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Authors

Chernyavsky, Alex I
Arredondo, Juan
Wess, Jürgen
[et al.](#)

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Novel signaling pathways mediating reciprocal control of keratinocyte migration and wound epithelialization through M₃ and M₄ muscarinic receptors

Alex I. Chernyavsky,¹ Juan Arredondo,¹ Jürgen Wess,² Evert Karlsson,³ and Sergei A. Grando¹

¹Department of Dermatology, University of California, Davis, Sacramento, CA 95817

²Laboratory of Bioorganic Chemistry, Molecular Signaling Section, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

³Section of Experimental Geriatrics, Neurotec, Karolinska Institute, 141 86 Huddinge, Sweden

To test the hypothesis that keratinocyte (KC) migration is modulated by distinct muscarinic acetylcholine (ACh) receptor subtypes, we inactivated signaling through specific receptors in *in vitro* and *in vivo* models of reepithelialization by subtype-selective antagonists, small interfering RNA, and gene knockout in mice. KC migration and wound reepithelialization were facilitated by M₄ and inhibited by M₃. Additional studies showed that M₄ increases expression of “migratory” integrins $\alpha_5\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$, whereas M₃ up-regulates “sedentary” integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Inhibition of migration by M₃ was mediated through

Ca²⁺-dependent guanylyl cyclase–cyclic GMP–protein kinase G signaling pathway. The M₄ effects resulted from inhibition of the inhibitory pathway involving the adenylyl cyclase–cyclic AMP–protein kinase A pathway. Both signaling pathways intersected at Rho, indicating that Rho kinase provides a common effector for M₃ and M₄ regulation of cell migration. These findings offer novel insights into the mechanisms of ACh-mediated modulation of KC migration and wound reepithelialization, and may aid the development of novel methods to promote wound healing.

Introduction

Cell migration is an essential step during normal and invasive growth. Proper regulation of the crawling locomotion of epithelial cells is critical during reepithelialization of skin wounds (Donaldson and Mahan, 1988). To cover the defect, keratinocytes (KCs), the stratified squamous epithelial cells comprising the epidermis, migrate laterally over the denuded dermis a few hours after wounding, and then differentiate, forming a new epidermis. KCs are thought to migrate both individually and as a cellular sheet (Donaldson and Mahan, 1988). The speed of locomotion of human KCs migrating as a sheet is $\sim 0.4 \mu\text{m}/\text{min}$ (Grando et al., 1993b). Different integrins mediate different types of interactions of KCs with the ECM. To attach to fibronectin, vitronectin, thrombospondin, and other proteins comprising the provisional matrix, crawling KCs express the $\alpha_5\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrins (Larjava et al., 1993). In contrast, the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are present at the sites of cell–cell

contacts (Marchisio et al., 1991). Thus, based on their differential expression in wounded versus intact epidermis and their relationship to KC migration, the integrin proteins expressed by KCs can be tentatively divided into two groups: “sedentary,” or nonmotile (α_2 and α_3), versus “migratory,” or motile (α_5 , α_v , and β_5) integrins. Wound KCs also express α_9 integrin, which is not maintained in cultured KCs under standard conditions (Stepp et al., 2002).

To characterize the physiologic control of crawling locomotion of KCs, we developed an *in vitro* model of skin reepithelialization, termed agarose gel KC outgrowth system (AGKOS), which allows accurate evaluation of effects of pharmacologic compounds on lateral migration of KCs (Grando et al., 1993b). AGKOS measures a large cell population response ($\sim 50,000$ cells) in a milieu that approximates a physiologic one because KCs move over the ECM proteins

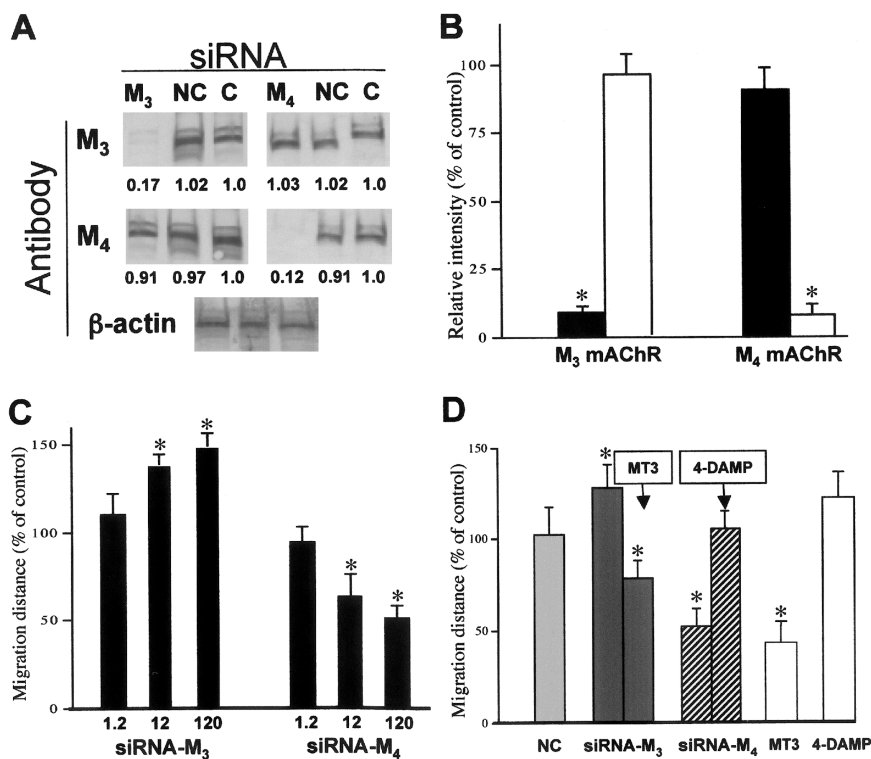
A.I. Chernyavsky and J. Arredondo contributed equally to this paper.

Address correspondence to Sergei A. Grando, Dept. of Dermatology, University of California, Davis, 4860 Y St., #3400, Sacramento, CA 95817. Tel.: (916) 734-6057. Fax: (916) 734-6793. email: sagrando@ucdavis.edu

Key words: integrins; cyclic GMP; cyclic AMP; knockout mice; acetylcholine

Abbreviations used in this paper: AC, adenylyl cyclase; ACh, acetylcholine; AGKOS, agarose gel keratinocyte outgrowth system; $[\text{Ca}^{2+}]_i$, intracellular-free calcium; cGMP, cyclic GMP; GC, guanylyl cyclase; GPCR, G protein-coupled receptor; IF, immunofluorescence; KC, keratinocyte; KGM, KC growth medium; KO, knockout; mAChR, muscarinic ACh receptor; PKA, protein kinase A; PKG, protein kinase G; ROK, Rho-associated protein kinase; siRNA, small interfering RNA; WT, wild-type.

Figure 1. In vitro experiments with anti-mAChR siRNA. (A) Representative results of Western blot analysis of the effects of siRNA-M₃ and siRNA-M₄ on M₃ and M₄ mAChR expression in human KCs transfected with anti-mAChR siRNAs or negative control (NC) siRNA. On the third day after transfection, the cellular proteins were analyzed by Western blotting. The results were compared with those obtained with control (C) KCs exposed to TransIT-TKO (Mirus Corp.) transfection reagent mixed in Opti-MEM medium (GIBCO BRL) alone. The numbers underneath the bands are ratios of the densitometry value of each mAChR receptor to that of β -actin compared with the values obtained in control KCs (taken as 1). (B) IF analysis of the effects of anti-M₃ and anti-M₄ siRNAs on the expression of the M₃ and M₄ mAChR subtypes on the cell membrane of human KCs. The cells were cultured and treated with the siRNA-M₃ (black bars), siRNA-M₄ (white bars), or negative control siRNA (not depicted) as described in A, fixed to avoid cell membrane permeabilization, and stained with anti-M₃ or anti-M₄ antibodies. (C) Dose-dependent effects of receptor-selective siRNA on KC migration. Second passage human KCs were loaded into AGKOS plates, transfected with 1.2, 12, or 120 nM of negative control M₃ or M₄ siRNA, or exposed to TransIT-TKO transfection reagent mixed in Opti-MEM medium alone (control), and then used in AGKOS assay. (D) Opposing alterations in KC crawling locomotion due to M₃ and M₄ receptor gene silencing. (B–D) Asterisk indicates significant ($P < 0.05$) differences from untreated control. NC, negative control siRNA.



produced and deposited on the dish surface by KCs during long-term incubations (Nickoloff et al., 1988; Marchisio et al., 1991). A shorter, 24-h assay that allows study of the initiation of KC outgrowth is a “scratch assay” (Savani et al., 1995). Compared with the AGKOS assay, it measures migration of a smaller, yet representative number of cells, i.e., up to 300 per a microscopic field at the magnification of 10.

