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Molecular mechanisms of synergy of corneal muscarinic and nicotinic acetylcholine receptors in upregulation of E-cadherin expression

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ABSTRACT

Corneal epithelial erosion is one of the most common problems in clinical ophthalmology. Despite significant progress in understanding how the cornea heals, clinically available pharmacological therapies that can promote repair and prevent visual complications remain limited. We have recently demonstrated that the acetylcholine (ACh) axis of corneal epithelium plays an important role in regulation and coordination of distinct activities of corneal epithelial cells (CEC) mediating re-epithelialization, but mechanisms remained unclear. We hypothesized that the grounds for synergistic effects of corneal ACh receptors lie within the signaling pathways linking different receptors to specific elements of the CEC pro-epithelialization activities. In this study, we sought to elucidate the molecular mechanisms of cooperation of corneal muscarinic and nicotinic ACh receptors (mAChRs and nAChRs) in upregulation of E-cadherin expression. The roles of individual corneal mAChRs and nAChRs subtypes were investigated by in-cell western assay of the ACh-treated CEC, in which different ACh receptor genes were silenced by receptor-specific shRNAs. Functional inactivation of M_3, but not M_4, mAChR subtype, or α3 or α7, but not α9, nAChR subunit significantly inhibited E-cadherin expression. To gain a mechanistic insight, we blocked the key steps of the downstream signaling pathways. Results demonstrated that cholinergic agonists can upregulate E-cadherin expression by activating M_3 mAChR, and α3β2 and α7 nAChRs via the common signaling cascade Ca^{2+}–CaMKII–PKC–Ras–Raf–MEK–ERK. Activation of α7 nAChR can launch the Ras–Raf–MEK–ERK cascade both indirectly, through the Ca^{2+}–CaMKII–PKC step, and directly, perhaps, due to its direct interaction with Ras. Although the biological significance of such redundancy remained to be elucidated, results of the present study point to a new direction to pharmacologically accelerate corneal re-epithelialization, and should have salient clinical implication.

1. Introduction

Despite significant progress in understanding how the cornea heals, clinically available pharmacological therapies that can promote repair and prevent visual complications remain limited [1]. Improving the understanding of cellular and molecular mechanisms of corneal epithelialization, therefore, has a potential for development of novel modalities for prevention and treatment of persistent corneal epithelial defects [2]. The purpose of our research of corneal re-epithelialization is to elucidate novel clinically relevant molecular circuitries regulating repair of corneal epithelial barrier in order to facilitate development of novel therapeutic strategies. The emerging picture is that a diversity of molecular signaling circuitries regulating corneal re-epithelialization signifies cross-talk interactions between receptors to various autocrine and paracrine mediators (reviewed in [3,4]). The intracellular signal cross-talks may either amplify or inhibit biological effects of the partnering receptor molecule. The binary signaling networks regulating corneal re-epithelialization may, therefore, be a novel target for pharmacological intervention.

We have recently demonstrated that the acetylcholine (ACh) axis of corneal epithelium plays an important role in regulation and coordination of distinct functions of corneal epithelial cells (CEC) mediating re-epithelialization but mechanisms remained unclear [5]. The ACh signaling network of the corneal epithelium is an example of the more general neuroendocrine-like mechanisms that mediate peripheral responses to environmental factors and of evolutionary conservation of neuroendocrine systems in the periphery [6,7]. ACh is produced by practically all types of living cells [8], and its concentration is remarkably high in the corneal epithelium, even exceeding that in the neural tissue (reviewed in [9]). ACh and cholinergic drugs elicit biological effects through binding to muscarinic and nicotinic classes of ACh receptors, mAChRs and nAChRs, respectively. The mAChR family is comprised

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of five, M_1–M_5, receptor subtypes preferentially coupled to distinct signal transduction pathways via specific G proteins. The M_1, M_3 and M_5 subtypes activate protein kinase C (PKC) by elevating intracellular Ca^{2+} and diacylglycerol, whereas M_2 and M_4 inhibit protein kinase A by diminishing adenylyl cyclase activity, resulting in the reduction of intracellular levels of cAMP (reviewed in [10]). The nAChRs are ACh-gated ion channels that mediate the influx of Na^+ and Ca^{2+} and efflux of K^+, and can also elicit downstream signaling events by modulating activities of protein kinases and phosphatases (reviewed in [11]). The nAChR pentamers are comprised by different combinations of α1–α10, β1–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 comprised by 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 [11].

Corneal epithelium expresses the ACh synthesizing enzyme choline acetyltransferase, the ACh-degrading enzyme acetylcholinesterase, two mAChRs subtypes, M_3 and M_4, and several nAChRs subtypes, including both α7- and α9-made homomeric, and α3-made heteromeric receptors [5]. We have also demonstrated that the pro-epithelialization signals of auto/paracrine ACh can be implemented by both corneal mAChRs and nAChRs acting synergistically. Carbachol (CCh), an agonist of both mAChRs and nAChRs, the muscarinic agonist muscarine and the nicotinic agonist nicotine all could upregulate E-cadherin expression in CEC, whereas the cholinergic antagonists abolished effects of the agonists. Combining antagonists of specific ACh receptor subtypes amplified their inhibitory effects, indicating that upregulation of E-cadherin in CEC was implemented 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 permeability of the CEC monolayer [5].

