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Cornea

The Acetylcholine Signaling Network of Corneal Epithelium and Its Role in Regulation of Random and Directional Migration of Corneal Epithelial Cells

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Purpose. Because cholinergic drugs are used in ophthalmology and cholinergic stimulation has been shown to facilitate epithelialization of mucocutaneous wounds, we performed a systematic analysis of components of the cholinergic network of human and murine corneal epithelial cells (CECs) and determined the role of autocrine and paracrine acetylcholine (ACh) in regulation of CEC motility.

Methods. We investigated the expression of ACh receptors at the mRNA and protein levels in human immortalized CECs, localization of cholinergic molecules in normal and wounded murine cornea, and the effects of cholinergic drugs on CEC directional and random migration in vitro, intercellular adhesion, and expression of integrin α_V and E-cadherin.

Results. We demonstrated that corneal epithelium expresses the ACh-synthesizing enzyme choline acetyltransferase, the ACh-degrading enzyme acetylcholinesterase, two muscarinic ACh receptors (mAChRs), M3 and M4, and several nicotinic ACh receptors (nAChRs), including both α7- and α9-made homomeric nAChRs and predominantly the $\alpha3\beta2 \pm \alpha5$ subtype of heteromeric nAChRs. Wounding affected the expression patterns of cholinergic molecules in the murine corneal epithelium. Constant stimulation of CECs through both muscarinic and nicotinic signaling pathways was essential for CEC survival and both directional and random migration in vitro. Both α7 and non-α7 nAChRs elicited chemotaxis, with the α7 signaling exhibiting a stronger chemotactic effect. Cholinergic stimulation of CECs upregulated expression of the integrin and cadherin molecules involved in epithelialization. We found synergy between the proepithelialization signals elicited by different ACh receptors expressed in CECs.

Conclusions. Simultaneous stimulation of mAChRs and nAChRs by ACh may be required to synchronize and balance ionic and metabolic events in a single cell. Localization of these cholinergic enzymes and receptors in murine cornea indicated that the concentration of endogenous ACh and the mode of its signaling differ among corneal epithelial layers. Elucidation of the signaling events elicited upon agonist binding to corneal mAChRs and nAChRs will be crucial for understanding the mechanisms of ACh signaling in CECs, which has salient clinical implications.

Keywords: acetylcholine, muscarinic receptors, nicotinic receptors, corneal epithelial cells, corneal epithelialization, chemotaxis

Corneal epithelial erosion is one of the most common problems in clinical ophthalmology. The loss of continuous epithelial integrity can result in infection, stromal edema, or corneal ulcer, whereas impaired corneal wound healing may lead to a compromised ocular surface.[1,2] Most corneal abrasions heal in 24 to 72 hours and rarely progress to corneal erosion or infection. Reepithelialization of corneal defects starts with migration of corneal epithelial cells (CECs) over the denuded surface toward the center of the wound bed to initiate repair and restore epithelial integrity. No proliferation of CECs is observed during this initial phase. However, despite significant progress in understanding how the cornea heals, clinically available pharmacologic therapies that can promote repair and prevent visual complications from persistent corneal wounds remain limited.[3] Increased understanding of cellular and molecular mechanisms of corneal reepithelialization has the potential to lead to the development of new modalities of prevention and treatment for persistent corneal epithelial defects.[1]

A number of approaches have been tried previously in the clinical and experimental settings for treating corneal wounds, including use of cholinergic drugs.[4–10] Several independent studies have demonstrated that the cholinergic (i.e., parasym-

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pathetic) agonist acetylcholine (ACh), which activates both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs), the acetylcholinesterase (AChE)-resistant mixed muscarinic and nicotinic agonist carbachol (CCh), and the irreversible AChE inhibitor echothiophate, which increases tissue levels of endogenously produced ACh, can accelerate corneal reepithelialization.[4–7] The data on the proepithelialization activities of the muscarinic agonist pilocarpine and the muscarinic antagonists atropine and homatropine, which exhibit the cycloplegic/mydriatic action, are controversial. Both positive and negative effects, as well as absence of effects, have been reported.[8–11] The reason for this controversy may lie in the complexity of the nonneuronal cholinergic network that operates in the nonkeratinized stratified squamous epithelium lining the cornea, analogous to that operating in the keratinized and nonkeratinized stratified squamous epithelia enveloping the skin and oral cavity (see Ref. 12 for review).

ACh is produced by practically all types of living cells,[13] and its concentration is remarkably high in the corneal epithelium, exceeding that in the neural tissue (see Ref. 14 for review). The amount of free ACh is a function of its synthesis by choline acetyltransferase (ChAT) and hydrolysis by AChE. ACh and related compounds elicit biological effects through binding to mAChRs and nAChRs. The mAChR family is composed of five receptor subtypes, M_1 through M_5, preferentially coupled to distinct signal transduction pathways via specific G proteins. The mAChRs can be grouped according to their functionality. The M_1, M_3, and M_5 subtypes activate protein kinase C by elevating intracellular Ca^{2+} and diacylglycerol, whereas the M_2 and M_4 inhibit protein kinase A by diminishing adenylyl cyclase activity, resulting in the reduction of intracellular levels of cyclic adenosine monophosphate (see Ref. 15 for review). The nAChRs are ACh-gated ion channels that mediate the influx of Na^+ and Ca^{2+} and efflux of K^+ and elicit downstream signaling events by modulating activities of protein kinases and phosphatases (see Ref. 16 for review). The nAChR pentamers are composed of different combinations of α1 through α10, β1 through β4, γ, δ, and ε subunits. Each of α7, α8 (not found in humans), and α9 subunits is capable of forming the homomeric nAChR channels. The heteromeric receptors can be composed of various combinations of α1, α2, α3, α4, α5, α6, β1, β2, β3, and β4 subunits, e.g., α3(β2/β4) ± α5, and α9 can form a heteromeric receptor with α10.[16]

In this study, we demonstrated that (1) corneal epithelium expresses ChAT, AChE, two mAChR subtypes, and several nAChR subtypes; (2) constant stimulation of CECs through both muscarinic and nicotinic signaling pathways is essential for CEC survival and both directional and random migration; (3) the proepithelialization signals of autocrine/paracrine ACh and its muscarinic and nicotinic congeners can be implemented through activation of odd- and even-numbered mAChR subtypes and heteromeric and homomeric nAChRs; and (4) cholinergic stimulation of CECs upregulates expression of the integrin and cadherin molecules involved in reepithelialization. The potential translational significance of these observations lies in the novel approach to facilitate corneal healing.

Materials and Methods

Cells and Reagents

The telomerase-immortalized human CEC line (hTCEpi)[17] was grown in KGM-2 medium (Lonza, Inc., Allendale, NJ, USA), referred to as growth medium (GM), at 37°C in a humid atmosphere of a 5% CO_2 incubator until approximately 80% confluence and then used in experiments. The AChE-resistant, mixed muscarinic and nicotinic agonist CCh, the muscarinic agonist muscarine (Mus), the nicotinic agonist nicotine (Nic), the pan-muscarinic antagonist atropine (Atr), the preferential inhibitor of non-α7 nAChRs mecamylamine (Mec), the preferred antagonist of α7 nAChR methyllycaconitine (MLA), and the metabolic inhibitor of ACh synthesis hemicholinium-3 (HC-3) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Agarose type A was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY, USA). Mouse monoclonal antibody to human E-cadherin was purchased from BD Biosciences (Woburn, MA, USA), to human integrin α_V from EMD Millipore Corporation (Temecula, CA, USA), and AChE from Thermo Fisher Scientific (Rockford, IL, USA). The antibodies to human α1 and β1 nAChR subunits were purchased from Sigma-Aldrich Corp., antibodies to α2, γ, and ε subunit from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and antibodies to M_1 through M_5 mAChR subtypes and α3, α4, α5, α7, α9, α10, β2, β3, and β4 nAChR subunits were raised and characterized in our previous studies.[18–21] All secondary antibodies were from Sigma-Aldrich Corp.