Using AGKOS, we demonstrated that acetylcholine (ACh) is required to initiate outgrowth of KCs in vitro and that cholinergic drugs have profound effects on the motility of these cells (Grando et al., 1995; Zia et al., 2000). Human KCs functionally synthesize, store, release, and degrade ACh both in vivo and in vitro (Grando et al., 1993c). In the intact epidermis, KCs constantly move along an upward concentration gradient of ACh (Nguyen et al., 2001). The effects of ACh on cell motility are complex and include both stimulatory and inhibitory pathways regulating crawling locomotion (Zheng et al., 1994; Clancy and Abramson, 2000). Recent results strongly suggest that many of these effects are mediated by members of the muscarinic ACh receptor (mAChR) family (Varker and Williams, 2002; Chernyavsky et al., 2003). The mAChRs are prototypical members of the superfamily of G protein-coupled receptors (GPCRs; Caulfield, 1993; Wess, 1996). The M₁, M₃, and M₅ mAChRs are preferentially coupled to activation of pertussis toxin-insensitive G proteins of the G_{αq/11} family and stimulate phospholipase C, with resulting activation of PKC and the release of Ca²⁺ from intracellular stores. The other two mAChR subtypes, M₂ and M₄, are selectively coupled to pertussis toxin-sensitive G proteins of the G_{i/o} class and mediate the inhibition of

adenylyl cyclase (AC) activity and the activation of inward rectifier K⁺ channels. Stimulation of the M₃ mAChR has been shown to up-regulate the binding activity of β_1 integrin, which is associated with increased cell adhesion to ECM proteins (Quigley et al., 1998; Williams, 2003) and inhibited cell migration (Varker and Williams, 2002). Although G_i signaling also alters integrin activity/expression, it leads to an up-regulated cell migration (Wang et al., 1999). Signaling from both G_{αq/11}- and G_{αi/o}-coupled mAChRs alters activity of the effector proteins of Rho family, with subsequent alterations in the Rho-associated protein kinase (ROK) activity (Harhammer et al., 1996). It has been demonstrated that the Rho family GTPases are essential downstream elements in the signaling pathway, linking mAChR stimulation to regulation of cell motility (Linseman et al., 2000).

The purpose of this study was to explore the role of KC M₃ and M₄ mAChRs in regulating KC migratory function and elucidate the responsible mechanism(s). We used a combination of three overlapping approaches to inhibit mAChR-coupled signaling pathways: (1) pharmacologic blockade with receptor antagonist and regulatory enzyme inhibitors; (2) mAChR gene silencing with small interfering RNA (siRNA); and (3) mAChR gene knockout (KO) in mice. We found that ACh stimulates the crawling locomotion of KCs by activating M₄ receptors, but inhibits this activity through stimulation of M₃ receptors. The M₃ effects were mediated through the intracellular Ca²⁺-dependent guanylyl cyclase (GC)-cyclic GMP (cGMP)-protein kinase G (PKG) signaling upstream from the Rho-ROK pathway. The M₄ effects resulted from inhibition of the inhibitory pathway AC-cAMP-pro-

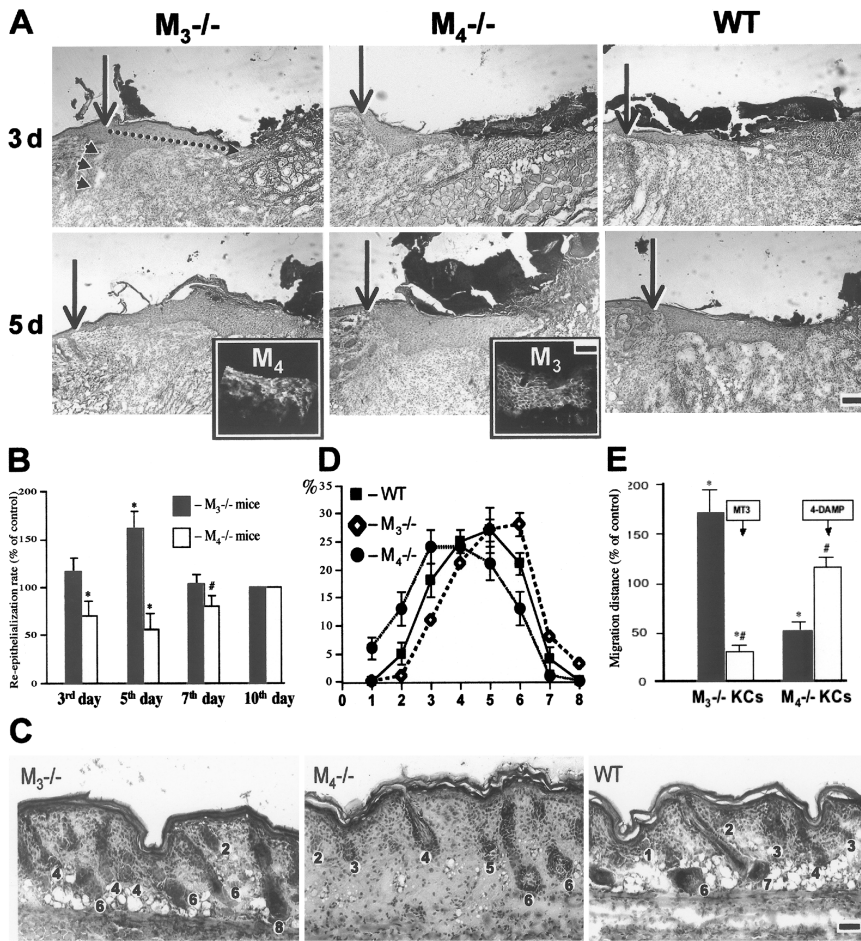


Figure 2. Effects of mAChR gene KO on crawling locomotion of murine KCs.

(A) The representative images of wound tissue of WT and mAChR KO mice harvested on the third and fifth day after wounding. The edge of the full thickness wound extending from the epidermis down to the dermis is depicted by arrowheads. The direction of forward movement of the epithelial tongue from the excision site (arrow) is indicated with a dotted line with an arrowhead. Bar, 150 μ m. Insets show immunolocalization of M₃ and M₄ on the epithelial tongues of M₄^{-/-} and M₃^{-/-} mice, respectively, on the fifth day after wounding. Bar, 20 μ m. (B) Alterations in epithelialization rate of skin wounds in M₃^{-/-} and M₄^{-/-} mice. The data are means \pm SD of the rate of epithelialization computed in six M₃^{-/-} and M₄^{-/-} mice compared with that determined in six WT animals, taken as 100%. *, significant ($P < 0.05$) differences from the epithelialization rate in WT mice. #, $P = 0.05$. (C) Representative images of hair follicles in the skin of 1-d-old WT and mAChR KO mice. An apparent stage of morphogenesis is shown as numbers underneath each hair follicle. Hematoxylin and eosin staining of cryostat sections of the longitudinal sections harvested from the upper back skin. Bar, 50 μ m. (D) Quantitative morphometric analysis of hair follicle development in mAChR KO mice. The percentage of hair follicles in each stage of morphogenesis (x axis) was determined in 1-d-old WT, M₃^{-/-}, and M₄^{-/-} mice. (E) Alterations of migration rate of KCs from M₃^{-/-} and M₄^{-/-}

mice in AGKOS plates. The results are means \pm SD of the migration distance of intact WT KCs (taken as 100%). *, significant ($P < 0.05$) differences from the migration distance of WT KCs; #, significant ($P < 0.05$) differences from the migration distances of nontreated KCs in each subgroup.

tein kinase A (PKA). The opposing mechanistic effects of M₃ and M₄ were accompanied by their reciprocal actions on the expression levels of specific integrins. These findings may aid the development of novel muscarinic drugs that are useful in facilitating wound healing.

