholinergic control of KC chemokinesis

Diverse effects of nicotinic agonists on random migration of human KCs

The nicotinic agonists epibatidine, nicotine and choline produced diverse effects on RMD of intact human KCs that were dose dependent (Fig. 2A). Although epibatidine stimulated migration, showing maximal effect at 10^{-7} M (P<0.05), both nicotine and choline inhibited migration and these inhibitory effects became significant (P<0.05) starting from 10^{-7} M nicotine and 10^{-3} M choline. At lower doses of choline, the RMD values were slightly increased (P>0.05). These results suggested that different subtypes of the 'neuronal' nAChR types expressed in KCs exhibit differential regulation of crawling locomotion.

Endogenous ACh is essential for KC migration

Human KCs synthesize and secrete ACh (Grando et al., 1993b). Addition of the polyvalent ACh congener CCh (1 mM) to human KCs in AGKOS plates did not alter their RMD (P>0.05) (Fig. 2B), suggesting that the amount of ACh secreted by KCs in the culture medium was sufficient to saturate all KC ACh receptors. To determine whether endogenously produced and secreted ACh is essential for KC migration, we exposed cells to the metabolic inhibitor of ACh synthesis HC-3 (20 μM). In the presence of HC-3, KC migration was completely blocked (P<0.05), which could be abolished by the presence of exogenously added ACh (data not shown) or the acetylcholinesterase-resistant mixed nicotinic-and-muscarinic agonist CCh (Fig. 2B). These data indicate that synergistic stimulation of KCs through both the nicotinic and muscarinic signaling pathways is essential for KC survival.

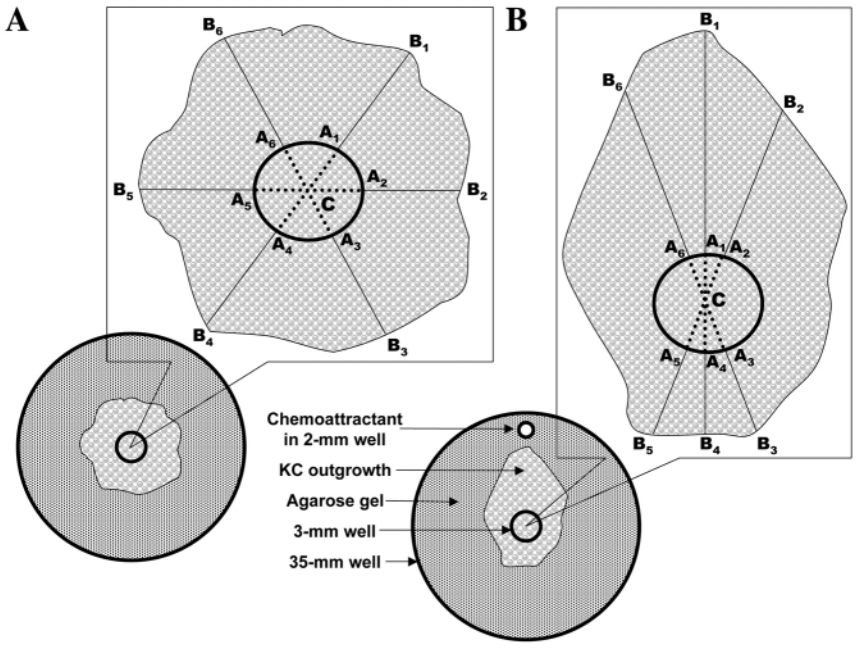

Fig. 1. Schemes of the AGKOS plates used for studying cholinergic effects on KC migration. (A) Chemokinesis AGKOS assay. (B) Chemotaxis AGKOS assay.

Receptor-selective nicotinic antagonists produce diverse effects on random migration of human KCs

To identify the nAChRs that mediate nicotinergic control of KC crawling locomotion, we tested the effects of subtype-preferring nAChR antagonists. Whereas strychnine (an inhibitor of KC α9 nAChRs) produced no significant changes in RMD (P>0.05), the inhibitors of α3- and α7-containing channels altered KC motility in diverse ways (Fig. 2B). The selective antagonist of α7 nAChR αBTX (1 μM) significantly (P<0.05) increased RMD. In marked contrast to this, α3 antagonists decreased RMD (P<0.05). The preferring blocker of the α3β2 channel αCtxMII (100 nM) was the most efficient inhibitor of KC migration, decreasing RMD by 75%. The RMD values of KCs exposed to αCtxMII were significantly (P<0.05) less than those of KCs exposed to the non-selective inhibitors of the neuronal types of nAChRs mecamylamine (50 μM) and tubocurarine (50 μM) and the preferring blocker of the α3β4 channel αCtxAuIB (1 μM). These results suggested strongly that nicotinergic stimulation of KC chemokinesis is predominantly mediated by the signaling pathway downstream of α3β2.

nAChR-selective AsOs reveal distinct roles of nAChR types in mediating nicotinergic chemokinesis of KCs

We used nAChR-specific AsOs to inhibit α3, α5, α7, α9, β2 or β4 subunit expression in migrating human foreskin KCs. The uptake of AsOs by cultured KCs was confirmed in experiments using FITC-conjugated AsOs (data not shown). Maximum inhibition of nAChR protein expression was achieved after 72 hours of incubation, as determined by western-blot analysis of KC proteins harvested at different time points. Transfection with different anti-receptor AsOs decreased the relative amounts of respective nAChR subunits in the range 82-92% (Fig. 2C).