Identification of the mAChR Subtypes and nAChR Subunits Expressed in CECs

The profile of nAChR subunits expressed in hTCEpi cells at the mRNA level was determined by us in a standard RT-PCR assay using primer sets for human M_1 through M_5 mAChR subtypes and α1 through α7, α9, α10, β1 through β4, γ, δ, and ε nAChR subunits (Operon, Alameda, CA, USA) and amplification conditions described by us elsewhere.[18,22] The hTCEpi cell lysate was used to probe the presence of ACh receptors at the protein level using an established technique.[20] The cryostat sections of normal eye tissues from BALB/c mice were stained with our M_3, M_4, α3, α5, α7, α9, β2, and β4 antibodies reacting with mouse ACh receptor proteins as well as anti-mouse ChAT (EMD Millipore Corp.) and AChE (Thermo Fisher Scientific) antibodies, using standard protocols described by us elsewhere.[20]

In-Cell Western (ICW) Experiments

The ICW, a high throughput quantitative assay of cellular proteins, was performed as described in detail elsewhere,[23] using the reagents and equipment from LI-COR Biotechnology (Lincoln, NE, USA). Corneal epithelial cells were fixed in situ, washed, permeabilized with Triton solution, incubated with the LI-COR Odyssey Blocking Buffer for 1.5 hours, and then treated overnight at 4°C with a primary antibody to human integrin α_V or E-cadherin. After that, the cells were washed and stained for 1 hour at room temperature with a secondary LI-COR IRDye 800CW antibody and Sapphire700 to normalize for cell number per well. The protein expression was quantitated using the LI-COR Odyssey Imaging System.

Cell Viability Assay

The exclusion test for trypan blue dye (TBD) was used to determine the number of viable cells present in CEC suspensions. This standard assay is based on the principle that live cells possess intact cell membranes that exclude TBD, whereas dead cells do not. The control (nontreated) and experimental CECs in suspension were mixed with 0.4% TBD (Sigma-Aldrich Corp.) in PBS, and then visually examined in a hemocytometer to determine the number of viable cells with clear cytoplasm and nonviable cells with a blue cytoplasm, as detailed elsewhere.[24]

Cell Migration Assays

A scratch assay was performed to assess reepithelialization of the corneal wound in vitro. We used our modification[25] of the original assay.[26] Briefly, confluent CEC monolayers in six-well dishes were scratched with a 100-μL pipette tip and incubated

Investigative Ophthalmology & Visual Science

Table 1. Relative Numbers (%) of TBD-Positive Cells After Incubation of CECs With HC-3 (Mean ± SD)

Incubation, h	No Drugs	CCh	Mus	Nic	Mus + Nic	HC-3 Alone	HC-3 + CCh	HC-3 + Mus	HC-3 + Nic	HC-3 + Mus + Nic
12	5.1 ± 2	8.3 ± 2	9.9 ± 2	12.0 ± 2	6.1 ± 2	7.9 ± 2	8.2 ± 2	10.2 ± 3	5.9 ± 2	7.0 ± 2
24	7.3 ± 3	7.6 ± 1	9.7 ± 2	11.0 ± 2	8.5 ± 2	10.1 ± 3	8.0 ± 2	8.3 ± 2	12.3 ± 3	10.0 ± 3
36	10.5 ± 3	5.6 ± 2	8.4 ± 2	10.2 ± 1	7.9 ± 2	40.0 ± 4*	14.4 ± 2†	20.5 ± 2*†	18.3 ± 2*†	16.0 ± 2†
48	9.2 ± 3	6.2 ± 1	8.3 ± 2	9.4 ± 1	7.5 ± 2	60.4 ± 11*	27.8 ± 6*†	30.5 ± 8*†	32.2 ± 5*†	29.8 ± 6*†
60	8.2 ± 3	10.5 ± 2	8.4 ± 2	11.3 ± 2	9.3 ± 2	80.9 ± 16*	30.2 ± 8*†	35.0 ± 9*†	27.7 ± 5*†	40.6 ± 10*†
72	12.1 ± 3	10.3 ± 2	9.4 ± 2	12.0 ± 3	10.4 ± 2	100.0 ± 19*	28.1 ± 7*†	35.3 ± 8*†	32.2 ± 5*†	50.1 ± 13*†

The TBD-exclusion test was used to determine the number of viable cells present in CEC suspensions as described in Materials and Methods. The suspended CECs mixed with 0.4% TBD solution were examined in a hemocytometer to count viable and nonviable cells. The results were expressed as percentage of TBD-positive (dead) cells in each experiment ($n = 3$). Drug concentrations used were HC-3, 20 μM; CCh, 50 μM; Mus, 1 μM; and Nic, 1 μM.

* $P < 0.05$ compared to intact control.

† $P < 0.05$ compared to HC-3 given alone.

at 37°C and 5% CO_2 in air until there was complete reepithelialization of wounded monolayer in one of the cultures, but for no longer than 24 hours. To inhibit proliferation, for the first 2 hours of incubation CECs were fed with the GM containing 10 μg/mL mitomycin C (Sigma-Aldrich Corp.). The extent of reepithelialization was documented by photography. The residual gap between CECs migrating toward each other from the opposing sides of the in vitro wound, that is, the area of the scratch remaining unfilled, was quantitated by the computer-assisted image analysis software IP Lab (Scanalytics, Fairfax, VA, USA) and the results expressed as percentage of reepithelialization determined in a control, nontreated monolayer.

Under-agarose assays were used to measure random and directional migration of CECs, in accordance with our original protocols detailed elsewhere.[27] Briefly, to evaluate cholinergic effects on random migration, CECs were suspended in GM, counted in a hemocytometer, loaded at a high density (1 × 10^4 cells/10 μL) into each 3-mm well in an agarose gel, fed with GM containing various concentrations of test drugs versus no treatment (control), and incubated for 10 days in a humid CO_2 incubator with daily changes of GM. Random migration distance, that is, the distance outward from the original 3-mm well to the leading edge, was measured at the end of each experiment and the results expressed as the percentage of control. In the chemotaxis assay, CECs in GM were loaded into a 3-mm well in agarose gel, as described above, and incubated overnight (to allow cells to settle), after which, a test cholinergic agonist diluted in 10 μL PBS was inoculated in a 2-mm chemoattractant well cut on one side of the 3-mm well containing CECs.[27] The incubation was continued for 10 days with daily changes of drug-containing solutions. After migration was terminated, a blueprint of the outgrowth was obtained and used to compute the directional migration distance. To control for possible changes in the rate of CEC proliferation that could affect measurements of migration distances in the under-agarose assays, we exposed some CECs in agarose plates to test compounds in the presence of the growth-arresting agent mitomycin C, 10 μg/mL. While 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 ($P > 0.05$).

Assay of Cell–Cell Adhesion

The effects of cholinergic drugs on spreading of the cytoplasmic aprons of CECs and formation of intercellular junctions were measured using the monolayer permeability assay detailed elsewhere.[28,29] Briefly, a confluent CEC monolayer was formed in the Costar Transwell cell culture chambers (Sigma-Aldrich Corp.) inserted into the 24-well culture plates 2 to 3 days after CECs were seeded at a cell density of 1 × 10^4/100 μL GM into the chambers and cultivated at 37°C in humid atmosphere with 5% CO_2. The monolayers were dissociated by brief (30 seconds) exposure to 0.53 mM EDTA, washed, and fed with GM containing test cholinergic drugs. After 3 hours of incubation, the permeability of the monolayer was measured by adding 100 μL GM containing [^{3}H]thymidine ([^{3}H]dT; 1 μCi/insert; 6.7 Ci/mM; Du Pont-NEN, Boston, MA, USA) to each culture. Five minutes later, 100-μL aliquots of solution containing [^{3}H]dT were taken in triplicate from each lower chamber. The more CECs are separated from each other, the more the tracer penetrates to the lower chamber through the porous membrane of the upper chamber and the higher the permeability coefficient (PC) values are obtained:

$$PC = \frac{\textit{Counts per minute in experimental culture}}{\textit{Counts per minute in control culture.}} \times 100.$$

Statistical Analysis

Each experiment was performed in triplicate or quadruplicate, the results expressed as mean ± SD, and the statistical significance determined by ANOVA with a Dunnett post hoc test using the GraphPad Prism software (GraphPad Prism Software, Inc., San Diego, CA, USA). The differences were deemed significant when the calculated P value was <0.05.