Results

Effects of mAChR subtype-selective siRNA on the migration distances of human KCs

Transfection with anti-M₃ siRNA decreased the relative amount of M₃ receptor protein by \sim 83% without altering M₄ receptor expression and, vice versa, the anti-M₄ siRNA decreased the amount of M₄ receptor protein by \sim 88% without affecting M₃ receptor expression (Fig. 1 A). Very similar findings were obtained in semiquantitative immunofluorescence (IF) assays (Fig. 1 B).

The siRNA-M₄ significantly ($P < 0.05$) decreased and the siRNA-M₃ significantly ($P < 0.05$) increased KC migration distance in a dose-dependent manner compared with that exhibited by KCs treated with negative control siRNA sequences (Fig. 1 C). The enhanced migration of siRNA-M₃-treated KCs could be inhibited when the cells were fed with serum-free KC growth medium (KGM) containing 10 nM

of the M₄-preferring antagonist MT3. Conversely, the inhibited migration of the siRNA-M₄-treated KCs could be prevented in the presence of 10 nM 4-DAMP, which blocks M₃ receptors as well as other mAChR subtypes with high affinity (Dorje et al., 1991; Fig. 1 D).

When given alone, 10 nM MT3 reduced the migration distance of cultured human KCs by $>$ 50% ($P < 0.05$), whereas 4-DAMP did not produce any significant effect ($P > 0.05$), as could be expected when the two receptors, M₃ and M₄, which exhibit reciprocal effects on KC migration, are blocked simultaneously (Fig. 1 D).

To elucidate the role of mAChRs in regulation of KC migration speed, we used a scratch assay that measures predominantly linear migration (Savani et al., 1995). The results obtained through both assays were always coherent.

Effects of M₃ and M₄ mAChR gene KO on KC migration in vivo and in vitro

1-cm² excisional wounds were inflicted in the back skin of M₃^{-/-}, M₄^{-/-}, and wild-type (WT) mice. The mice were killed 3, 5, 7, or 10 d later, and the rate of epithelialization was assessed in specimens of wound tissue stained with hematoxylin and eosin. The representative images used for quantitative histomorphometry, illustrating the time course of wound reepithelialization, are shown in Fig. 2 A. The mor-

phometric analysis revealed that on the third day after wounding, $M_3^{-/-}$ mice exhibited a small increase ($P > 0.05$), whereas $M_4^{-/-}$ mice showed a significant ($P < 0.05$) decrease of epithelialization rate (Fig. 2 B). These changes became more pronounced on the fifth day after wounding, when the epithelialization rate in $M_3^{-/-}$ mice increased by $\sim 60\%$ ($P < 0.05$) and that in $M_4^{-/-}$ mice decreased by $\sim 50\%$ ($P < 0.05$).

To eliminate possible confounding effects of proliferation and apoptosis on the measurements of epithelialization rate through morphometric analysis, both proliferating and apoptotic KCs were visualized within the epithelial tongues covering the wounds using antibody to the proliferation marker Ki-67 and the TUNEL technique, respectively. We found no statistical differences ($P > 0.05$) between the numbers of positive cells among the mutant and WT mice. In each strain, the Ki-67-positive KCs constituted $\sim 10\%$ of total cells seen in a microscopic field at the magnification of 10, and that of TUNEL-positive KCs never exceeded 5%.

Taken together, these data indicated that the early stage of wound epithelialization, mediated by crawling locomotion of KCs (Woodley et al., 1993), was facilitated in the absence of M_3 and delayed in the absence of M_4 receptors.

To elucidate differences in hair follicle morphogenesis, a physiological process where massive KC migration occurs, we performed quantitative morphometric analysis of hair follicle development in mAChR KO mice. 1-d-old $M_3^{-/-}$ pups displayed accelerated hair follicle morphogenesis, as could be judged from a significantly ($P < 0.05$) increased number of hair follicles in advanced stages of perinatal follicle morphogenesis (stages 6–8) and a reciprocal decrease of the percentage of follicles at the early stages of their development (stages 1–3) compared with WT controls (Fig. 2, C and D). In marked contrast, the $M_4^{-/-}$ neonates showed a retardation of hair follicle morphogenesis evidenced by a significant ($P < 0.05$) increase in the number of hair follicles at the immature developmental stages and a corresponding reduction of the percentage of hair follicles at more mature stages (Fig. 2, C and D).

The roles of M_3 and M_4 mAChRs in regulating KC crawling locomotion were further investigated in AGKOS plates loaded with KCs grown from the epidermis of WT and $M_3^{-/-}$ and $M_4^{-/-}$ mutant mice. As expected, the lack of M_3 receptors was associated with an increase of KC migration distance by $\sim 85\%$ (Fig. 2 E). The $M_4^{-/-}$ KCs showed a significantly reduced migration distance (by $> 50\%$; $P < 0.05$). 10 nM of the M_4 -preferring antagonist MT3 significantly ($P < 0.05$) decreased the migration distances of $M_3^{-/-}$ KCs, but had no effects on the migration distance of $M_4^{-/-}$ cells (not depicted). 10 nM 4-DAMP increased the migration distance of $M_4^{-/-}$ KCs compared with nontreated $M_4^{-/-}$ cells ($P < 0.05$).

Role of intracellular-free calcium ($[Ca^{2+}]_i$) in the signaling pathways mediating reciprocal effects of M_3 and M_4 on KC migration

Because elevation of $[Ca^{2+}]_i$ can mediate both M_3 (Carroll and Peralta, 1998) and M_4 (Ransom et al., 1991) signaling, we sought to determine if the inhibitors of Ca^{2+} ATPase in endoplasmic reticulum with thapsigargin or cyclopiazonic acid alter the stimulatory effect of siRNA- M_3 and the inhibi-

tory effect of siRNA- M_4 on KC migration. The siRNA- M_3 - or siRNA- M_4 -transfected versus intact KCs were exposed to either 1 μ M thapsigargin (Fig. 3) or 20 μ M cyclopiazonic acid (not depicted) dissolved in KGM for 30 min daily during the 10-d duration of the migration assay in AGKOS plates. Mobilization of $[Ca^{2+}]_i$ produced a mild inhibitory effect on migration distance of intact KCs ($P > 0.05$), abolished an up-regulated migration of siRNA- M_3 -transfected KCs ($P < 0.05$), decreasing it slightly below the normal level ($P > 0.05$), and produced no significant alterations of the down-regulated migration of siRNA- M_4 -transfected cells ($P > 0.05$).

Role of the GC-cGMP-PKG pathway in the mechanism of M_3 -dependent inhibition of KC migration

Among the known signaling mechanisms engaged upon M_3 -dependent mobilization of $[Ca^{2+}]_i$, a possible role for the PKC-dependent pathway in mediating the inhibitory effect of M_3 on KC migration has been ruled out by the results showing that although activation of M_3 inhibits cell migration, the direct activation of PKC with phorbol 12-myristate 13-acetate actually stimulates migration (Varker and Williams, 2002). Therefore, we investigated the role of the cGMP cascade, which is an alternative pathway that can be engaged in the muscarinic signaling upon mobilization of $[Ca^{2+}]_i$ (Michikawa et al., 1998). Treatment of intact or thapsigargin-pretreated KCs with 10 μ M of the GC inhibitor LY-83,583, significantly ($P < 0.05$) up-regulated KC migration (Fig. 3). These results indicated that the GC-dependent activation of cGMP cascade is engaged in the signaling pathway linking M_3 to inhibition to KC migration downstream from the $[Ca^{2+}]_i$ mobilization step. This hypothesis was tested in an AGKOS assay in which the siRNA- M_3 -transfected KCs were incubated in the presence of 5 μ M of the cGMP analogue 8-pCPT-cGMP, which significantly ($P < 0.05$) decreased an up-regulated migration distance of siRNA- M_3 -transfected KCs ($P < 0.05$). Because 8-pCPT-cGMP is a selective activator of PKG type I α , I β , and II (Sirotkin et al., 2000), we tested a role for the PKG-mediated signaling pathway downstream from M_3 -dependent cGMP production. PKG was irreversibly blocked with the selective inhibitor of PKG type I and II Rp-8-pCPT-cGMP (Butt et al., 1994). In the presence of 1 μ M Rp-8-pCPT-cGMP, intact KCs migrated significantly ($P < 0.05$) farther than nontreated control cells. However, this up-regulated migration could be abolished by 10 μ g/ml C3 exoenzyme as well as by 5 μ M of the ROK inhibitor Y-27632 (Fig. 3). These findings indicated that in the signaling cascade linking M_3 to inhibition of KC migration, the PKG activation step is situated proximally to the interception with the RhoA-ROK pathway maintaining KC migration.