AGKOS assays showed that transfection of KCs with receptor-selective AsOs, but not control oligonucleotides, altered their RMD. Functional inactivation of α7 nAChR significantly (P<0.05) upregulated the CCh-dependent KC migration. Inhibition of expression of α3, as well as β2 and β4 subunits (both of which can contribute to the α3-containing channels), significantly (P<0.05) downregulated migration (Fig. 2D). A decrease of RMD values of KCs transfected with AsOs-β2 significantly (P<0.05) exceeded that of KCs transfected with AsOs-β4. Functional inactivation of α5 expression only slightly altered KC migration (P=0.05). Transfection of KCs with a combination of subunit-selective AsOs further supported this supposition. The RMD values of KCs transfected with a combination AsOs-β2 and AsOs-β4 with or without AsOs-α5 did not

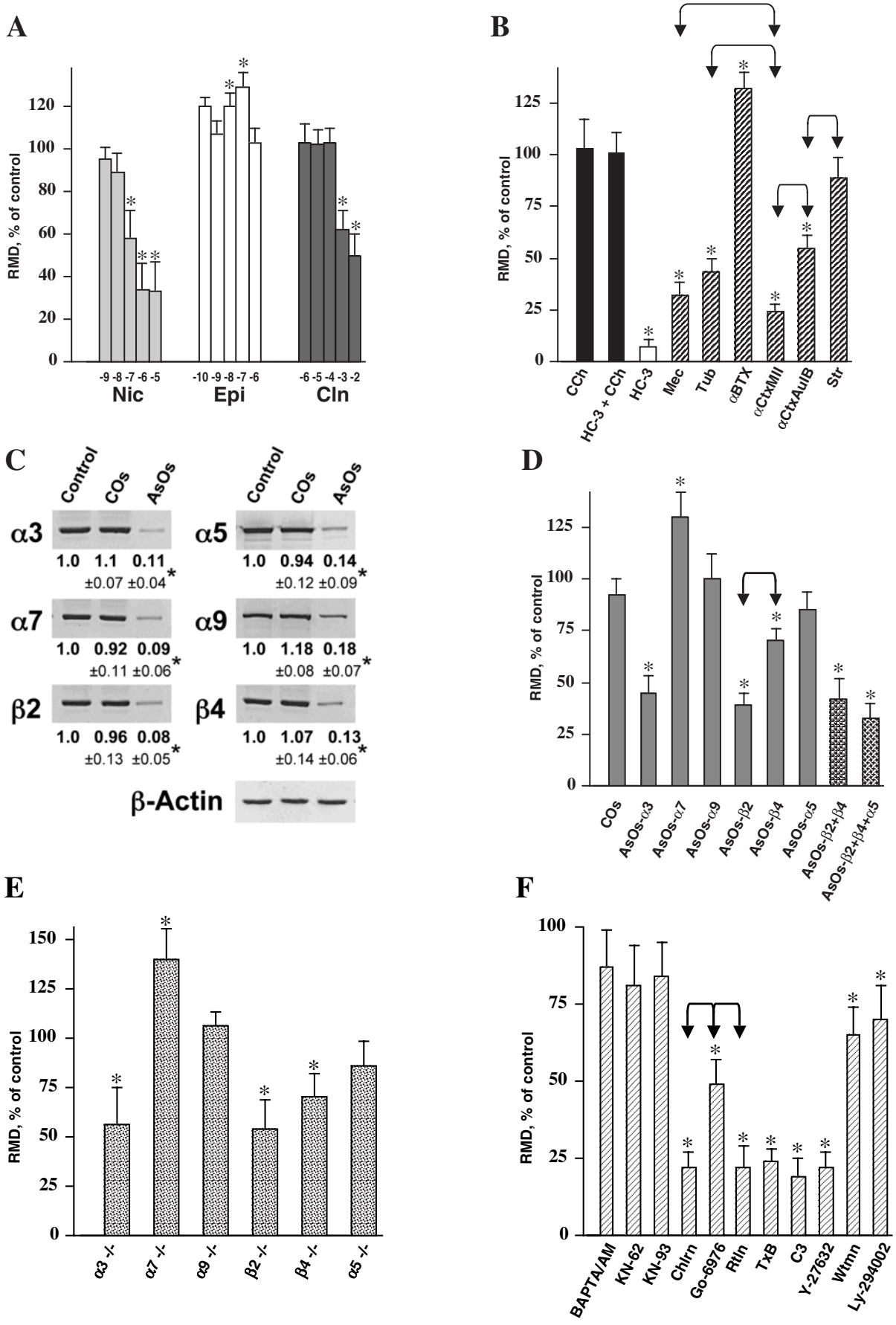
A
RMD, % of control
Nic
Epi
Cln
B
RMD, % of control
CCh
HC-3 + CCh
HC-3
Mec
Tub
αBTX
αCtxMII
αCtxAuIB
Str
C
Control
COs
AsOs
α3
1.0 1.1 0.11
±0.07 ±0.04*
α5
1.0 0.94 0.14
±0.12 ±0.09*
α7
1.0 0.92 0.09
±0.11 ±0.06*
α9
1.0 1.18 0.18
±0.08 ±0.07*
β2
1.0 0.96 0.08
±0.13 ±0.05*
β4
1.0 1.07 0.13
±0.14 ±0.06*
β-Actin
D
RMD, % of control
COs
AsOs-α3
AsOs-α7
AsOs-α9
AsOs-β2
AsOs-β4
AsOs-α5
AsOs-β2+β4
AsOs-β2+β4+α5
E
RMD, % of control
α3 -/-
α7 -/-
α9 -/-
β2 -/-
β4 -/-
α5 -/-
F
RMD, % of control
BAPTA/AM
KN-62
KN-93
Chlrn
Go-6976
Rtln
TxB
C3
Y-27632
Wtmn
Ly-294002