Results

Endogenous ACh Is Essential For CEC Survival in Culture

Deprivation of cultured CECs from autocrine and paracrine ACh by treating the cells with HC-3, which inhibits ACh synthesis by blocking cellular reuptake of its metabolic precursor choline,[30] decreased CEC viability measured by the TBD-exclusion assay (Table 1). The effect of HC-3 became significant ($P < 0.05$) after 36 hours of incubation. Addition of the mixed muscarinic and nicotinic agonist CCh to the GM containing HC-3 lessened the deleterious effect of the latter (Table 1). Since CCh action could be mediated through both muscarinic and nicotinic pathways of ACh signaling, we tested effects of the muscarinic agonist Mus and the nicotinic agonist Nic. Both agonists, given alone or in combination, reduced the

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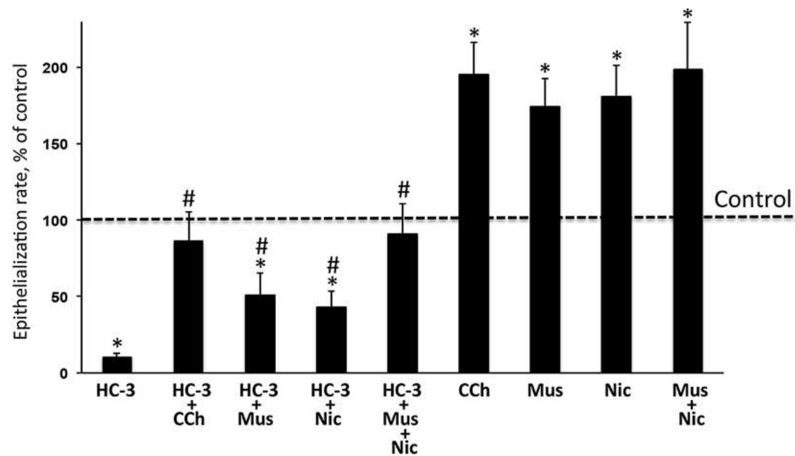

Figure 1. Requirement of autocrine/paracrine ACh for epithelialization of corneal wound in vitro. The role of autocrine/paracrine ACh in corneal epithelialization was evaluated in the scratch assay described in Materials and Methods. Briefly, confluent CEC monolayers in six-well dishes were scratched with a 100-μL pipette tip and incubated with test drugs until complete reepithelialization of wounded monolayer in one of the cultures, but no longer than 24 hours. The following drug concentrations were used: HC-3, 20 μM; CCh, 50 μM; Mus, 1 μM; and Nic, 1 μM. The results are expressed as percent of control, that is, values obtained in untreated cultures and taken as 100%. $^*P < 0.05$ compared to intact control; $\#P < 0.05$ compared to HC-3 given alone.

number of TBD-positive cells in the ACh-deprived CEC cultures (Table 1).

These results indicated that constant stimulation of CECs through both mAChRs and nAChRs is essential for CEC survival.

Autocrine/Paracrine ACh Is Essential For Cornea Reepithelialization In Vitro

To evaluate the role of autocrine and paracrine ACh in the physiological control of epithelialization of corneal defects, we employed an in vitro scratch assay that allows visualization and measurement of outward migration of CECs from the edges of a linear defect created in a confluent monolayer. To deprive CECs of their endogenously produced and secreted ACh, we employed HC-3 because the number of TBD-positive CECs exposed to HC-3 did not exceed normal levels after 24 hours of incubation (Table 1), which corresponded to the duration of the scratch assay. In the presence of HC-3, CEC migration was almost completely blocked (Fig. 1). CCh restored the ability of ACh-deprived CECs to epithelialize the defect in the monolayer (Fig. 1). Incubation of the CECs deprived of ACh in the presence of either Mus or Nic significantly ($P < 0.05$) increased cell migration rate, which, however, did not reach that of CCh-exposed cells (Fig. 1). Combining Mus and Nic increased migration to the extent seen in CEC cultures exposed to HC-3 in the presence of CCh (Fig. 1).

The agonists CCh, Mus, Nic, and a mixture of Mus and Nic significantly ($P < 0.05$) accelerated reepithelialization in the monolayers that did not receive HC-3 (Fig. 1), thus showing a feasibility to accelerate migration rate of CECs beyond the physiological level, apparently due to pharmacologic stimulation of mAChRs and nAChRs in cooperation with autocrine/paracrine ACh.

These data indicated that synergistic stimulation of CECs through both muscarinic and nicotinic signaling pathways is indispensable for normal CEC migration.

The mAChR and nAChR Subtypes Expressed in CECs

Next, we sought to identify the types of cholinergic receptors that could mediate muscarinic and nicotinic regulation of CECs. Using RT-PCR and Western blotting, we determined the expression of mAChR subtypes and nAChR subunits in CECs at the mRNA and protein levels, respectively. The results of RT-PCR assay demonstrated the presence of mRNAs encoding M_3, M_4, and M_5 mAChR subtypes, as well as α3, α4, α5, α7, α9, α10, β2, and β4 nAChR subunits (Table 2). However, the expression of M_5 mAChR and α4 and α10 nAChR subunits at the protein level could not be confirmed by immunoblotting (Table 2), indicating that genes for these receptors are either not translated and/or receptor proteins are expressed at very low (undetectable) levels.

These results demonstrated that CECs predominantly express two mAChR subtypes, M_3 and M_4, two homomeric nAChRs, α7 and α9, and the heteromeric nAChRs that can be composed of various combinations of α3, β2, β4, and α5 subunits, that is, α3(β2/β4) ± α5.

Localization of Corneal ChAT, AChE, and mAChRs and nAChRs in Intact Cornea

Both the ACh synthesizing and degrading enzymes, ChAT and AChE, respectively, were visualized in the mouse cornea, but

Table 2. The mAChR Subtypes and nAChR Subunits Expressed in Human CECs

	mAChR Subtypes					nAChR Subunits															
	M_1	M_2	M_3	M_4	M_5	α1	α2	α3	α4	α5	α6	α7	α9	α10	β1	β2	β3	β4	γ	δ	ε
RT-PCR	−	−	+	+	+	−	−	+	+	+	−	+	+	+	−	+	−	+	−	−	−
WB	−	−	+	+	−	−	−	+	−	+	−	+	+	−	−	+	−	+	−	−	−

The profile of mAChR subtypes and nAChR subunits at the mRNA and protein levels were determined in CECs grown to approximately 80% confluence using the standard RT-PCR Western blot assays, respectively, described in Materials and Methods. WB, Western blot.

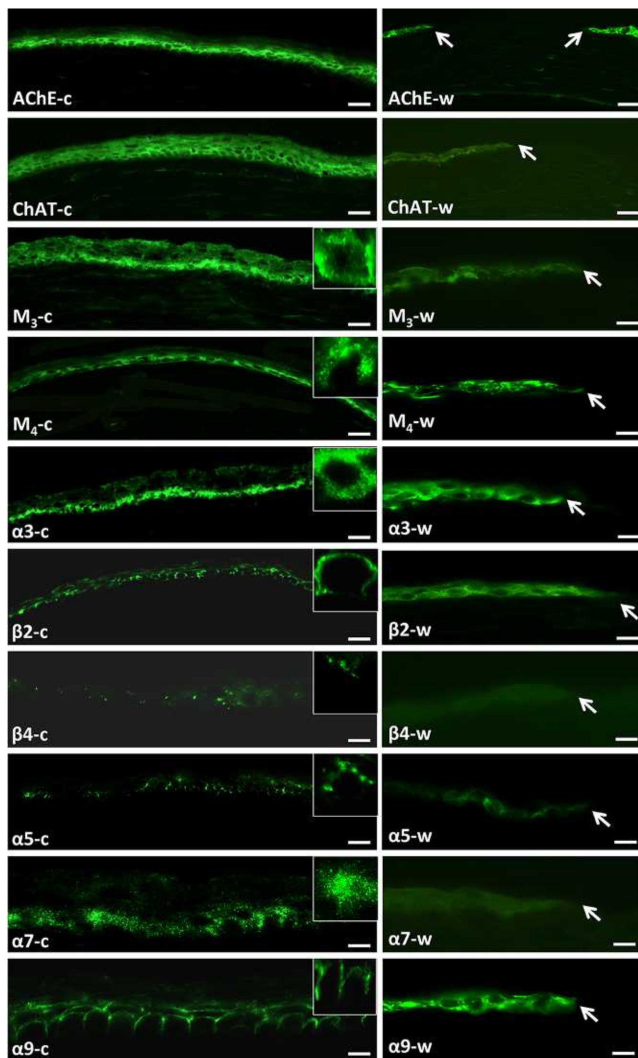

FIGURE 2. Immunolocalization of cholinergic enzymes and receptors in mouse cornea. The cryostat sections of freshly frozen control (c; *left column*) and wounded (w; *right column*) eyes were enucleated from a 8-week-old mouse 18 hours after a 2-mm diameter manual corneal debridement and stained for ChAT, AChE, and the mAChR subtypes, and nAChR subunits were found to be expressed by CECs by Western blot (Table 2). All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Binding of the primary antibodies was visualized using the corresponding secondary, FITC-labeled antibodies (see Materials and Methods). Both preincubation of the antipeptide immune sera with the synthetic peptides used for immunization and omission of the primary antibody abolished the fluorescent staining. These images are representative of those obtained in several independent experiments. Note, *insets* illustrate a typical staining pattern of individual cells with different anti-ACh receptor antibodies, and *arrows* indicate the leading edge of the neoepithelium reepithelializing corneal wound. *Scale bars*: 50 μm for AChE-w, ChAT-w, and M4-c; 30 μm for AChE-c and β2-c; 20 μm for ChAT-c, M3-c, α3-c, α5-c, M3-w, and β2-w; and 10 μm for other.