Role of the AC-cAMP-PKA pathway in the mechanism of M_4 -dependent stimulation of KC migration

The immediate effect of M_4 activation is inhibition of AC constitutively activated through Gs-coupled receptors, such as KC β_2 adrenergic receptors stimulated by autocrine epinephrine (Schallreuter, 1997). Activation of the cAMP signaling cascade is associated with inhibition of migration of various cell types (Johnson et al., 1972; McCawley et al., 2000). Hence, the

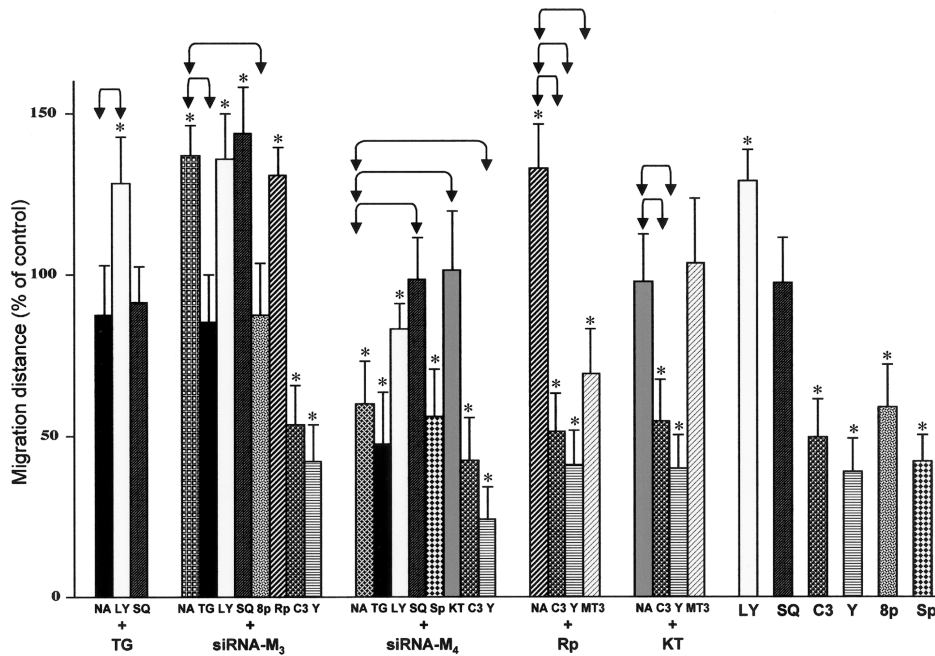


Figure 3. **Effects of the mAChR signaling modifiers on migration of human KCs.** The migration assays of intact or siRNA-M₃- or siRNA-M₄-transfected KCs were performed in AGKOS plates. The cells were exposed to 1 μ M thapsigargin (TG), 10 μ M LY-83,583 (LY), 1 mM SQ 22536 (SQ), 50 μ M Sp-8-Br-cAMPS (Sp), 5 μ M 8-pCPT-cGMP (8p), 1 μ M Rp-8-pCPT-cGMP (Rp), 25 nM KT5720 (KT), 10 μ g/ml C3 exoenzyme (C3), 5 μ M Y-27632 (Y), or 10 nM MT3 dissolved in KGM. Some cells were pretreated for 30 min with thapsigargin, Rp-8-pCPT-cGMP, or KT5720 before exposing them to a second test agent versus no additional (NA) treatment. The results are expressed as means \pm SD of nontreated control, taken as 100%. *, significant ($P < 0.05$) differences from control. Significant differences between specific experimental conditions are indicated in the graph with square brackets with arrows.

functional inactivation of M₄ that decreased KC migration could result from the inhibitory action of a Gs-coupled signaling pathway released from the M₄ control by MT3 and siRNA-M₄. The AC inhibitors up-regulated migration of siRNA-M₄-transfected KCs ($P < 0.05$), indicating that AC, rather than $[Ca^{2+}]_i$, plays a key role in the M₄-coupled signaling pathway.

To elucidate the mechanism of M₄ signaling in KCs downstream from inhibition of AC, we tested the effect of the cAMP analogue Sp-8-Br-cAMPS, which decreased the migration distance ($P < 0.05$; Fig. 3), suggesting further that M₄ stimulates KC migration by putting a brake on the signaling pathway that includes the cAMP activation step. Because Sp-8-Br-cAMPS, just like naturally occurring cAMP, exhibits its biological action via activation of PKA (Seebeck et al., 2002), we sought to determine the role for PKA in the signaling pathway linking M₄ to KC migration. 25 nM of the PKA inhibitor KT5750 increased the migration distance of siRNA-M₄-transfected KCs to the normal level ($P > 0.05$; Fig. 3). C3 exoenzyme and Y-27632 inhibited the migration of both of the KT5750-pretreated and siRNA-M₄-transfected KCs, indicating that in the signaling cascade linking M₄ to stimulation of KC migration, the Rho-ROK pathway is situated distally from the cAMP-dependent events.

Relationship between the M₃- and M₄-coupled pathways of the physiologic regulation of KC migration by ACh

We tested the effect of 10 nM of the M₄ blocker MT3 on the migration distances of KCs pretreated with Rp-8-pCPT-

cGMP or KT5750. As seen in Fig. 3, although MT3 significantly ($P < 0.05$) decreased the migration distance of Rp-8-pCPT-cGMP-pretreated cells, it had no effect on the KT5750-pretreated KCs. Thus, when the M₃-dependent inhibition of migration was blocked pharmacologically or due to the M₃ gene silencing, crawling locomotion of KCs was up-regulated owing to their unopposed stimulation through the M₄-coupled stimulatory pathway.

Effects of mAChR siRNAs on the integrin expression levels in human cultured KCs

Transfection of human foreskin KCs with siRNA-M₃ resulted in a decrease of the relative amounts of α_2 and α_3 integrins by ~ 50 and 40%, respectively ($P < 0.05$), and an increase in α_5 , α_v , and β_5 expression by ~ 30 , 100, and 70%, respectively ($P < 0.05$; Fig. 4). KCs transfected with siRNA-M₄ showed changes in the integrin expression pattern that were, in most cases, reciprocal to those observed in KCs transfected with siRNA-M₃, suggesting that knock-down of M₃ receptors favors the expression of the migratory integrins, whereas the silencing of M₄ receptors leads to overexpression of sedentary integrins.

Signaling mechanisms leading to integrin switch in human cultured KCs

We measured integrin expression in KCs migrated for 10 d in AGKOS plates in the presence of the PKG inhibitor Rp-8-pCPT-cGMP and compared it with that in KCs exposed to Rp-8-pCPT-cGMP only for 6 h, during which no appreciable migration can occur (Grando et al., 1993b). KCs

Integrin	Effects of mAChR siRNAs				Effects of mAChR Signaling Modifiers										MW		
	Control	siRNA-NC	siRNA-M ₃	siRNA-M ₄	Short term (6h)					Long-term (10 d)							
					Control	Rp	Rp + MT3	KT	KT + MT3	MT3	Control	Rp	Rp + MT3	KT		KT + MT3	MT3
α_2	1.0	0.86 ± 0.11	0.51 $\pm 0.13^*$	1.58 $\pm 0.15^*$	1.0	0.93 ± 0.08	0.99 ± 0.05	1.07 ± 0.11	1.14 ± 0.11	1.06 ± 0.14	1.0	0.55 $\pm 0.11^*$	1.22 ± 0.08	1.07 ± 0.07	0.94 ± 0.11	2.83 $\pm 0.10^*$	165
α_3	1.0	1.14 ± 0.15	0.62 $\pm 0.1^*$	1.45 $\pm 0.11^*$	1.0	0.97 ± 0.08	1.01 ± 0.08	1.05 ± 0.15	1.08 ± 0.13	1.12 ± 0.13	1.0	0.46 $\pm 0.11^*$	1.18 ± 0.10	1.0 ± 0.12	0.79 $\pm 0.06^*$	2.11 $\pm 0.08^*$	130
α_5	1.0	0.99 ± 0.21	1.33 $\pm 0.11^*$	0.84 ± 0.06	1.0	1.83 $\pm 0.12^*$	1.10 ± 0.11	1.07 ± 0.13	1.15 ± 0.09	1.07 ± 0.16	1.0	2.81 $\pm 0.06^*$	0.89 ± 0.09	1.36 $\pm 0.08^*$	1.65 $\pm 0.10^*$	0.36 $\pm 0.09^*$	135
α_v	1.0	1.18 ± 0.06	2.05 $\pm 0.23^*$	0.93 ± 0.08	1.0	2.51 $\pm 0.2^*$	1.16 ± 0.10	1.06 ± 0.13	1.02 ± 0.13	1.0 ± 0.11	1.0	3.04 $\pm 0.27^*$	0.88 ± 0.08	1.33 $\pm 0.06^*$	0.87 ± 0.13	0.26 $\pm 0.11^*$	125
β_5	1.0	1.07 ± 0.11	1.69 $\pm 0.09^*$	0.85 $\pm 0.04^*$	1.0	2.15 $\pm 0.14^*$	0.90 ± 0.11	0.93 ± 0.08	1.0 ± 0.1	1.08 ± 0.1	1.0	3.2 $\pm 0.19^*$	0.87 ± 0.15	1.00 ± 0.11	1.40 $\pm 0.1^*$	0.29 $\pm 0.11^*$	110
Actin																	42