Fig. 2. Cholinergic effects on KC random migration. (A) Effects of cholinergic agonists on random migration of human KCs. Second-passage foreskin KCs were loaded into the chemokinesis AGKOS plates, incubated overnight, to allow cells to settle, after which the increasing concentrations of the agonists nicotine (Nic), epibatidine (Epi) and choline (Cln) diluted in KGM vs KGM alone (control) were added. The agonist concentrations are shown on the ordinate axis as 10^{-x} M. The plates were incubated for 10 days with daily refreshing of the culture medium. The random migration distance (RMD) was measured in μm (Fig. 1A). Triplicate experiments were performed with KCs from each cell donor and the combined results were averaged. An asterisk denotes statistical significance (P<0.05) compared with control, taken as 100%. (B) Different effects of cholinergic antagonists on random migration of human KCs. Second-passage foreskin KCs were loaded into the chemokinesis AGKOS plates and incubated in KGM (baseline migration distance) or in KGM containing test agents. The medium containing test agents was replaced every day during the 10-day course of the migration assay. Experimental KCs were exposed to 20 μM hemicolinium-3 (HC-3), 1 mM carbachol (CCh), a combination of HC-3 and CCh, 50 μM mecamylamine (Mec), 50 μM tubocurarine (Tub), 1 μM α-bungarotoxin (αBTX), 100 μM α-conotoxin MII (αCtxMII), 1 μM α-conotoxin AuIB (αCtxAuIB) or 5 μM strychnine (Str). The results are expressed as means±s.d. of nontreated control, taken as 100%. Asterisks indicate significant (P<0.05) differences from control. Significant differences between specific experimental conditions are indicated in the graph with arrows at the top. (C) Effects of functional inhibition of nAChR expression on random migration of human KCs. Representative results of western-blot analysis of the effect of antisense oligonucleotides (AsOs) vs control oligonucleotides (COs) on the expression of α3, α5, α7, α9, β2 or β4 nAChR subunits in human KCs. Cells were seeded in 24-well plates at a density of 5×10^4 per well and incubated overnight to allow cell adherence to the dish bottom. KCs were then transfected with COs and AsOs (Table 1). The receptor bands appeared at the expected molecular weights. (D) Alterations in KC random migration because of nAChR subunit gene silencing. Second-passage human KCs were loaded into AGKOS plates, incubated for 18 hours to allow cells to settle and transfected with COs or anti-nAChR-subunit AsOs and incubated for 10 days in KGM containing 20 μM HC-3 and 1 mM CCh, with daily changes of culture medium. Functional inactivation of α3, β2 and β4 significantly (P<0.05) decreased RMD compared with the values determined in the control cultures that were exposed to HC-3 and CCh without transfection with control or experimental AsOs (taken as 100%). The results are expressed as means±s.d. of control, taken as 100%. Significant differences are indicated with asterisks and arrows at the top. (E) Effects of nAChR gene knockout on random migration of murine KCs. Second-passage KCs grown from the epidermis of at least three neonatal $\alpha3^{-/-}$, $\alpha5^{-/-}$, $\alpha7^{-/-}$, $\alpha9^{-/-}$, $\beta2^{-/-}$, $\beta4^{-/-}$ or their +/+ littermates were loaded into AGKOS plates, exposed to a combination of 20 μM HC-3 and 1 mM CCh, and incubated for 10 days, after which migration was stopped and RMD values were measured. The results are means±s.d. of RMD of wild-type KC littermates, taken as 100%. Asterisks indicate significant (P<0.05) differences from control values in each subgroup. The lack of α3, β2 and β4 significantly (P<0.05) decreased RMD, whereas lack of α7 significantly (P<0.05) increased RMD. (F) Effects of the nAChR signaling modifiers on random migration of human KCs. The migration assay of human KCs was performed in the chemokinesis AGKOS plates as described for A. The ACh synthesis was inhibited with 20 μM HC-3 and the cells were stimulated to migrate with 1 mM CCh in the absence (control) or presence (experiment) of 10 μM BAPTA/AM, 10 μM KN-62 or KN-93, 1 μM chelerythrine (Chlrn), 1 μM Gö-6976, 5 μM rottlerin (Rtln), 100 pg ml^{-1} toxin B (TxB), 10 μg ml^{-1} C3 exoenzyme (C3), 5 μM Y-27632, 100 nM wortmannin (Wtmn) or 10 μM Ly-294002. The results are expressed as means±s.d.% of the control cultures that were not exposed to signal modifiers, taken as 100%. Asterisks indicate significant (P<0.05) differences from control. Significant differences between specific experimental conditions are indicated in the graph by arrows at the top.

significantly differ from those of KCs transfected with AsOs-β2 alone (P>0.05) (Fig. 2D). Taken together, these results indicated that α3 nAChR and α7 nAChR play opposing roles in nicotinergic control of KC chemokinesis.

Effects of nAChR gene KO on KC random migration

The physiological relevance of nicotinergic effects on KC crawling locomotion was investigated in chemokinesis AGKOS plates loaded with murine KCs lacking the nAChR subunit α3, α5, α7, α9, β2 or β4 vs wild-type KCs. In keeping with the results obtained with nAChR antagonists and AsOs, the lack of α7 channels was associated with an increase of CCh-dependent KC migration by approximately 25% (P<0.05) (Fig. 2E). The $\alpha3^{-/-}$ and $\beta2^{-/-}$ KCs exhibited minimal migration. The RMD of KCs deficient in the β4 subunit was also decreased (P<0.05). These results confirmed the important role of α3-containing nAChRs, predominantly α3β2, in mediating nicotinergic control of KC chemokinesis and further demonstrated an inhibitory role of α7-containing nAChRs.

Signaling pathways mediating nicotinergic chemokinesis of KCs

To gain a mechanistic insight into nicotinergic control of KC motility, we pharmacologically blocked key steps of the signaling pathways known to mediate chemokinesis. Chelation of intracellular free Ca^{2+} with 10 μM BAPTA/AM or inhibition of CaMKII with either 10 μM KN-62 or 10 μM KN-93 slightly decreased CCh-dependent KC migration (P>0.05) (Fig. 2F). The PI3K inhibitors wortmannin (100 nM) and Ly-294002 (10 μM) decreased RMD by approximately 25% (P<0.05). The preferential inhibitor of the PKC-α and -β isoforms Gö-6976 (1 μM) caused approximately 50% inhibition of KC migration. The most profound inhibitory effect, however, was exhibited by the isoenzyme-nonselective PKC inhibitor chelerythrine (1 μM) and the PKC-δ-preferring inhibitor rottlerin (5 μM). The RMD values in the presence of these inhibitors were significantly (P<0.05) lower than those of KCs treated with Gö-6976 and controls. These results suggested that PKC-δ is a key mediator of the CCh-dependent KC chemokinesis.

Effector molecules mediating nicotinergic chemokinesis of KCs

The CCh-dependent migration of KCs could be abolished equally efficiently by 100 pg ml^{-1} TxB, which inhibits Rho, Rac and Cdc42 (Just et al., 1996), and the selective Rho inhibitor C3 (10 μg ml^{-1}) (P<0.05) (Fig. 2F). To discern the role of Rho-mediated pathway, some KCs were exposed to the ROK inhibitor Y-27632 (5 μM). A decrease of RMD in KCs treated with Y-27632 was similar to that induced by TxB and C3, pointing toward an important role of the Rho/ROK pathway in the signaling cascade mediating CCh-dependent KC chemokinesis.