the staining patterns of these two cholinergic enzymes differed from one another. While ChAT immunoreactivity appeared to spread diffusely throughout the epithelium, the intensity of staining produced by anti-AChE antibody appeared greater in the basal cell layer (Fig. 2). The anti-AChE antibody produced both intercellular and cortical/membrane-associated staining patterns. The M_3 and M_4 mAChR antibody stainings were strongest in the basal cell layer, but M_3 antibody also stained the suprabasal epithelial cells (Fig. 2). Anti-α3 antibody also stained basal epithelial cells. The non–ACh-binding subunits capable of forming heteromeric nAChRs in combination with the α3 subunit were also visualized in the corneal epithelium, each featuring a unique expression pattern. While CEC labeling with anti-β2 nAChR antibody was localized to the basal epithelial cells, staining by anti-β4 antibody was limited to punctate regions of the epithelial cell membrane (Fig. 2, inset). The extent and intensity of staining produced by anti-α5 antibody approached that of anti-β4 antibody (Fig. 2). Both the α7 and α9 subunits, each capable of assembling a homomeric nAChR, were also visualized, showing distinct expression patterns. The anti-α7 antibody stained the entire epithelium, with stronger basal cell localization, whereas α9 was seen almost exclusively within the basal cell compartment (Fig. 2).

These results indicated that the concentration of endogenous ACh, which is a function of its synthesis by ChAT and degradation by AChE, as well as the mode of its signaling, which depends on the repertoire of mAChR and nAChR subtypes expressed by individual CECs, differ among corneal epithelial layers.

Localization of Corneal ChAT, AChE, and mAChRs and nAChRs in Wounded Cornea

Using a 2-mm-diameter limbus-to-limbus manual corneal debridement wound model,[31] we have examined expression of the cholinergic molecules 18 hours after wounding. The CECs composing the leading edge of the migrating epithelial sheet stained brightly for AChE, M_4 mAChR, α3, β2, and α9 nAChR subunits, but not for ChAT, M_3, β4, α5, and α7 (Fig. 2). An apparent increase in the AChE/ChAT ratio suggests that ACh level is decreased in the corneal wound.

These results indicated that wounding results in a change in the localization of ACh receptor subtypes at the leading edge of the migrating epithelial sheet. These changes may underlie facilitated or altered ACh signaling, suggesting a potential role in the physiological control of corneal reepithelialization.

Synergistic Muscarinic and Nicotinic Regulation of Random Migration of CECs

To evaluate involvement of the muscarinic and nicotinic pathways of ACh signaling in the physiological regulation of CEC migration, we employed the long-term under-agarose migration assay lasting 10 days. Carbachol accelerated migration by almost 2-fold, and its effect could be significantly ($P < 0.05$) decreased by the mAChR subtype nonselective antagonist Atr, as well as by Mec, a nicotinic antagonist that preferentially inhibits non-α7 nAChR subtypes (Fig. 3A). MLA—a preferring antagonist of α7 nAChR—also decreased the CCh-dependent accelerated migration, albeit insignificantly ($P > 0.05$). A combination of Atr with Mec and MLA not only completely abolished CCh effect, but significantly ($P < 0.05$) decreased CEC migration below the intact control levels (Fig. 3A). The migration rate of CECs was also significantly ($P < 0.05$) upregulated by the muscarinic agonist Mus and the nicotinic agonist Nic, and the respective antagonists abolished these effects of agonists. In contrast to Mec, MLA decreased CCh-stimulated migration insignificantly ($P > 0.05$) (Fig. 3A).

To further characterize regulation of CEC migration by endogenous agonist ACh, we treated cells with antagonists without adding an exogenous agonist. As expected, each antagonist decreased CEC migration distance. Atr and Mec significantly ($P < 0.05$) decreased random migration when given alone or in combination, whereas the inhibitory effect of MLA did not reach statistical significance (Fig. 3B).

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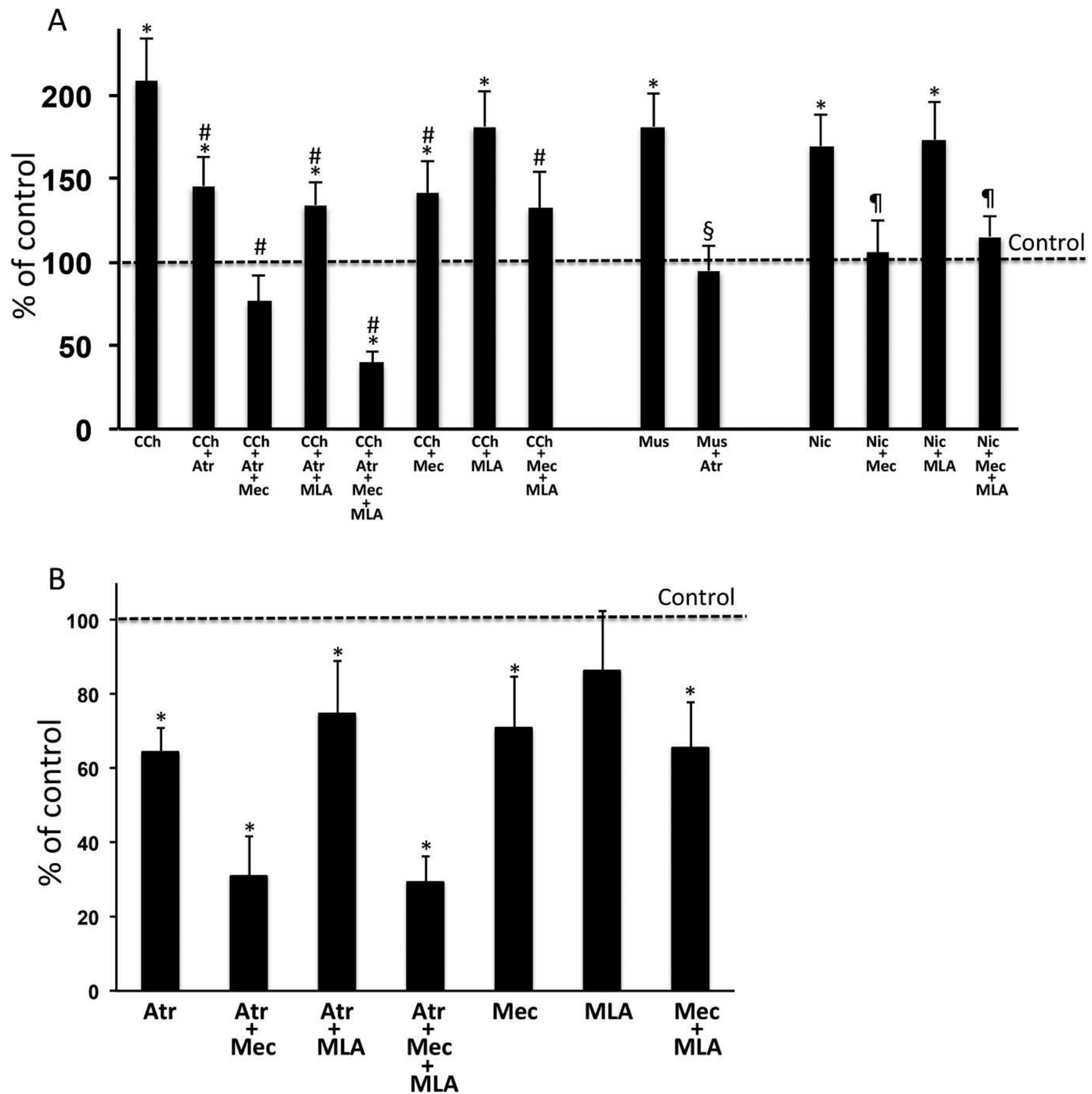

FIGURE 3. Regulation of random migration of CECs through the muscarinic and nicotinic pathways. The under-agarose migration assay was used to evaluate effects of stimulation of cholinergic receptors by agonists (**A**) and inhibition by antagonists (**B**) on random migration of CECs, as described in Materials and Methods. Briefly, CECs were loaded into each 3-mm well in an agarose gel, exposed to test drugs versus no treatment (control), and incubated for 10 days with daily changes of GM containing drugs. Random migration distance was measured at the end of each experiment. The results are expressed as the percentage of intact control. The following drug concentrations were used: CCh, 50 μM; Mus, 1 μM; Nic, 1 μM; Atr, 10 μM; Mec, 50 μM; and MLA, 100 nM. $^*P < 0.05$ compared to untreated control, taken as 100% in both (**A**) and (**B**). In (**A**), $\#P < 0.05$ compared to CCh given alone; $\S P < 0.05$ compared to Mus given alone; and $\P P < 0.05$ compared to Nic given alone.