Figure 4. **Muscarinic effects on integrin expression in human KCs.** Relative amounts of α_2 , α_3 , α_5 , α_v , and β_5 integrins were analyzed by Western blotting of total protein isolated from human KCs 72 h after transfection with siRNA-M₃, siRNA-M₄, or negative control siRNA (siRNA-NC), and from control KCs treated with TransIT-TKO transfection reagent mixed in Opti-MEM medium alone (control). Changes in the expression of integrins in human cultured KCs treated with mAChR signaling modifiers were similarly analyzed after incubation for 6 h or 10 d in the absence (control) or presence of 1 μ M Rp-8-pCPT-cGMPS (Rp), 25 nM KT5720 (KT) or 10 nM MT3, or a combinations of these agents shown in the table. The images show typical bands appearing at the expected molecular weights (MW). *, significant ($P < 0.05$) differences from control.

exposed to Rp-8-pCPT-cGMPS for 10 d showed reciprocal changes in the expression of the migratory and sedentary integrins, which included both a decrease of the relative amount of α_2 and α_3 by ~ 45 and 55%, respectively, and an increase of α_5 , α_v , and β_5 by approximately, 280, 300, and 320%, respectively ($P < 0.05$). However, significant changes in the integrin expression pattern, featured by an up-regulated expression of the migratory integrins α_5 , α_v , and β_5 ($P < 0.05$), could be seen already after 6 h of exposure (Fig. 4). The shifts in the integrin expression pattern were abolished when KCs were exposed to Rp-8-pCPT-cGMPS in the presence of the M₄ inhibitor MT3 (Fig. 4). Thus, a decrease of the sedentary integrin expression after 10 d of incubation might be secondary to outside-in signaling occurring in actively moving KCs (Longhurst and Jennings, 1998). The rapid shift in the integrin expression after 6 h of incubation suggested that signaling from M₄ up-regulates the migratory integrin gene expression, thus launching crawling locomotion of KCs.

To elucidate the role of PKA in the KC integrin shift, the cells were exposed for 6 h or 10 d to the PKA inhibitor KT5750, M₄ antagonist MT3, or a combination of both. The short-term exposure produced no significant changes in the integrin phenotype ($P > 0.05$; Fig. 4). After the long-term incubation, which provided for KC migration, the cells exposed to KT5750 demonstrated slightly increased levels of the migratory integrins α_5 , α_v , and β_5 , which could be a result of the outside-in signaling characteristic of crawling KCs. The effect of a long-term exposure to MT3 was very

similar to that of transfection with siRNA-M₄ (Fig. 4) and included both up-regulation of the sedentary integrins α_2 and α_3 , and down-regulation of the migratory integrins α_5 , α_v , and β_5 ($P < 0.05$), which is consistent with migration inhibition by both MT3 and siRNA-M₄ (Fig. 1 D). The cells exposed to both KT5750 and MT3 for 10 d had a decreased level of α_3 and increased levels of α_5 and β_5 ($P < 0.05$; Fig. 4).

Role of integrins in altered KC migration in mAChR KO mice

In WT mice, wounding down-regulated the expression of the sedentary integrins α_2 and α_3 in WT mice, as detected by Western blotting (Fig. 5 A) and semiquantitative IF (Fig. 5 B). In marked contrast, the protein levels of the migratory integrins α_5 , α_v , and β_5 in wounded skin exceeded those found in the intact skin. In M₃^{-/-} mice, the relative expression levels of all sedentary integrins were decreased in both intact and wounded skin, whereas those of migratory integrins were increased compared with WT mice (Fig. 5, A and B). In M₄^{-/-} mice, the relative amounts of the sedentary integrins α_2 and α_3 were found to be significantly ($P < 0.05$) increased in the intact skin by both Western blotting and IF. Although wounding down-regulated expression of the sedentary integrins and up-regulated α_5 and α_v expression, the β_5 integrin levels were found to be below the control values in samples from both intact and wounded skin (Fig. 5, A and B). Although the relative amounts of the integrins under consideration in KCs residing in the epithelial tongues of