Studies of cholinergic control of KC chemotaxis

Nicotinic agonists are chemotactic for human KCs

To determine whether nAChR agonists are chemoattractive for human KCs, we measured directional migration toward

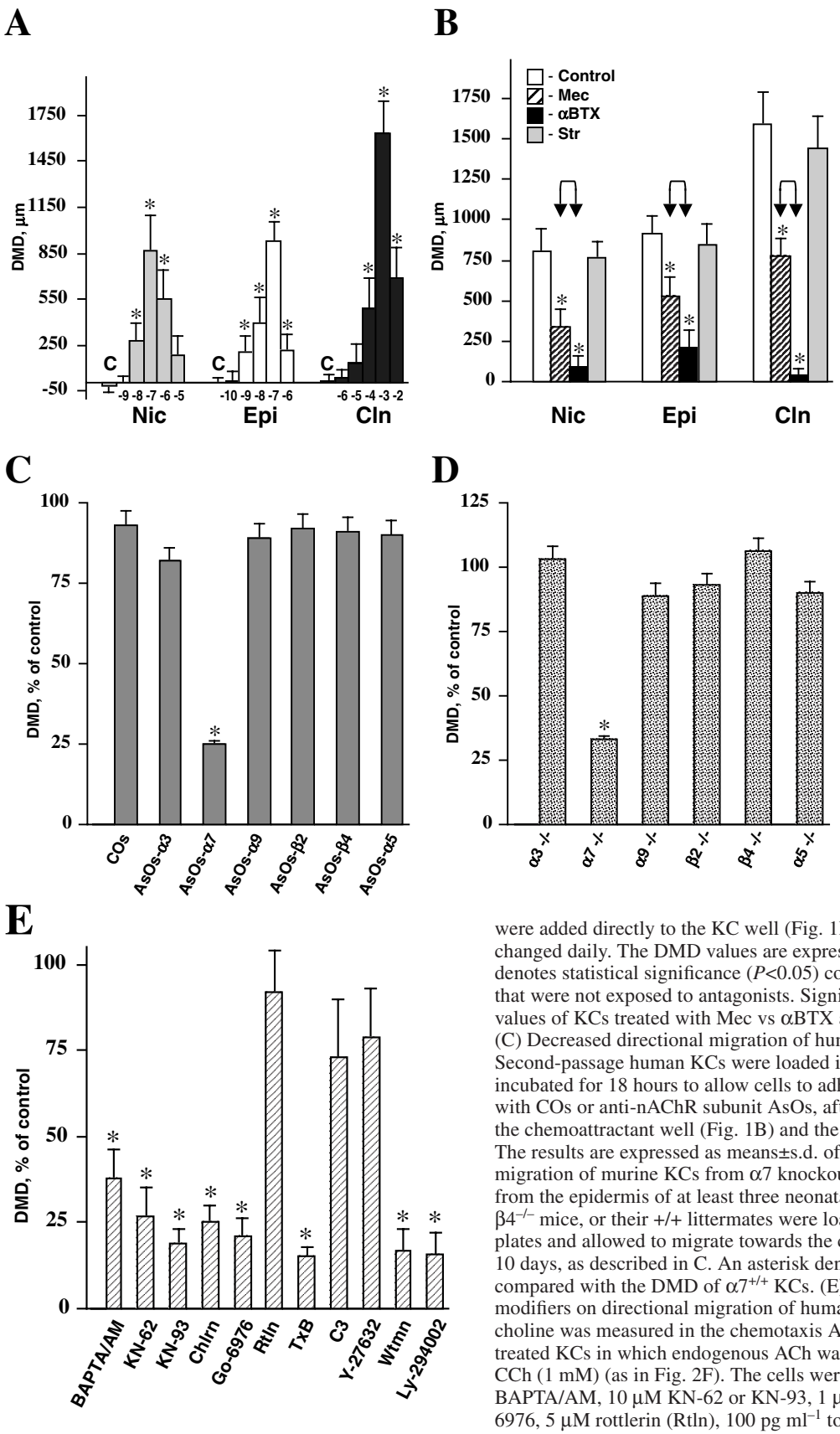

Fig. 3. Cholinergic effects on KC directional migration. (A) Chemotactic effects of cholinergic agonists on human KCs. Second-passage foreskin KCs were loaded into the chemotaxis AGKOS plates and incubated overnight to allow cells to settle, after which increasing concentrations of the agonists nicotine (Nic), epibatidine (Epi) and choline (Cln) diluted in PBS vs PBS alone (control; C) were added to the chemoattractant well (Fig. 1B). The agonist concentrations are shown on the ordinate axis as 10^{-x} M. The plates were incubated for 10 days with daily refreshment of the chemoattractant solution. A statistically significant (P<0.05) increase in the directional migration distance (DMD) was observed starting at 10 pM nicotine, 1 pM epibatidine and 0.1 mM choline. The DMD of control and experimental cells are expressed as means±s.d. µm. An asterisk denotes statistical significance, P<0.05, compared with control. (B) Subtype-selective antagonists of nAChRs exhibit differential inhibitory effects on directional migration of KCs. The chemotaxis of human KCs was elicited using the most efficient concentration of each agonist shown in A. The antagonists 50 µM mecamylamine (Mec), 1 µM α-bungarotoxin (αBTX) and 5 µM strychnine (Str) were added directly to the KC well (Fig. 1B), being dissolved in KGM that was changed daily. The DMD values are expressed as means±s.d. µm. An asterisk denotes statistical significance (P<0.05) compared with DMD of the control KCs that were not exposed to antagonists. Significant differences between DMD values of KCs treated with Mec vs αBTX are indicated by arrows at the top. (C) Decreased directional migration of human KCs with silenced α7 nAChR. Second-passage human KCs were loaded into the chemotaxis AGKOS plates, incubated for 18 hours to allow cells to adhere to the dish bottom and transfected with COs or anti-nAChR subunit AsOs, after which 1 mM choline was added to the chemoattractant well (Fig. 1B) and the incubation was continued for 10 days. The results are expressed as means±s.d. of control. (D) Decreased directional migration of murine KCs from α7 knockout mice. Second-passage KCs grown from the epidermis of at least three neonatal $\alpha3^{-/-}$, $\alpha5^{-/-}$, $\alpha7^{-/-}$, $\alpha9^{-/-}$, $\beta2^{-/-}$ or $\beta4^{-/-}$ mice, or their +/+ littermates were loaded into the chemotaxis AGKOS plates and allowed to migrate towards the concentration gradient of choline for 10 days, as described in C. An asterisk denotes statistical significance (P<0.05) compared with the DMD of $\alpha7^{+/+}$ KCs. (E) Effects of the nAChR signaling modifiers on directional migration of human KCs. The chemokinetic response to choline was measured in the chemotaxis AGKOS plates using HC-3 (20 µM) treated KCs in which endogenous ACh was substituted by exogenously added CCh (1 mM) (as in Fig. 2F). The cells were fed with KGM containing 10 µM BAPTA/AM, 10 µM KN-62 or KN-93, 1 µM chelerythrine (Chlrn), 1 µM Gö-6976, 5 µM rottlerin (Rtln), 100 pg ml^{-1} toxin B (TxB), 10 µg ml^{-1} C3 exoenzyme (C3), 5 µM Y-27632, 100 nM wortmannin (Wtmn) or 10 µM Ly-294002. The results are expressed as means±s.d. of untreated control, taken as 100%. Asterisks indicate significant (P<0.05) differences from control.