These results indicated that random migration of CECs is stimulated by a synergistic signaling through the mAChRs and non-α7 nAChR subtypes.

Synergistic Muscarinic and Nicotinic Regulation of Directional Migration of CECs

Using the chemotaxis under-agarose assay, we evaluated the ability of cholinergic agonists to initiate and guide directional migration of CECs. The muscarinic and nicotinic agonist CCh, the muscarinic agonist Mus, and the nicotinic agonist Nic induced directional migration of CECs toward their concentration gradients (Fig. 4). All three agonists exhibited comparable chemotactic activities, which could be inhibited by respective antagonists (Fig. 4). A combination of Atr with Mec and/or MLA completely abolished CCh-induced chemotaxis. Atr blocked the chemotactic action of Mus, and the nicotinic antagonists Mec and MLA blocked that of Nic, with MLA being slightly more

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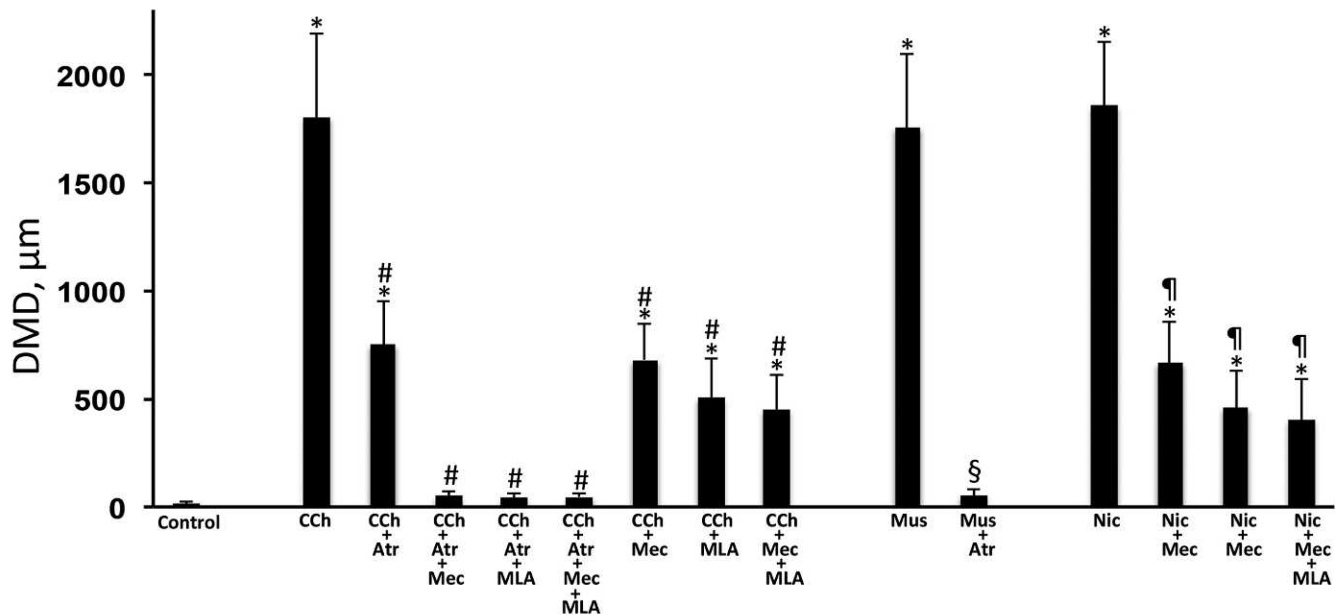

Figure 4. Regulation of directional migration of CECs through the muscarinic and nicotinic pathways. The directional migration distance (DMD) of CECs toward the concentration gradient of the cholinergic agonists CCh, 50 µM; Mus, 1 µM; and Nic, 1 µM, diluted in PBS and added to the chemoattractant well, were measured in the chemotaxis under-agarose plates as described in Materials and Methods. Briefly, CECs in GM were loaded into a 3-mm well in agarose gel, incubated overnight (to allow cells to settle), after which a test cholinergic agonist diluted in 10 µL PBS, with or without antagonist(s), was inoculated in a 2-mm chemoattractant well cut on one side of the 3-mm well containing CECs. The antagonists Atr, 10 µM; Mec, 50 µM; and MLA, 100 nM, were added directly to the well containing CECs. The plates were incubated for 10 days, with daily refreshment of solutions of test drugs. The DMD of control (PBS without an agonist) and experimental cells are expressed as means ± SD in micrometers. *$P < 0.05$ compared to control CECs migrating in the chemotaxis plates containing PBS without an agonist; #$P < 0.05$ compared to CCh given alone; §$P < 0.05$ compared to Mus given alone; ¶$P < 0.05$ compared to Nic given alone.

effective than Mec. No directional migration of CECs could be observed when an antagonist or a combination of antagonists was placed in the chemoattractant well (data not shown).

These results demonstrated that the cholinergic chemotaxis may be initiated via muscarinic and nicotinic pathways independently from each other and that both α7 and non-α7 nAChRs can stimulate chemotaxis, with the α7 signaling exhibiting a stronger chemotactic action.

Cholinergic Stimulation of Intercellular Adhesion of Cultured CECs

To evaluate the role of cholinergic signaling in assembly of the cell–cell adhesion complexes required for reestablishment of the corneal epithelial barrier, we employed a quantitative assay of cell monolayer permeability. After the confluent monolayer had been dissociated due to a brief exposure to EDTA and then exposed to cholinergic agonists, the permeability of the monolayer significantly ($P < 0.05$) decreased, and the effects of agonists could be abolished in the presence of respective antagonists (Fig. 5A). Stimulation of with CCh, Mus, or Nic decreased the PC values equally efficiently. A mixture of Mec and MLA showed a more potent antagonistic effect against Nic compared to each antagonist given alone (Fig. 5A). When dissociated CECs were exposed to antagonist(s) without an agonist, the PC values increased (Fig. 5B). This was expected since the antagonists interrupted constant stimulation of CEC cholinergic receptors by autocrine/paracrine ACh, which elicited CEC reattachment in control monolayers.

These results indicated that ACh can stimulate cell–cell adhesion and formation of a confluent monolayer by activating both mAChRs and nAChRs, with each signaling pathway being equally important, and that simultaneous activation of α7 or non-α7 nAChR produces an additive (synergistic) effect.

Cholinergic Regulation of Expression of the Migratory Integrin α_V

The expression of α_V integrin by cultured CECs incubated with test drugs for 24 hours was measured by ICW directly in the cells attached to the bottom of 24-well plate. The agonists CCh, Mus, and Nic upregulated α_V integrin expression by approximately 7- to 8-fold (Fig. 6A). These effects were diminished in the presence of antagonists. Atr decreased the CCh-dependent upregulated expression of α_V integrin, albeit insignificantly ($P > 0.05$). The significant ($P < 0.05$) changes were achieved in the presence of Mec given alone or in combination with Atr (Fig. 6A). The effect of Mus was completely abolished by Atr, whereas that of Nic was abolished by Mec or a combination of Mec and MLA, but not MLA given alone (Fig. 6A). Given without an agonist, each antagonist, except for MLA, or a mixture of antagonists significantly ($P < 0.05$) decreased α_V integrin expression below the control level (Fig. 6B), perhaps due to interference with the physiological stimulation of CECs with autocrine/paracrine ACh.