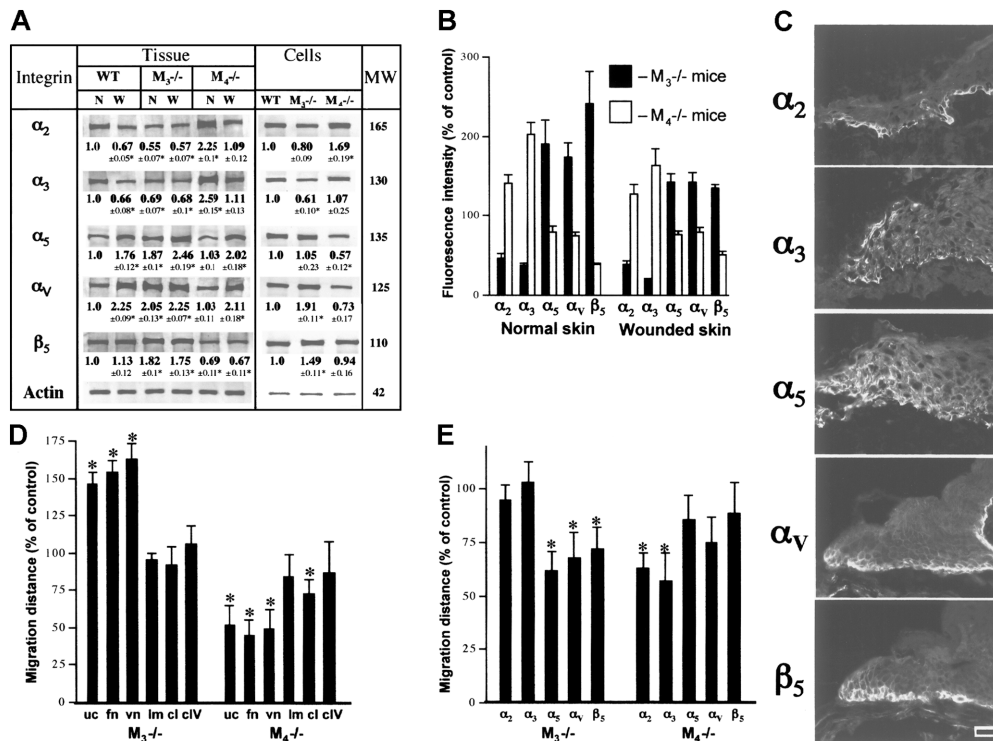


Figure 5. Role of integrins in mediating effects of M₃ and M₄ mAChRs on KC migration in vivo and in vitro. (A) Western blotting analysis of integrin gene expression. Equal samples of normal (N), i.e., intact, and wound (W) tissues were harvested from two wounds inflicted in six mice of each subgroup 24 h after wounding. Monolayers of murine KCs were established from the skin of neonatal WT and M₃^{-/-} and M₄^{-/-} mutant mice, and grown to ~70% confluence in KGM. The results are expressed as ratios of the densitometry value of each integrin to that of β-actin in the same lane, compared with the values obtained in either intact skin or KCs grown from WT mice (taken as 1). (B) Semiquantitative IF analysis of KC integrin expression in the skin of WT and M₃^{-/-} and M₄^{-/-} mutant mice. Cryostat sections of intact and wounded skin from killed mice were stained with the antibodies targeting integrins indicated on the x axis. Fluorescence intensities of normal (intact) and wounded skin in the KO mice are expressed relative to levels determined in the intact and wounded skin, respectively, of WT mice (taken as 100%). Data are means ± SD from experiments performed with the tissue from two wounds in each of six mice in each subgroup. All changes are statistically significant (P < 0.05). (C) Immunolocalization of integrin receptors in KCs comprising the epithelialization tongue of WT mice on the third day after wounding. Bar, 25 μm. (D) Effects of the mAChR gene null mutation on the ability of murine KCs to migrate over ECM proteins. The migration distance was measured using a scratch assay. The M₃^{-/-} and M₄^{-/-} versus WT (control) KCs were freshly seeded in the culture dishes either uncoated (uc) or coated with fibronectin (fn), vitronectin (vn), laminin (lm), collagen I (cl) or IV (cIV), and the formed monolayers were wounded with a 100-μl pipette tip. The amount of migration was quantitated, and the results were expressed relative to migration distance of WT KCs (taken as 100%). (E) Effects of anti-integrin antibodies on migration distance of mAChR KO and WT KCs. The migration distance was measured using a scratch assay. The M₃^{-/-} and M₄^{-/-} KCs were grown to confluence in uncoated culture dishes, and the monolayers were wounded with a 100-μl pipette tip and incubated with (experiment) or without (control) antibodies targeting integrins identified on the x axis. The amount of migration was quantitated, and the results were expressed relative to migration distance of control KCs (taken as 100%). (A, D, and E) Asterisks indicate significant (P < 0.05) differences from control.

WT versus mAChR KO mice were different, the immunostaining patterns were similar (Fig. 5 C).

We performed an additional series of experiments using single cell-type cultures of murine KCs from the epidermis of neonatal M₃^{-/-}, M₄^{-/-}, and WT mice. While M₃^{-/-} KCs featured decreased amounts of sedentary integrins and increased amounts of migratory integrins, the M₄^{-/-} KCs exhibited increased expression of sedentary integrins and decreased expression of migratory integrins (Fig. 5 A and Table I).

Next, we tested the effects of mAChR null mutations on the ability of receptor KO KCs to move over the ECM proteins that represent known ligands for the integrin types under consideration. α_vβ₆ and α₅β₁ integrin receptors preferentially bind to fibronectin, α₃β₁ and α₆β₄ bind to laminin 5, α₂β₁ binds to collagen types I and IV, and α₅β₅ binds to vitronectin (for review see Yamada et al., 1996). Compared with migration of WT KCs over fibronectin and vitronectin, the migra-

tion of M₃^{-/-} KCs was significantly (P < 0.05) up-regulated and that of M₄^{-/-} KCs was down-regulated (Fig. 5 D).

Finally, the role for integrins in providing a mechanistic link between M₃ and M₄ mAChR signaling pathways and crawling locomotion was investigated by measuring the rate of reepithelialization of in vitro wounds in the monolayers of WT and mutant KCs in the presence or absence of a specific anti-integrin antibody. Compared with WT KCs, the migration of M₃ KO cells was significantly (P < 0.05) reduced in the presence of antibodies to migratory integrins, and that of M₄ KO cells was reduced in the presence of antibodies to sedentary integrins (Fig. 5 E).

Discussion

We demonstrated for the first time that M₃ and M₄ mAChRs exhibit reciprocal effects on the lateral migration of KCs. Ac-

Table 1. Summary of changes of integrin gene expression in mAChR KO versus WT mice

Integrin	M ₃ ^{-/-}			M ₄ ^{-/-}		
	In vivo (IF)		In vitro (WB)	In vivo (IF)		In vitro (WB)
	N	W		N	W	
α ₂	↓ ^a	↓ ^a	↓	↑ ^a	↑	↑ ^a
α ₃	↓ ^a	↓ ^a	↓ ^a	↑ ^a	↑	↑
α ₅	↑ ^a	↑ ^a	↑	↓ ^a	↑ ^a	↓ ^a
α _v	↑ ^a	↑ ^a	↑ ^a	↓ ^a	↑ ^a	↓ ^a
β ₅	↑ ^a	↑ ^a	↑ ^a	↓ ^a	↓ ^a	↓

IF, immunofluorescence; N, normal (intact) skin; W, wound tissue; WB, Western blot.

^aP < 0.05 and the rest are P > 0.05 compared to WT mice.

tivation of M₃ receptors inhibited KC migration and led to the preferential expression of sedentary integrins. On the other hand, activation of M₄ receptors stimulated KC migration and favored the expression of migratory integrins. The inhibitory effect of M₃ was mediated through the [Ca²⁺]_i-dependent GC–cGMP–PKG signaling pathway. The M₄ effects resulted from inhibition of the inhibitory pathway involving the AC–cAMP–PKA pathway. Both muscarinic signaling pathways intersected at Rho, indicating that ROK provides a common effector mechanism for regulation of cell migration by ACh through M₃ and M₄ mAChRs.

In this study, we observed a pronounced inhibition of KC crawling locomotion with the M₄ receptor–selective blocker MT3 (Jolkkonen et al., 1994). Moreover, the pivotal role of M₄ receptors in facilitating KC crawling locomotion was convincingly demonstrated in experiments in which M₄ receptor expression was either knocked down with siRNA or completely abolished in transgenic M₄^{-/-} mice. Studies with anti-M₃ siRNA and M₃^{-/-} mice demonstrated that reduced or absent signaling through M₃ receptors increases KC migration. Although the siRNA-M₃ and siRNA-M₄ used in this study specifically inhibited M₃ and M₄ expression, respectively, this technology does not allow permanent gene silencing. Hence, the changes in the migration distances observed in transfected cultures might represent the initial reduction in the M₃ and M₄ levels.

Because stimulation of migration of siRNA-M₃–transfected KCs could be suppressed with the M₄ receptor–preferring antagonist MT3, the elimination of the inhibitory M₃ receptor activity apparently unmasked the stimulatory activity of M₄ receptors on the KC migratory function. It is well documented that other signaling molecules, such as growth factors, also play important roles in stimulating KC migration. Therefore, not surprisingly, compared with WT and M₃^{-/-} mice, the epithelialization of M₄^{-/-} mice was significantly delayed, but not completely blocked, as it would occur if M₄ was the only signaling pathway regulating migration in KCs. The fact that wound epithelialization was significantly different 3–5 d after wounding reflects differences in KC migration because reepithelialization is predominantly mediated by the migratory function of KCs (Woodley, 1996).

In human epidermis, M₄ receptors are predominantly expressed by the lowermost prickle cells that continuously mi-

grate upward, whereas terminally differentiated KCs lack M₄ receptors (Nguyen et al., 2001). In marked contrast, M₃ receptors are predominantly expressed by basal KCs that establish hemidesmosomal contact with the basal membrane (Ndoye et al., 1998). Hence, M₃ receptor–induced downstream signaling may support the establishment and/or maintenance of stable connections of KCs with the substrate in culture, and of the epidermis to the underlying dermis in the skin. It has been proposed that activation of M₃ receptors is coupled to both up-regulation of β₁ integrin–mediated cell adhesion (Quigley et al., 1998; Williams, 2003) and inhibition of cell migration (Varker and Williams, 2002). Therefore, the stimulation of KC migration caused by suppression of M₃ receptor signaling might be mediated by decreased attachment of KCs to ECM proteins, such as laminin, which are known to decrease crawling locomotion (Decline and Rousselle, 2001).

The mechanism of muscarinic regulation of KC motility characterized in this study is schematically shown in Fig. 6. GPCRs play an important role in mediating biological effects of different hormones, growth factors, cytokines, and cytotransmitters on cell motility. Cell migration can be increased by activating a variety of heterotrimeric GPCRs, including those for adenosine (Woodhouse et al., 1998), angiotensin II (for review see Demoliou-Mason, 1998), lysophosphatidic acid (Lummen et al., 1997), opioids (Arai et al., 1997), sphingosine 1-phosphate (Graeler and Goetzl, 2002), thrombin (Arai et al., 1997; Lummen et al., 1997), and various peptide chemoattractants and cytokines (for review see Arai et al., 1997). Some of these GPCRs stimulate G_{αq/11}, whereas others stimulate G_{αi/o}, leading to specific biological responses (Lummen et al., 1997; for review see Demoliou-Mason, 1998). For instance, stimulation of mAChRs in oligodendrocytes activates α_v integrin on the cell surface (Gudz et al., 2002).