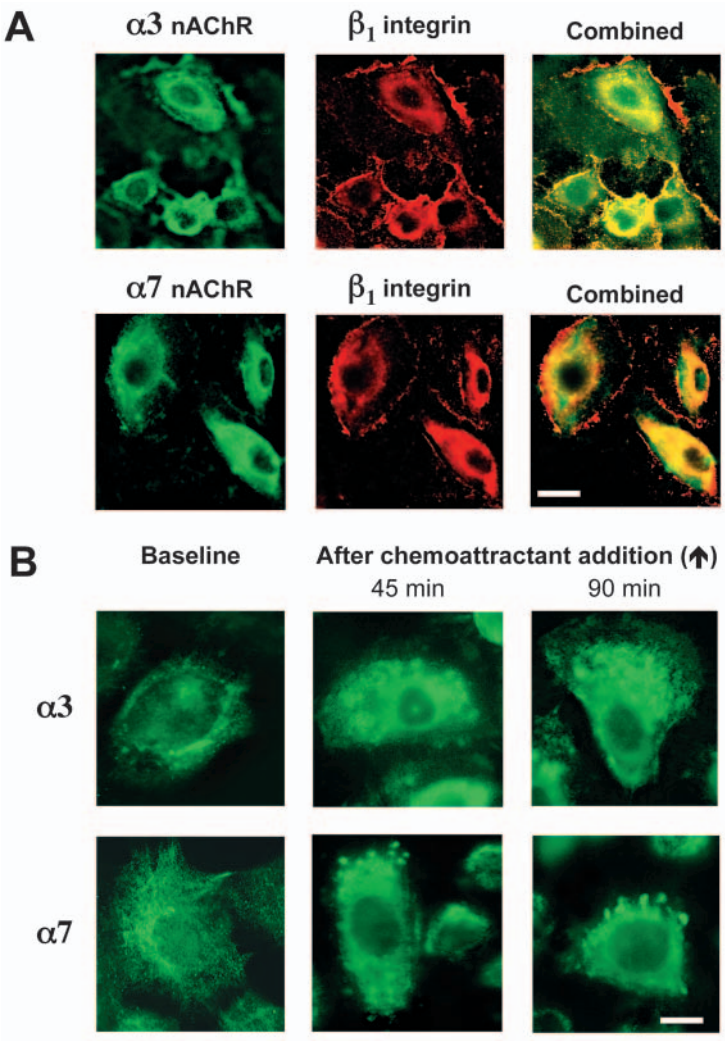

Fig. 4. Immunolocalization of α3 and α7 nAChRs in chemotaxing KCs. Colonies of second-passage human foreskin KCs grown on coverslips inserted underneath the agar gel of the chemotaxis AGKOS plates (Fig. 1B) were fixed to avoid cell-membrane permeabilization. (A) Dual immunolabeling with antibody to nAChR subunit and β1 integrin analysed by deconvolution microscopy. The images show cell-membrane colocalization (yellow) of α3 or α7 nAChR subunit (green) with the integrin β1 (red). The images of nAChR subunits and β1 integrin were acquired in a single plane. Bar, 30 μm. (B) Immunostaining with the α3- or α7-specific rabbit antibodies before and 45 minutes and 90 minutes after addition of 1 mM choline to the chemoattractant well. The vertical arrow indicates the position of the chemoattractant well. Notice that the haphazard pattern of receptor distribution on the cell membrane of intact KC changes after exposure to a chemoattractant. The α7 receptor accumulated at the leading edge (lamellipodium), decorating the filopodia (anterior cytoplasmic spikes). The α3 receptor immunoreactivity was abundant at the frontal cell area behind the leading edge. Specific staining was eliminated when the primary rabbit anti-receptor antibody was omitted or when the rabbit antiserum was preincubated with the peptide used for immunization. No immunostaining was observed when the KCs were treated with pre-immune sera obtained from the same rabbits (data not shown). Bar, 25 μm.

concentration gradient of the agonists nicotine, epibatidine and choline using a chemotaxis AGKOS assay (Fig. 1B). All three agonists induced KC chemotaxis, with choline being the most powerful chemoattractant (Fig. 3A). At the highest concentration of each agonist, the chemoattractive effect was attenuated, suggesting a role for receptor desensitization.