These results indicated that expression of α_V integrin in CECs can be stimulated through mAChRs and non-α7 nAChR subtypes independently from each other, as was observed in the studies of cholinergic regulation of random migration of CECs (Fig. 3).

Cholinergic Regulation of Expression of E-cadherin

Expression of E-cadherin by the CECs stimulated with cholinergic agonists in the absence or presence of antagonists was measured by ICW using the same assay setting as for measurement of α_V integrin expression. The agonists CCh, Mus, and Nic upregulated E-cadherin expression by approximately 5- to 6-fold. (Fig. 7A). Each antagonist significantly ($P < 0.05$) decreased the effect of relevant agonist, and combining

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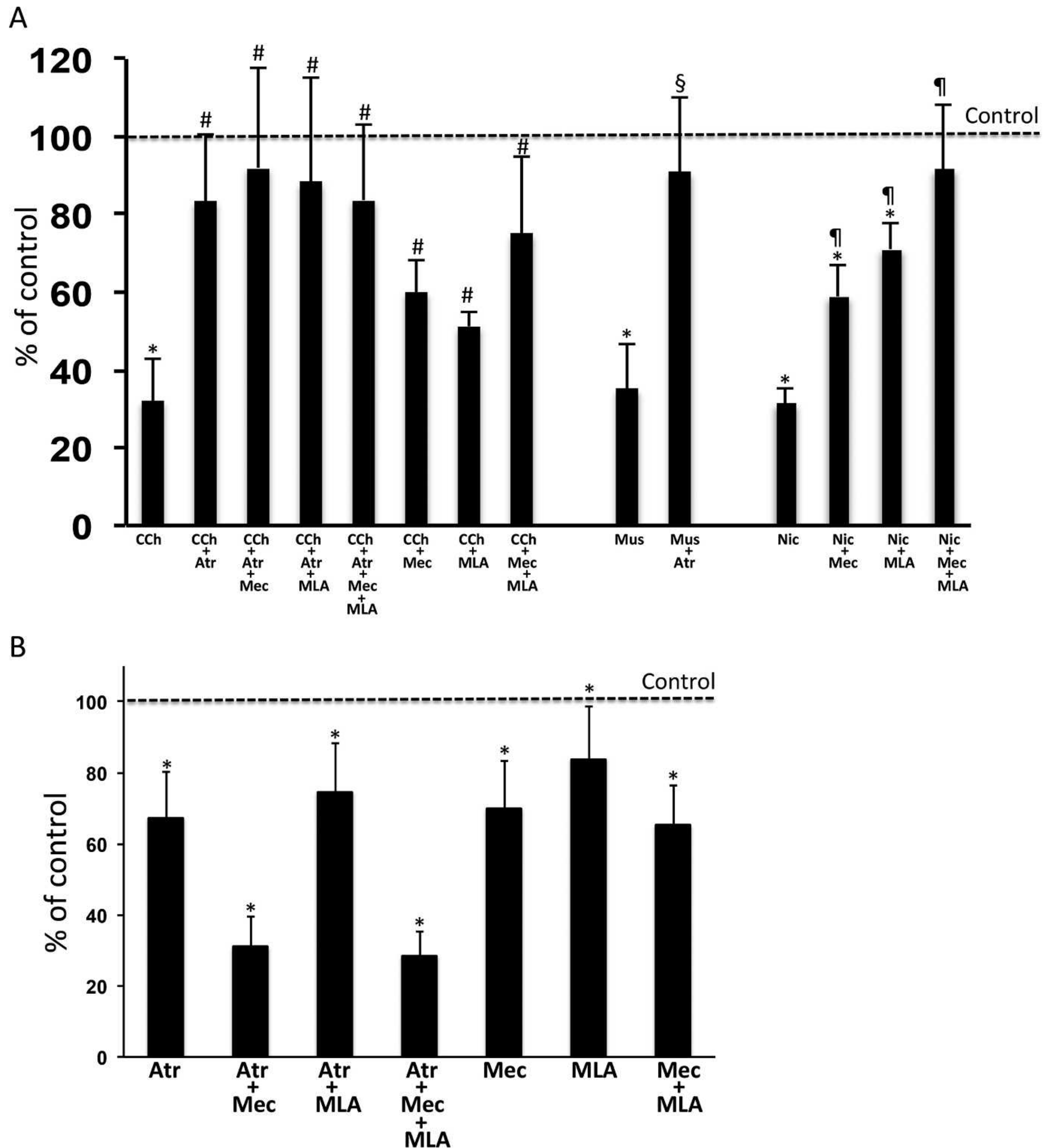

Figure 5. Regulation of intercellular adhesion of CECs through the muscarinic and nicotinic pathways. The effects of cholinergic agonists and antagonists on the ability of CECs to spread their cytoplasmic aprons and form intercellular attachments were measured in the cell monolayer permeability assay described in Materials and Methods. Briefly, the cells dissociated by a brief, 30-second exposure to 0.53 mM EDTA were incubated for 3 hours in the presence of test agonist with or without antagonist(s) (**A**) or antagonist(s) without agonists (**B**) dissolved in PBS, after which monolayer PC was measured. The results are expressed as percentage of the PC value determined in the control CEC monolayer exposed to PBS without test drugs and taken as 100%. The following drug concentrations were used: CCh, 50 μM; Mus, 1 μM; Nic, 1 μM; Atr, 10 μM; Mec, 50 μM; and MLA, 100 nM. All experimental results significantly ($P < 0.05$) differ from controls. *$P < 0.05$ compared to control CECs exposed to PBS without test drugs taken as 100% in both (**A**) and (**B**). In (**A**), #$P < 0.05$ compared to CCh given alone; §$P < 0.05$ compared to Mus given alone; and ¶$P < 0.05$ compared to Nic given alone.