The opposing effects of M₃ and M₄ receptor activation on KC migratory function were mirrored by reciprocal changes in the integrin gene expression patterns. An M₄-dependent shift in the integrin phenotype to a migratory pattern might be sufficient to launch KC crawling locomotion. On the other hand, a shift toward the sedentary phenotype in the KCs with inactivated M₄ activity is probably a secondary phenomenon, resulting from an outside-in signaling mechanism operating in a slowly migrating KC. It has been demonstrated that phosphorylation of paxillin and focal adhesion kinase by activation of M₃ receptors is dependent on the outside-in signaling downstream from integrin receptor binding to the ECM ligand (Rosado et al., 2000).

Results of this study strongly suggest that elevation of [Ca²⁺]_i plays a role in the signaling cascade linking M₃ to inhibited KC migration and that this cascade involves activation of the cGMP-dependent pathway. GC activation is mediated via an M₃ mAChR coupled to a pertussis toxin–insensitive G protein, whereas the GC inhibition can result from a pertussis toxin–sensitive G_{i/o} protein–coupled mAChR (Tonnaer et al., 1991; Alfonzo et al., 1998). We have demonstrated previously that activation of M₄ receptors results in a decrease in [Ca²⁺]_i, whereas activation of M₃ receptors increases Ca²⁺ in KCs (Nguyen et al., 2001), and that a nicotinic ACh receptor–mediated increase of [Ca²⁺]_i is

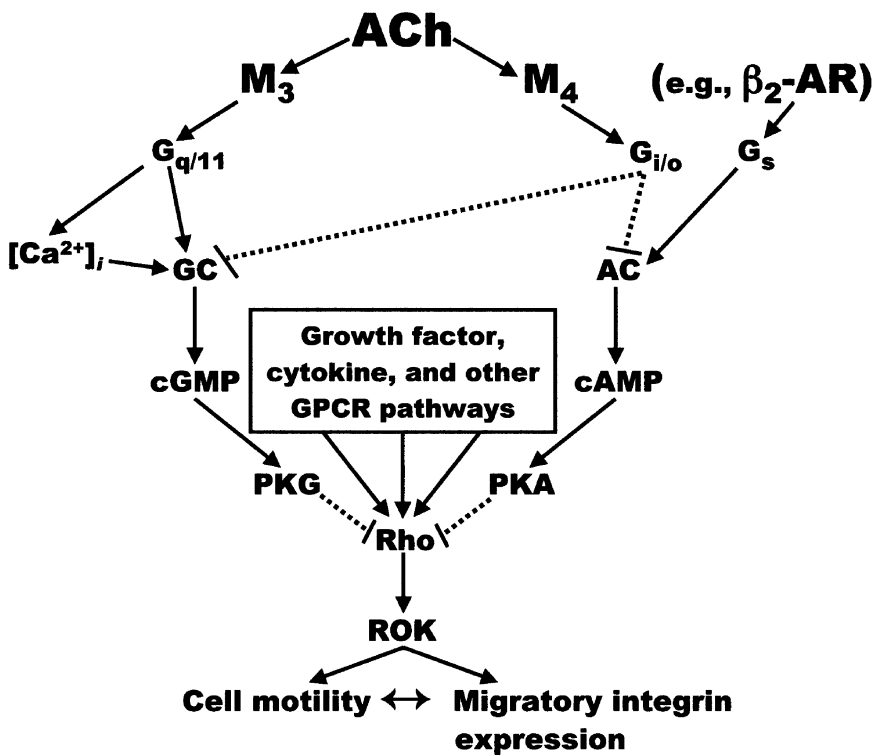


Figure 6. Hypothetical scheme of the signaling mechanisms linking M_3 and M_4 to regulation of KC motility and integrin expression. The signaling cascades originating at M_3 and M_4 include distinct stimulatory (\rightarrow) and inhibitory ($\bullet\bullet\bullet$) steps and converge at the common effector pathway involving Rho proteins.

associated with decreased migration of human KCs in AGKOS (Zia et al., 2000).

To address a possibility that the two types of mAChRs, M_3 and M_4 , exhibit reciprocal control of KC migration owing to competition of their effectors for common regulators of cell motility, Rho and ROK, we studied the effects of Rho and ROK inhibitors on the migration of intact and siRNA- M_3 - or siRNA- M_4 -transfected KCs in AGKOS plates. The obtained results indicate that regulation of the Rho-ROK pathway is a common endpoint in the signaling mechanism, mediating muscarinic control of KC migration. Because cAMP inhibits activation of Rho (Gratacap et al., 2001), mostly owing to its PKA-dependent phosphorylation (Dong et al., 1998), one of the mechanisms mediating the stimulatory effect of M_4 signaling on KC migration, in addition to inactivation of GC, could be inhibition of the cAMP cascade resulting in PKA activation. This supposition was corroborated by the ability of the PKA inhibitor KT5750 to reverse inhibited migration of siRNA- M_4 -transfected or MT3-treated KCs. Thus, the stimulatory effect of M_4 on KC migration stems from M_4 -dependent inhibition of AC activation through a G_{α_s} protein coupled by one of the KC GPCRs that are known to inhibit KC migration, such as β_2 -adrenergic receptors that can inhibit KC migration via β_2 -adrenergic receptor-mediated elevation of cAMP (Donaldson and Mahan, 1984).

In conclusion, the available data indicate that KC migration is regulated by ACh through both stimulatory and inhibitory signaling pathways, involving different populations of cell surface ACh receptors. The diversity of ACh receptors expressed by KCs in the course of their maturation would allow ACh to exert different effects at various stages of cell development. We propose that simultaneous stimulation of M_3 and M_4 receptors by endogenously secreted ACh is re-

quired to synchronize the development of ionic and metabolic events in migrating KCs. These findings offer novel mechanistic insights underlying the cholinergic control of KC crawling locomotion. They also suggest that mAChR-selective drugs and siRNA may serve as novel tools to modulate abnormal KC migration, such as in wounds that fail to heal.

Materials and methods

Chemicals and tissue culture reagents

The muscarinic receptor blocker 4-DAMP was purchased from Sigma-Aldrich. The M_4 inhibitor MT3 was prepared as detailed elsewhere (Karls-son et al., 2000). Cell permeable inhibitors of Ca^{2+} -ATPases cyclopiazonic acid and thapsigargin, competitive inhibitor of GC LY-83,583, irreversible inhibitor of AC MDL-12,330A, membrane permeant, selective inhibitor of PKG 1a, 1b and type II Rp-8-pCPT-cGMPs, selective inhibitor of PKA KT5720, membrane permeant cAMP and cGMP agonists Sp-8-Br-cAMPS and 8-pCPT-cGMP, respectively, functional inhibitor of Rho proteins C3 exoenzyme, and cell-permeable and selective inhibitor of ROK Y-27632 were purchased from Axxora, LLC, whereas the cell-permeable AC inhibitor SQ 22536 was from Calbiochem-Novabiochem. KGM containing 5 ng/ml of epidermal growth factor and 50 μ g/ml of bovine pituitary extract were purchased from GIBCO BRL. Agarose type A was obtained from Accurate Chemical & Scientific Corporation. Heat-inactivated newborn calf serum, 0.05% trypsin, trypan blue dye solution, and Wright's stain were obtained from Sigma-Aldrich. Rabbit polyclonal antibodies to α_2 , α_3 , α_5 , α_v , and β_1 integrins were purchased from Chemicon International, Inc. Anti- β -actin antibody and secondary, FITC-labeled goat anti-rabbit antibodies were from Sigma-Aldrich. The antibodies to M_3 and M_4 mAChRs that we characterized previously (Ndoye et al., 1998) are commercially available from Research and Diagnostic Antibodies. Rat anti-mouse antibody to the proliferation marker Ki-67 and a secondary, FITC-conjugated antibody were purchased from DakoCytomation. The DeadEnd™ Fluorometric TUNEL System allowing visualization of apoptotic KCs was purchased from Promega.

mAChR mutant mice

Mice deficient in M_3 or M_4 mAChRs ($M_3^{-/-}$ and $M_4^{-/-}$ mice, respectively) were generated as described previously (Gomez et al., 1999; Yamada et al., 2001). In all experiments, age- and gender-matched WT mice of the

same genetic background (129/SvEv × CF1) were used as controls. Mouse genotyping was performed by PCR analysis of mouse tail DNA.