Receptor-selective nicotinic antagonists exhibit differential effects on nicotinergic chemotaxis of KCs

In a series of experiments testing the effects of nicotinic antagonists, KC chemotaxis was stimulated with a single, most efficient dose of each agonist. The chemotaxis could be abolished in the presence of the antagonists of the neuronal-type nAChR mecamylamine and αBTX ($P<0.05$) but not the α9-preferring blocker strychnine ($P>0.05$) (Fig. 3B). This observation indicated that type(s) of ACh-gated ion channels other than α9 nAChR mediated nicotinergic chemotaxis of KCs. As seen in Fig. 3B, the α7-preferring antagonist αBTX inhibited nicotinergic chemotaxis more efficiently than mecamylamine ($P<0.05$). Taken together, these results suggested strongly that α7-containing nAChR plays a central role in mediating nicotinergic chemotaxis of KCs. Therefore, in all subsequent chemotaxis experiments, we used the α7 agonist choline to induce directional migration of KCs.

α7-nAChR-selective AsOs inhibit nicotinergic chemotaxis of KCs

Transfection of human KCs with AsOs-α7 decreased DMD by approximately 75% ($P<0.05$) (Fig. 3C). The DMD values of KCs transfected with AsOs targeting other nAChR subunits (α3, α5, α9, β2 and β4) did not significantly differ from those of KCs transfected with control AsOs or intact KCs ($P>0.05$). Thus, α7-containing channels mediate nicotinergic chemotaxis of human KCs.

Effects of nAChR gene KO on nicotinergic chemotaxis of KCs

The use of nAChR-subunit KO mice in chemotaxis AGKOS assays further demonstrated the importance of α7 signaling in directional migration of KCs (Fig. 3D). Null mutation of the *Acra7* gene caused an approximately 70% decrease of DMD values ($P<0.05$). Lack of other types of nAChR subunits did not significantly alter the ability of KC to move toward the concentration gradient of choline ($P>0.05$) (Fig. 3D).

Signaling mechanisms of nicotinergic chemotaxis of KCs

To elucidate the signaling pathways linking activation of α7 nAChR to the subcellular machinery of directional migration, we studied the impact of inhibition of the intracellular signaling pathways on DMD of human KCs moving toward choline gradient in the chemotaxis AGKOS plates. In these cells, endogenous ACh was substituted with CCh to allow comparison of the results with those determined in similar experiments using the chemokinesis AGKOS assay (Fig. 2F).

The chemotaxis was significantly ($P<0.05$) inhibited by the chelator of intracellular free Ca^{2+} BAPTA/AM, the CaMKII inhibitors KN-62 and KN-93, the PI3K inhibitors wortmannin and Ly-294002, and the PKC inhibitors chelerythrine and Gö-6976, but not by rottlerin (Fig. 3E). These results suggested that nicotinergic chemotaxis of human KCs is regulated via the Ca^{2+}-dependent pathway that involves CaMKII, PI3K and certain PKC isoenzymes, and that PKC-δ is not involved.

Effector molecules mediating nicotinergic chemotaxis of KCs

The multipotent inhibitor of small GTPases TxB significantly ($P<0.05$) reduced chemotaxis of human KCs toward choline (Fig. 3E). To elucidate the role for Rho/ROK cascade in KC chemotaxis, we exposed cells to C3 and Y-27632. The observed changes in the DMD values did not reach significance ($P>0.05$), indicating that in a chemotaxing KC, Rac and Cdc42 are the major effectors downstream of α7 nAChR and arguing against an important role for Rho in this signaling pathway.

Relocation of α7 nAChRs to the leading edge of a chemotaxing KC

Using high-resolution deconvolution microscopy, we found that, on the KC plasma membrane, the α3 and α7 nAChR subunits colocalize with the integrin β1 (Fig. 4A). Before addition of a chemoattractant, both α3 and α7 had haphazard distribution on the KC plasma membrane (Fig. 4B). Some 30-45 minutes after chemoattractant had been added to the system, when most KCs started to extend their cytoplasmic aprons (lamellipodia) crowned by filopodia towards the direction of a chemoattractant, the α3 immunoreactivity became most abundant at the cell front behind the leading edge, leaving the leading edge unstained. By marked contrast, the α7 immunoreactivity localized to tips of the edge of the cell facing the chemoattractant (filopodia), even though, in most KCs, the full extension of the pseudopodia or acquisition of the classical migratory phenotype (such as the crescent shape) could not be fully appreciated at this point in time (Fig. 4B). These patterns of α3 and α7 expression on the cell membrane of the chemotaxing KCs became more apparent some 90-120 minutes after addition of the chemoattractant (Fig. 4B). The above observations indicated that relocation of α7 channels to the pole of the cell facing chemoattractant precedes extension of the pseudopodia, suggesting a role for α7 in rerouting the cell toward the direction of a chemoattractant.

Discussion

The results obtained in this study identify the types of nAChR that regulate chemokinesis and chemotaxis of KCs, and define signaling pathways mediating each function. The α3 nAChR and α7 nAChR played opposing roles in nicotinergic control of KC chemokinesis, which was predominantly mediated by signaling pathway downstream of α3β2 and involved PKC-δ and Rho/ROK-dependent events. The KC α7 nAChR played a central role in mediating nicotinergic chemotaxis via the Ca^{2+}-dependent pathway, which involved CaMKII, PI3K, conventional PKC isoenzymes and the Rac/Cdc42 pathway.

The advantage of AGKOS assay for 10 days is twofold: it measures a large cell population response (~50,000 cells) in a milieu that approximates a physiological one. In wounded skin, KCs are thought to migrate both individually and as a cellular sheet (Donaldson and Mahan, 1988). In the AGKOS assay, which uses standard plastic dishes, KCs move over the extracellular matrix proteins (i.e. integrin-receptor ligands) laid down by cultured KCs themselves (Marchisio et al., 1991; Nickoloff et al., 1988). Using AGKOS assay, we have previously demonstrated that ACh is required for KC outgrowth initiation (Grando et al., 1993a).

The importance of nAChR-coupled signaling pathways for the physiologic regulation of the subcellular machinery of crawling locomotion has been underscored by the results of the studies involving different cell types. Interestingly, both stimulatory (Dwivedi and Long, 1989; Grando et al., 1995; Yong et al., 1997) and inhibitory (Owen and Bird, 1995; Thomas et al., 1981; Zia et al., 2000) effects have been reported, as have lack of effects (Drell et al., 2003; Grando et al., 1993a; Sasagawa et al., 1985), suggesting that either different cells express different combinations of cell surface receptors to ACh and/or in different cells the same types of ACh receptors are coupled to different signaling pathways. The reports of lack of effects of ACh and its congeners on migration suggested that one type of ACh receptors stimulates and another inhibits migration and that simultaneous activation of both groups of the receptors in a single cells results in a zero net effect on motility. The grounds for diverse effects of nicotinic agonists on KC motility were revealed in the present study.