In the present study, we sought to elucidate the molecular mechanisms of cooperation of corneal mAChRs and nAChRs in upregulation of E-cadherin expression, which is essential for normal re-epithelialization of corneal wounds. We demonstrated that auto/paracrine ACh and cholinergic agonists can upregulate E-cadherin expression by activating M_3 mAChR, and α3β2 and α7 nAChRs via the common signaling cascade Ca^{2+}–CaMKII–PKC–Ras–Raf–MEK–ERK. These results point to a new direction to pharmacological facilitation of corneal re-epithelialization, and, therefore, should have salient clinical implication.

2. Materials and methods

2.1. Cells and reagents

The telomerase-immortalized human CEC line (hTCEpi) [12] were grown in KGM-2 medium (Lonza Inc., Allendale, NJ) at 37 °C in a humid atmosphere of a 5% CO_2 incubator until approximately 80% confluence and then used in experiments. The pan-muscarinic agonist muscarine, the preferring M_3 mAChR antagonist darifenacin [13], epibatidine — the preferring agonist of the "ganglionic" nAChRs, such as α3-made nAChRs [14], the α7-preferring antagonist methyllycaconitine [15], the acetylcholinesterase (AChE)-resistant, mixed muscarinic-and-nicotinic agonist CCh, hemicholinium-3 (HC-3), which indirectly affects ACh synthesis by inhibiting high-affinity choline uptake [16,17], and the PKC activator phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Corp. Inc. (St. Louis, MO). The preferring blockers of α3β2 and α3β4 nAChRs α-conotoxins MII (αCtxMII) and AuIB (αCtxAuIB), respectively, which block their respective target receptors with the IC_{50} of 0.5 nM and 0.75 μM and other nAChR subunit combinations with 2–4 orders of magnitude less potent [18–20], were synthesized by Advanced ChemTech (Louisville, KY, USA). The preferential α7 nAChR agonist AR-R17779 [21] was a gift from AstraZeneca Pharmaceuticals (Wilmington, DE). The cell permeable chelator of intracellular free Ca^{2+} 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA/AM), the cell permeable inhibitor of Ca^{2+}-ATPases thapsigargin, the selective inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) KN-62 and the highly specific phosphatidylinositol 3-kinase (PI3K) inhibitor Ly-294002 were purchased from Axxora, LLC (San Diego, CA). The cell permeable inhibitor of all PKC isoforms chelerythrine, the inhibitor of the PKC δ isoform rottlerin, the specific inhibitor of p38 MAPK SB202190, the noncompetitive inhibitor of the Ras acceptor protein manumycin A [22], the cRaf-1 kinase inhibitor GW5074 [5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone] [23], and the cell-permeable, potent and selective inhibitor of MEK "MEK inhibitor I" [24] were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). The plasmids encoding the constitutively active MEK1 (CA-MEK) with 2 point mutations (S218E and S222E) and a deletion of amino acid residues 31–52, the dominant negative MEK1 mutant that contains 3 point mutations (K97R, S218A and S222A) and thus could neither be phosphorylated by its activators nor phosphorylate its downstream effectors ERKs were purchased from Biomyx Technology (San Diego, CA). The predesigned and tested small hairpin RNA (shRNA) targeting human M_3 and M_4 mAChRs and α3, α7 and α9 nAChR subunits as well as scrambled shRNA (negative control; shRNA-NC) were purchased from OriGene Technologies (Rockville, MD). Antibodies to M_3, M_4, α3, α7 and α9 were raised and characterized in our previous studies [25–27]. Mouse monoclonal antibody to human E-cadherin was from BD Biosciences (clone 36; Woburn, MA).

2.2. shRNA transfection experiments

For transfection of CEC with the HuSH-29™ predesigned shRNA plasmids specific for human M_3 and M_4 mAChR subtypes and α3, α7 and α9 nAChR subunit genes, we followed a standard protocol described by us in detail elsewhere [28]. Briefly, one day before transfection CEC were seeded at a density of 1×10^4 cells per well of the 96-well in-cell western (ICW) plates and exposed to experimental, i.e., mAChR subtype- or nAChR subunit gene-specific shRNA or shRNA-NC plasmids in GIBCO™ Opti-MEM I Reduced-Serum Medium (Invitrogen, Carlsbad, CA) with a transfection reagent from Mirus Bio LLC (Madison, WI). The transfection was continued for 24 h at 37°C in a humid, 5% CO_2 incubator. On the next day, the transfection medium was replaced by growth medium, and the cells were incubated for additional periods of time to determine by immunoblotting the relative protein levels of targeted receptors using respective anti-receptor antibodies and standard protocol detailed by us elsewhere [25,26]. The maximum inhibition was achieved at 72 h after transfection, at which point the shRNA-transfected cells were used in ICW experiments.

2.3. ICW experiments

The ICW — a high throughput quantitative assay of cellular proteins, was performed as described by us in detail elsewhere [29], using the reagents and equipment from LI-COR Biotechnology (Lincoln, NE). CEC were fixed in situ, washed, permeabilized with Triton solution, incubated with the LI-COR Odyssey Blocking Buffer for 1.5 h and then treated overnight at 4 °C with anti-E-cadherin antibody. The cells were then washed, and stained for 1 h at room temperature with a secondary LI-COR IRDye 800CW antibody, and Sapphire700 to normalize for cell number/well. The protein expression was quantitated using the LI-COR Odyssey Imaging System.

2.4. Statistical analysis

Each experiment was performed in a triplicate, the results expressed as mean ± SD, and the statistical significance was determined by ANOVA with a Dunnet post test using the GraphPad Prism software

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