Cultures of human and murine KCs

Human KCs were isolated from foreskin epidermis and grown in culture as detailed elsewhere (Grando et al., 1993a). Murine KCs were similarly isolated from the epidermis of WT and $M_3^{-/-}$ and $M_4^{-/-}$ mutant mice that were 1–2 d old and grown using the cell culture techniques optimized for mouse KCs (Arredondo et al., 2002).

siRNA preparation and KC transfection

To design target-specific siRNA duplexes, we selected sequences of the type AA (N_{19}) UU (N, any nucleotide) from the open reading frame of the target mRNAs to obtain a 21-nt sense and 21-nt antisense strand with symmetric 2-nt 3' overhangs of identical sequence (Elbashir et al., 2001). The siRNA- M_3 and siRNA- M_4 were custom synthesized by Dharmacon. The target sequences for the human *CHRM3* (NM_000740) and *CHRM4* (NM_000741) genes were as follows: *CHRM3*, 5'-GGTCAACAAGCAGCTGAAG-3' (corresponding to amino acids 280–300); and *CHRM4*, 5'-CCTCTACACCGTGATC-3' (corresponding to amino acids 259–279). The negative control siRNA-targeting luciferase gene was also purchased from Dharmacon. For transfection, human KCs were seeded at a density of 5×10^4 cells per well of a 24-well plate, and incubated for 16–24 h to achieve ~70% confluence. To each well, increasing concentrations (see Results) of siRNA duplex in the transfection solution were added, and the transfection was continued for 16 h at 37°C in a humid, 5% CO₂ incubator. On the next day, the transfection medium was replaced by KGM, and the cells were incubated for 72 h to achieve maximum inhibition of mAChR protein expression. After 72 h of incubation, the cells were harvested and the proteins were extracted and used in Western blotting assays of integrin expression. The siRNA transfection efficiency in KCs was also assayed using FITC-labeled luciferase GL2 duplex (Dharmacon) with the target sequence 5'-CGT ACG CGG AAT ACT TCG A-3'.

In vitro KC migration assays

AGKOS assay. Second passage human or murine KCs were suspended in KGM, counted in a hemocytometer, loaded at a high density (4×10^4 cells/10 μ l) into each 3-mm well in an agarose gel, and used in AGKOS assays as detailed elsewhere (Grando et al., 1993b; Zia et al., 2000). KCs were fed with KGM containing various concentrations of cholinergic drugs or no drugs (control), and incubated for 10 d in a humid CO₂ incubator with daily changes of medium. Some human KCs were first transfected with siRNA, and then exposed to drugs. To standardize results obtained in experiments using KCs from different donors, the mean values of the migration distances were converted into the percentage of control. The control value for KCs from each particular donor was determined by measuring the baseline migration distance (in millimeters) and taken as 100%. In parallel studies, to control for changes in the rate of KC proliferation on the migration distances measured by AGKOS assay, we exposed KCs in AGKOS plates to test compounds in the presence of 10 μ g/ml of the growth-arresting agent mitomycin C (Sigma-Aldrich).

Scratch assay. The original scratch assay (Savani et al., 1995) was adapted to measuring KC migration as detailed elsewhere (Cha et al., 1996). In brief, KCs were either grown to confluence or freshly seeded in 6-well tissue culture dishes. Some dishes were coated with fibronectin, laminin, collagen I or IV (all from BD Biosciences), or vitronectin (Sigma-Aldrich). The monolayers were scratched with a 100- μ l pipette tip and incubated at 37°C and 5% CO₂ in air until complete reepithelialization of wounded monolayers occurred in one of the cultures, but no longer than 24 h. To inhibit proliferation, for the first 2 h of incubation, KCs were fed with KGM containing 10 μ g/ml mitomycin C. Reepithelialization was documented by photography and the amount of migration was quantitated by computer-assisted image analysis software (IP Lab; Scanalytics). The residual gap between KCs migrating toward each other from the opposing sides of the in vitro wound, i.e., the area of the scratch remaining unfilled, was measured and the results were expressed as percentage of reepithelialization determined in control, nontreated monolayer. Each experiment was performed in triplicate.

In vivo wounding and morphometric assay of epithelialization rate

Because the wound healing process is dependent on the stage of the hair cycle, in each experiment we used the mice of exactly the same age and gender with hair cycles that were synchronized using the anagen induction technique (Paus et al., 1990). In brief, 6–7-wk-old WT and $M_3^{-/-}$ and $M_4^{-/-}$ mutant mice in the state of telogen, as judged by their pink skin color, were anesthetized and stripped of hair using a wax and rosin mix-

ture that was painted over the back of the mouse and after hardening was peeled off, inducing the resting follicles to enter anagen. 5 d later, full thickness excisions through the panniculus carnosus were made on the anesthetized skin using a uniform square template with a side length of 1 cm. Each animal received two wounds at the symmetric sites of the central back, 0.5 cm off the vertebral line. Wounds were left undressed and wounded animals were individually housed under aseptic conditions for 3, 5, 7, and 10 d, after which time the mice were killed by a CO₂ overdose. Equally sized pieces of the wounded and intact skin near the wound were excised. To optimize the yield of migrating KCs, we always harvested the wound border by shaving a narrow strip along the perimeter of the wound. For extraction of total proteins used in Western blotting assay, the panniculus carnosus was removed (confirmed microscopically), and tissue was weighed, diced, and sonicated. At least six animals per time point and genotype were used. The morphometric analysis on sections was always performed from the middle of the wound. The rate of epithelialization was assayed in hematoxylin and eosin-stained cryostat sections of the wound tissue by measuring the lengths of the tongues of new epithelium extending from either side of the wound, in accordance with standard protocols of morphometric analysis (Graves et al., 2001). In brief, three to five random microscopic fields were captured at a magnification of 4 in each skin section cut perpendicularly to the edge of the wound. The images were printed and the rate of epithelialization was computed by measuring directly on the prints the length of the epithelialization tongue advancing over the wound bed from its margin, which could be easily identified based on the abrupt change in the appearance of dermal ground substance at the excision line (Fig. 2 A). In each image, the length of epidermal outgrowth was measured in millimeters.

Morphometric analysis of hair follicle morphogenesis in mAChR KO mice

The morphometric analysis was performed in the longitudinal sections of dorsal skin of 1-d-old $M_3^{-/-}$ or $M_4^{-/-}$ mice versus WT littermates. The eight defined substages of hair follicle morphogenesis were identified based on the criteria defined by Paus et al. (1999). The percentage of hair follicles in each stage of morphogenesis was assessed using the established technique detailed elsewhere (Botchkarev et al., 1998). 100 follicles in each of the 50 microscopic fields (at a magnification of 10) of vertical skin sections derived from at least four different animals were calculated in each strain.

IF assay

The IF experiments were performed as detailed previously (Ndoye et al., 1998), using freshly frozen specimens of intact and wounded murine skin or cultured KC monolayers as a substrate, and computer-assisted image analysis with a software package purchased from Scanalytics. In tissue samples, the intensity of fluorescence was calculated pixel by pixel by dividing the summation of the fluorescence intensity of all pixels by the area occupied by the pixels (i.e., segment), and then subtracting the mean intensity of fluorescence of a tissue-free segment (i.e., background). For each specimen, a minimum of three different segments in at least three different microscopic fields were analyzed. The specimens were examined with a fluorescence microscope (model Axiovert 135; Carl Zeiss Microimaging, Inc.).

Western blotting assay

Proteins were isolated by adding 1.5 ml isopropyl alcohol per 1 ml TRIzol reagent (GIBCO BRL) to the phenol-ethanol supernatant of homogenates of the samples of human and murine KCs, washed, dissolved in a sample buffer, separated via 4–15% SDS-PAGE, and electroblotted onto a 0.2- μ m nitrocellulose membrane (Bio-Rad Laboratories). The membranes were developed using a chemiluminescent detection system (ECL Plus; Amersham Biosciences) and scanned (Storm™/FluorImager; Molecular Dynamics). The relative density of scanned bands was determined by area integration using ImageQuant software (Molecular Dynamics), and the results were expressed as integrated intensity of pixels of the spot excluding the background. The final results were expressed as ratios of the densitometry value of each integrin to that of β -actin in the same lane, compared with the values obtained in control samples. The protein content ratio in each control sample was always set equal to 1.

Statistics

The results of quantitative experiments were expressed as means \pm standard deviation. Significance was determined using Student's *t* test.

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