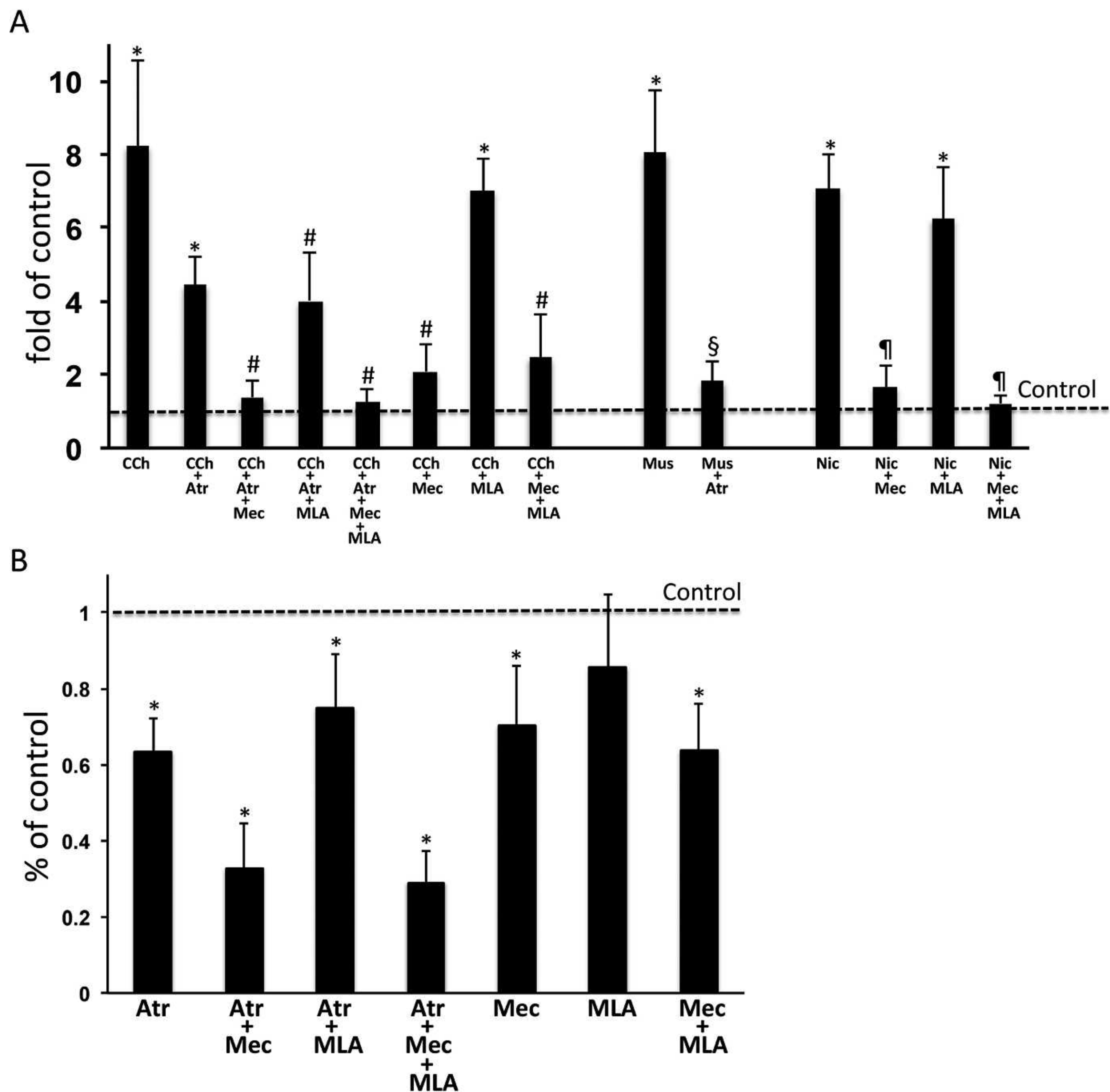

FIGURE 6. Regulation of expression of the migratory integrin α_V in CECs through the muscarinic and nicotinic pathways. The protein level of α_V integrin was measured by ICW in confluent CEC cultures in 96-well plates after incubation for 24 hours in absence (control) or presence of test cholinergic drugs as described in Materials and Methods. The effects of agonists with or without antagonists are shown in (**A**) and those of antagonists without agonists in (**B**). The protein expression level determined in the control cultures was taken as 1 and the results expressed as fold of control. The following drug concentrations were used: CCh, 50 μM; Mus, 1 μM; Nic, 1 μM; Atr, 10 μM; Mec, 50 μM; and MLA, 100 nM. $*P < 0.05$ compared to untreated control, taken as 100% in both (**A**) and (**B**). In (**B**), $\#P < 0.05$ compared to CCh given alone; $\S P < 0.05$ compared to Mus given alone; and $\P P < 0.05$ compare to Nic given alone.

different antagonists amplified their inhibitory effects (Fig. 7A). Likewise, each antagonist or any mixture of antagonists given without an agonist, significantly ($P < 0.05$) decreased E-cadherin expression below the basal level (Fig. 7B), thus further demonstrating the importance of constant stimulation of CEC cholinergic receptors with autocrine/paracrine ACh for normal E-cadherin expression.

These results indicated that upregulation of E-cadherin in CECs can be achieved through independent stimulation of mAChRs and α7 and non-α7 nAChRs and that simultaneous activation of these cholinergic pathways provides for a synergistic stimulatory effect. The pattern of cholinergic regulation of E-cadherin matched that of cholinergic regulation of CEC cell–cell attachment and monolayer permeability (Fig. 5).

DISCUSSION

Classic studies demonstrated that mammalian corneal epithelium contains a very high concentration of endogenous ACh, has high levels of activity of ChAT and AChE, and expresses M_1

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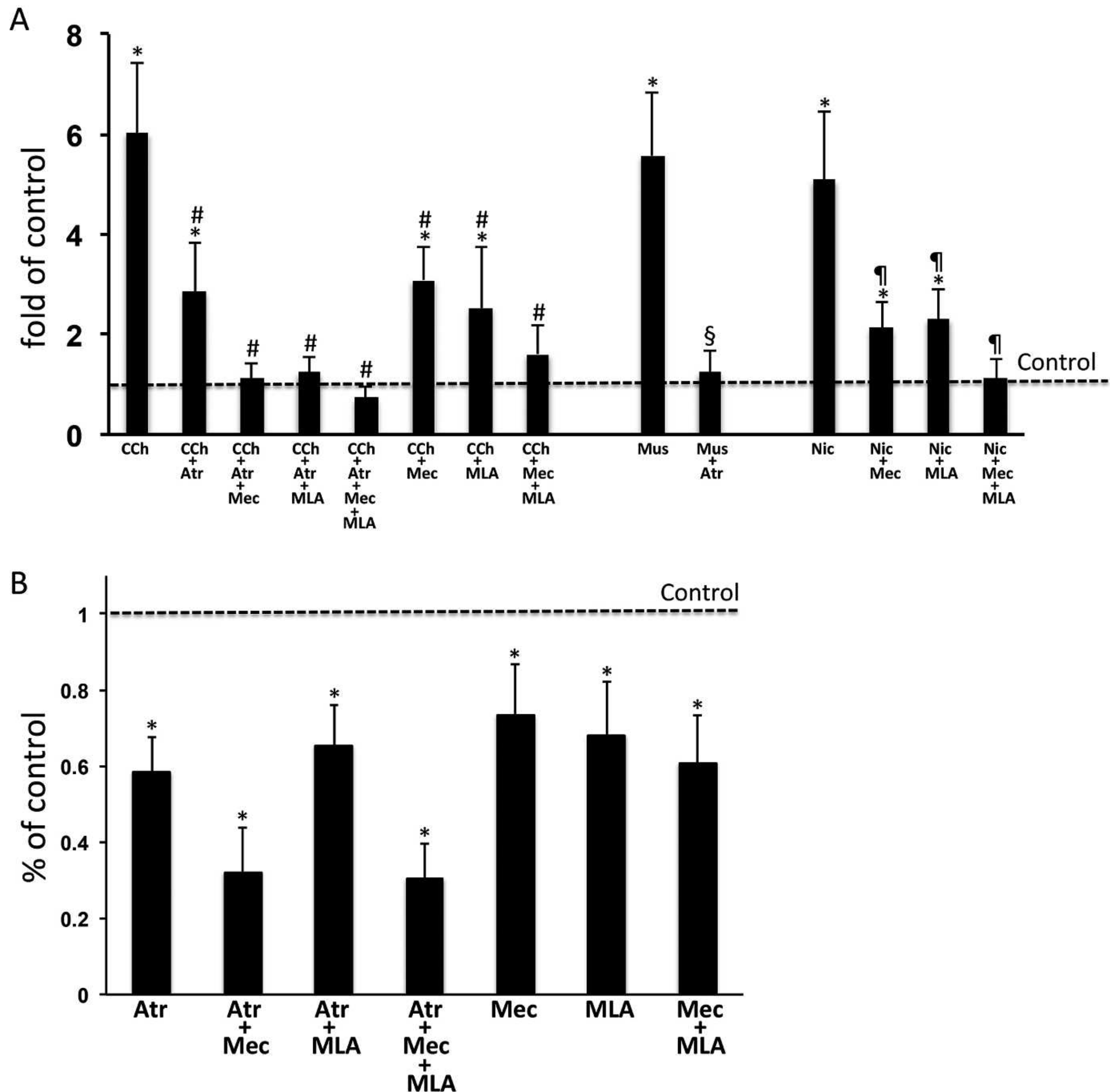

FIGURE 7. Regulation of expression of E-cadherin in CECs through the muscarinic and nicotinic pathways. The protein level of E-cadherin was measured by ICW in confluent CEC cultures in 96-well plates after incubation for 24 hours in absence (control) or presence of test cholinergic drugs as described in Materials and Methods. The effects of agonists with or without antagonists are shown in (**A**) and those of antagonists without agonists in (**B**). The control values were taken as 1, and the results expressed as multiples of control. The following drug concentrations were used: CCh, 50 μM; Mus, 1 μM; Nic, 1 μM; Atr, 10 μM; Mec, 50 μM; and MLA, 100 nM. *$P < 0.05$ compared to untreated control, taken as 100% in both (**A**) and (**B**). In (**B**), #$P < 0.05$ compared to CCh given alone; §$P < 0.05$ compared to Mus given alone; and ¶$P < 0.05$ compare to Nic given alone.

through M_5 mAChRs,[32–38] which was unrelated to corneal innervation, but the biological function of this nonneuronal cholinergic network remained unknown. Surprisingly, there were no reports addressing potential expression of nAChRs in the cornea. To identify a complete repertoire of the cholinergic receptors comprising the ACh signaling axis in the corneal epithelium and to explore its physiological significance, we investigated the structure and function of mAChRs and nAChRs in the telomerase-immortalized human CEC line hTCEpi. The results showed that the muscarinic signaling of autocrine/paracrine ACh and muscarinic agonists can be mediated by the M_3 and M_4 mAChR subtypes and the nicotinic signaling by the heteromeric nAChR channels composed of different combinations of α3, α5, β2, and β4 subunits, that is, α3β2, α3β4, α3β2α5, α3β4α5, or α3β2β4α5, and two homomeric nAChR subtypes, each composed of five α7 or α9 subunits. Thus, CECs possess a functional cholinergic system for signal transduction with ACh as a single cytotransmitter (or a local hormone) that exerts a proepithelialization activity. The system also includes the synthesizing enzyme ChAT and the degrading enzyme AChE. The expression patterns of cholinergic molecules vary between intact and wounded murine corneas. Indeed, the

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results obtained with hTCEpi cells and murine corneal epithelium need to be validated on normal human CECs and corneal tissue, respectively. Regulation of cell–matrix and cell–cell adhesion by ACh is one of the important biological functions of corneal ACh axis. The downstream targets of ACh include both the cell–matrix adhesion molecules, such as integrin α_V, and the intercellular adhesion molecules, such as E-cadherin. These findings advance our knowledge about the role of the corneal ACh axis in eye biology and have salient clinical implications because the integrity of corneal epithelial barrier protects the eye from environmental hazards and maintains normal vision by transmitting light onto the lens and retina.