The research results obtained in this study convincingly demonstrated that endogenously produced and secreted ACh is essential for KC migration. The metabolic inhibitor of ACh synthesis HC-3 blocks choline uptake and almost completely blocked KC movement, which could be reversed by CCh. When given alone, CCh did not significantly alter RMD, which might be explained by simultaneous activation of α3 and α7 nAChR types on the KC cell membrane. Perhaps, in an intact KC, that constantly synthesizes and releases ACh (Grando et al., 1993b), simultaneous activation of different receptor subtypes allows ACh to maintain distinct receptor-mediated signaling mechanisms in a state of dynamic equilibrium, as a baseline. Alterations in the migration rate are achieved by skewing the dynamic equilibrium of KC control by ACh owing to selective activation or inactivation of specific receptor subtypes coupled to stimulation or inhibition, respectively, of crawling locomotion. This hypothesis is supported by a significant increase of RMD by either epibatidine, which has the high affinity for α3β2±α5 nAChRs (Wang et al., 1996a), or the α7 blocker αBTX and a decrease of migration by the selective antagonists of α3β2 and α3β4. The important role of α3β2 was revealed by results of experiments with the neurotoxin αCTXMII that, in addition to α3β2, also blocks the α6- and β3-containing ACh channels (Cui et al., 2003; Evans et al., 2003), which, however, are not present in human KCs. The fact that migration of KCs harvested from mice lacking α3 or β2 was inhibited less than that of KCs transfected with corresponding AsOs might be explained by a recently demonstrated backup mechanism that compensates for the inactivated ACh receptor type in a KO mouse by rerouting the physiological control of vital cell functions through alternative cholinergic signaling pathways (Arredondo et al., 2002).

The inhibitory effect of α7 on KC random migration might be mediated by biological processes activated by an increase in intracellular Ca^{2+} (Zia et al., 2000), because it can launch the terminal differentiation program (Sharpe et al., 1993) and also upregulate cell adherence to the substrate (De Luca et al., 1992; Trinkaus-Randall and Gipson, 1984), both of which can slow locomotion (Obedencio et al., 1999; Stephenson, 1982). The effect of α7 signaling on KC chemotaxis, however, was the opposite to that on chemokinesis. The following results obtained in this study demonstrate a central role that α7 nAChR plays in initiation of KC chemotaxis: (1) nicotinergic chemotaxis of KCs was most prominent toward the concentration gradient of choline, a potent agonist of α7 nAChR; (2) the α7-preferring antagonist αBTX, AsOs-α7 and α7 gene KO most efficiently diminished KC DMD; and (3) formation of lamellipodia and filopodia followed relocation of α7 to the cell pole facing a chemoattractant. The last of these observations indicates that KCs are among those cell types that can sense extracellular directional cues and respond with asymmetric changes in cell morphology and motility (Devreotes and Janetopoulos, 2003).

The results showing opposite effects of α7 nAChR on chemotaxis (stimulation) and chemokinesis (inhibition) were largely unexpected, because chemokinesis and chemotaxis are highly related events. This seeming controversy can be resolved based on the following considerations. First, it should be noted that the chemokinesis AGKOS assay measures actual distance of random migration, which was increased by inhibition of α7, whereas the chemotaxis AGKOS assay measures the relative distance of cell outgrowth toward the chemoattractant vs other directions, which was decreased by α7 inhibitors. We reported previously that long-term exposures to nicotine significantly decrease RMD of KCs (Zia et al., 2000), whereas, in this study, we found that nicotine increases DMD. Apparently, both effects were mediated by α7 nAChR that 'senses' the chemoattractant and facilitates cell reorientation at the beginning of migration while inhibiting the machinery of crawling locomotion. Thus, the differences in migration distance measurements (i.e. actual vs relative) provide a first explanation of the differential effects nicotine (and α7 nAChR) on random and directional migration of KCs.

A second explanation is based on compartmentalized effects of the cell-surface receptors coupled to regulation of cell motility, such as localized activation of protein kinases at the front of moving cells (for reviews, see Devreotes and Janetopoulos, 2003; Firtel and Chung, 2000; Merlot and Firtel, 2003). Application of high-resolution deconvolution microscopy demonstrated that, on the KC plasma membrane, the nAChR subunits colocalize with the integrin β1. This is consistent with previous reports that integrins form clusters with AChRs on the plasma membranes (Bozyczko et al., 1989; Burkin et al., 2000). The exclusion of the nucleus from the combined staining seen in Fig. 4A does not indicate cytoplasmic localization of staining, because cultured KCs are not flat. The plasma membrane enveloping the cell compartment containing nucleus is greatly elevated above that enveloping the surrounding cytoplasmic apron (Bereiter-Hahn et al., 1981).

In this study, we found clustering of α7 nAChRs at the leading edge of KCs moving towards the concentration gradient of a chemoattractant. Clustering of nAChRs has been observed at the leading edge of other types of migrating cells (Luther and Peng, 1985; Peng et al., 1993; Poo, 1981; Stollberg and Fraser, 1990; Young and Poo, 1983). Relocation of α7 channels to the pole of a KC facing the chemoattractant preceded extension of the pseudopodium. This is consistent with the observation that turning of nerve growth cones induced by ACh depends on the localized activation of nAChRs, requires the presence of extracellular Ca^{2+} and appears to be mediated by CaMKII (Zheng et al., 1994). The α7-containing ACh-gated ion channels that are sensitive to αBTX exhibit the highest measured Ca^{2+} permeability of the nAChR subtypes expressed in KCs (for reviews, see Fucile, 2004; Grando, 2001). Furthermore, activation of α7 nAChR raises the concentration of intracellular free Ca^{2+} (Sharma and Vijayaraghavan, 2001). It has been demonstrated that CaMKII is involved in chemotaxis through the pathway that involves activation of Cdc42 (Chen et al., 2003; Kitani et al., 1998). Thus, relocation of α7 to the pole of the cell facing a chemoattractant might be required for local activation of Ca^{2+}-dependent kinases and, subsequently, engagement of Cdc42 in modification of KC shape and cytoskeleton, which is a prerequisite for chemotaxis initiation. In marked contrast to this, the cell-surface distribution of α3 channels [which have only minor Ca^{2+} permeability (Fucile, 2004)] was quite different from that of α7 channels. The differences in distribution of α3- and α7-containing channels were associated with different coupling to effectors of signal transduction such as PKC-δ and RhoA downstream of α3, and CaMKII and conventional PKC isoforms, PI3K, and Rac/Cdc42 downstream of α7. These effectors have been shown to mediate signaling downstream of the neuronal type nAChRs (Damaj, 2000; Gasman et al., 1999; Jorgensen et al., 2000; Kihara et al., 2001; Sharma and Vijayaraghavan, 2002) and exhibit different regulation of chemotaxis and chemokinesis in other cell types (for reviews, see Devreotes and Janetopoulos, 2003; Firtel and Chung, 2000; Fukata et al., 2003; Merlot and Firtel, 2003; Schoenwaelder and Burridge, 1999; Wojciak-Stothard and Ridley, 2003).