The newly discovered cholinergic effects of autocrine/paracrine ACh in the cornea may represent a general biological mechanism, since ACh has recently emerged as a candidate for a regulatory role in numerous biological processes that are intimately connected to each other, including viability, proliferation, differentiation, apoptosis, adhesion, and migration of nonneuronal cells (see Ref. 39 for review). It has been well documented that both mAChRs and nAChRs play important roles in regulation of reepithelialization of the mucocutaneous wounds, and cholinergic agonists reportedly have been employed to facilitate wound healing in the experimental and clinical settings (see Refs. 40 and 41 for reviews). However, lack of information about the corneal epithelial cholinergic receptor signaling makes it difficult to translate understandings from other tissue to the cornea to devise novel approaches to treat corneal wounds. For example, reports of negative results from the clinical studies of pilocarpine in corneal healing may be explained by the fact that this muscarinic agonist predominantly activates the odd-numbered mAChR subtypes,[42–44] such as M_3, which inhibits cell migration by upregulating the sedentary integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ through the Ca^{2+}-dependent guanylyl cyclase/cyclic GMP/protein kinase G signaling pathway.[25] At high doses, pilocarpine also inhibits synthesis of ACh,[45] which may completely deprive CECs of this local hormone because its production in the corneal epithelium is already reduced due to injury.[32] Identifying the role of ACh signaling in controlling normal corneal homeostasis and wound repair may facilitate development of new clinical solutions to treat corneal epithelial disorders.

Localization of functional elements of the corneal cholinergic network indicates that the concentration of endogenous ACh, which is a function of its synthesis by ChAT and degradation by AChE, as well as the mode of its signaling, which depends on the repertoire of mAChRs and nAChRs expressed by individual CECs, differ among corneal epithelial layers. This phenomenon may have biological meaning because it results in each layer having a unique combination of cholinergic signaling molecules. Since CECs express a unique combination of ACh receptors within different levels of the epithelial sheet, each receptor may regulate a specific cell function. Hence, a single cytotransmitter, ACh, or a cholinergic drug may exert unique effects on CECs at different stages of their differentiation.

The homogenous staining for ChAT is consistent with the intracellular localization of this ACh-synthesizing enzyme,[46] whereas combined, cytoplasmic and cell-surface staining for AChE is indicative of the presence in CECs of both the membrane-anchored and secreted molecular forms of this enzyme.[20] Likewise, the more diffuse staining patterns for M_3 mAChR and $\alpha3$ and $\alpha7$ nAChRs can be explained by expression of certain mAChR and nAChR subtypes on the nuclear and mitochondrial membranes,[47–51] in addition to the cell membrane. The receptor molecules expressed on the cell membrane and inside the cells can be activated by the extracellular[35] and intracellular,[52] respectively, pool of ACh. In addition to ACh, the intracellular cholinergic receptors can be ligated by certain cholinergic drugs that can penetrate the cell membrane, such as the alkaloids nicotine and atropine. The ability of intracellularly located nAChRs and mAChRs to bind ligands and elicit signaling cascades involving protein kinases has been demonstrated in other cell types.[51,53]

Comparison of the staining patterns produced by each specific anti-ACh receptor antibody (Fig. 2) suggests that CECs express the M_3 subtype of mAChR more abundantly then M_4 and the $\alpha7$-made homomeric nAChR—more than the $\alpha9$-made homomeric nAChR. Among heteromeric nAChRs composed of various combinations of $\alpha3$, $\beta2$, $\beta4$, and $\alpha5$ subunits, the most prevalent appears to be the $\alpha3\beta2 \pm \alpha5$ nAChR. The cytoplasmic staining patterns of CECs in murine cornea reflects the sites of expression of cholinergic enzymes and receptors. It will be important to determine in the future if the localization of cholinergic molecules in human corneal epithelium matches the pattern observed in the mouse tissue used in the present study.

Nicotinic and muscarinic drugs exhibited profound effects on cell migration and intracellular adhesion of CECs. These results demonstrated that ACh receptors expressed by CECs are functional and coupled to regulation of cell motility and expression of the adhesion molecules involved in the process of reepithelialization. Most importantly, we found synergy between the proepithelialization signals emanating from various ACh receptor subtypes expressed by CECs. The cholinergic chemotaxis of CECs could be independently initiated via muscarinic and nicotinic pathways. Both $\alpha7$ and non-$\alpha7$ nAChRs elicited chemotaxis, with the $\alpha7$ signaling exhibiting a stronger chemotactic effect. These findings suggest that individual corneal ACh receptors regulate the expression/function of specific molecules and that a group of ACh receptors may be jointly responsible for execution of a specific cellular function required to complete specific steps of reepithelialization. In the present study, the cholinergic effects on CEC motility were observed in the immortalized hTCEpi cells forming a monolayer, which mimics the advance of the CEC sheet epithelializing a corneal wound. A physiological cooperation (synergism) between ACh receptors, therefore, may be required for fine tuning of CEC functions mediating reepithelialization. The biological roles of these receptors in the stratified corneal epithelium could not be evaluated in the cell system used. They may differ depending on the level of CEC differentiation in the corneal epithelium, analogous to other types of stratified squamous epithelium (see Ref. 41 for review).

The molecular mechanisms of functional synergy among distinct ACh subtypes may be mediated by cross-activation of downstream signaling pathways coupled by individual receptors, such as activation of the Ras/Raf-1/MEK1/ERK cascade by M_1 and $\alpha7$,[54] as well as engagement in the cross talk with growth factor receptors. The former mechanism can be illustrated by aggravation of abnormalities of keratinocyte adhesion caused by simultaneous inhibition of several ACh receptor subtypes.[27,29] The latter mechanism is suggested by the evidence of functional interactions of mAChRs with receptors to epidermal growth factor, transforming growth factor-β, and vascular endothelial growth factor[55–57] and that of nAChRs with receptors to epidermal growth factor, fibroblast growth factor-2, and insulin-like growth factor-1 (see Ref. 16 for review). The crucial role of growth factors of these families in the physiological regulation of corneal reepithelialization is well documented (see Ref. 58 for review).

Simultaneous stimulation of mAChRs and nAChRs by ACh may be required to synchronize and balance ionic and metabolic events in a single cell, and the net biological

response is determined by a unique combination of the subtypes of muscarinic and nicotinic receptors expressed by an individual cell. In this model, binding of ACh to an individual CEC simultaneously elicits several diverse biochemical events, the biological sum of which, taken together with cumulative effects of other hormonal and environmental stimuli, determines a distinct step of reepithelialization. Simultaneous activation of distinct cholinergic signaling pathways may produce a kind of a yin and yang regulatory balance with the result that ACh takes the pacemaker function in the corneal epithelium. Unopposed activation or blockade of certain subtype(s) of corneal ACh receptors may alter the physiological signaling by autocrine/paracrine ACh and lead to the reciprocal alterations in cell functions. Therefore, elucidation of ACh receptor subtype selective control of the gene expression responsible for acquisition of a particular cell phenotype in the course of reepithelialization will provide a mechanistic insight into a general regulatory mechanism driving cornea epithelial turnover and will also help focus future mechanistic studies on either pre- or posttranscriptional events regulated by each particular ACh receptor subtype. Future studies should also identify the consequences of epithelial barrier disruption on the structure and function of the corneal cholinergic network.

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