PKC-δ has been reported to play a major role in initiating and sustaining cell migration (Andre et al., 1999; Keller et al., 2000; Kruger and Reddy, 2003; Li et al., 1980), and in tumor-cell metastasis (Kiley et al., 1999). The signaling pathway involving both PKC-δ and RhoA has been implicated in mediating regulation of cell motility downstream of other types of cell surface receptors, such as the leukotriene D_4, lysophosphatidic acid and bombesin receptors (Barry and Critchley, 1994; Massoumi et al., 2002). Both PKCs and Rho GTPases are known to mediate nicotinergic signaling in non-neuronal cells (Delouche et al., 1997; Gasman et al., 1999; Zidovetzki et al., 1999). Although the involvement of PKC-δ and RhoA in regulation of KC motility was independently reported (Cozzolino et al., 2003; Li et al., 2002), the newly discovered co-operation between PKC-δ and RhoA in mediating nicotinergic control of KC migration presents a novel paradigm of intracellular signaling from ACh-gated ion channels.

The essential role of PI3K in chemotaxis of different cell types (Pilkington et al., 1998; Sadhu et al., 2003) is determined by its ability to activate Rac and Cdc42 (Benard et al., 1999; Hawkins et al., 1995; Nakagawa et al., 2001). In neurons, too, α7 nAChR transduces signals to PI3K (Kihara et al., 2001).

The chemotaxis-related activity of PI3K is linked to intracellular Ca^{2+} (Siddiqui and English, 2000) and related to the activities of other Ca^{2+}-dependent kinases (CaMKII and PKC). Intracellular $Ca^{2+}$is required for Rac activation in platelets (Soulet et al., 2001). The Ca^{2+}-dependent Rac activation is dependent on the activation of a conventional PKC isoforms (Price et al., 2003). Furthermore, PKC-α has been implicated in the promotion of a migratory cell phenotype (Carnevale and Cathcart, 2003). Activation of both Rac and Cdc42 requires PKC activity (Buchanan et al., 2000; Shigeta et al., 2003).

Finally, the inverse effects of α3 and α7 on KC chemokinesis and distinct localization of these nAChR types on the cell membrane of chemotaxing cells might be also related to selective coupling of α3 and α7 to the cell motility effector molecules RhoA and Rac1/Cdc42, respectively. RhoA and Rac1/Cdc42 exert mutually antagonistic effects in the cell motility processes (for a review, see Fukata et al., 2003). RhoA induces the assembly of contractile actin-myosin filaments (stress fibers) and associated focal-adhesion complexes, Rac induces the assembly of a meshwork of actin filaments at the cell periphery, producing lamellipodia and membrane ruffling, and Cdc42 induces actin-rich surface protrusions, or filopodia (for reviews, see Fukata et al., 2003; Slater et al., 2001). In polarized motile cells, Rac and Cdc42 localize to the leading edge of the cells (Etienne-Manneville and Hall, 2001), whereas RhoA localizes mostly to the cytosol (for a review, see Fukata et al., 2003). During the nicotinergic-agent-induced exocytosis from adrenal chromaffin cells, Rho, Rac and Cdc42 play distinct role in coupling the actin cytoskeleton to the sequential steps underlying membrane trafficking at the site of exocytosis (Gasman et al., 1999). Inhibition of Rac or Cdc42 has been shown to disrupt polarity or chemotaxis in polarized epithelial cells (Kroschewski et al., 1999), fibroblasts (Nobes and Hall, 1999), T cells (Haddad et al., 2001) and macrophages (Allen et al., 1998).

The M_3 and M_4 subtypes of muscarinic ACh receptors, just like α3 and α7, produce inverse effects on cell motility (Chernyavsky et al., 2004). Their presence in KCs did not affect the outcome of experiments with CCh-treated KCs, because both M_3 and M_4 were simultaneously activated by CCh, thus preserving the physiological state of dynamic equilibrium between the inhibitory and stimulatory, respectively, muscarinic pathways. Other endogenous and exogenous chemokines, such as epidermal growth factor (Hudson and McCawley, 1998), did not alter the results because they were present in the culture medium of both experimental and control cells.

In conclusion, the results obtained in this study suggest that the nAChR types α3 and α7 are important regulators of the migratory function of KCs by triggering the activation of protein kinases and engagement of the effector GTPases RhoA and Rac1/Cdc42, respectively. These results have clinical implications because chemotaxis is central to wound healing (Abe et al., 2000; Gyulai et al., 1994; Sauder et al., 1990; Schelfhout et al., 2002; Wang et al., 1996b) and has been implicated in cancer metastasis (Moore, 2001; Murphy, 2001).

We thank D. Hsu (Department of Dermatology, University of California–Davis), for help with deconvolution microscopy. The α9 KO mice were bred from the original colony provided by D. E. Vetter (Department of Neuroscience, Tufts University, School of Medicine, Boston, MA). This work was supported by the NIH grants GM62136 and DE14173 and a research grant from Flight Attendant Medical Research Institute to S.A.G., and by NIH grant DA12661 to L.M.M.

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