Abstract: Acetylcholine and its receptors are not only essential for the nervous system but also act as mediators of cell communication between non-neuronal cells. Signal transduction after cholinergic stimulation is mediated by two types of receptors. The nicotinic acetylcholine receptors are ion channels, whereas the muscarinic receptors belong to the group of G protein-coupled receptors. Their activation can lead to initiation of the mitogen-activated protein (MAP) kinase cascade which contributes to cell survival, differentiation and other important cellular responses. In the human keratinocyte cell line HaCaT, muscarinic receptors mediate the transactivation of the epidermal growth factor receptor (EGFR) to facilitate the activation of the MAP kinases ERK1/2 and the protein kinase Akt. This transactivation process includes a triple-membrane-passing signal, the details of which are here studied in more detail. We show that the matrix metalloproteases MMP-2 and MMP-9 are not involved, neither are the members of the ADAM (a disintegrin and metalloproteinase) family ADAM10 and ADAM17. Furthermore, the EGFR most likely forms homodimers to facilitate its function in cholinergic signaling in HaCaT cells.

Keywords: muscarinic receptor; transactivation; epidermal growth factor receptor; matrix metalloprotease; a disintegrin and metalloproteinase; mitogen-activated protein kinase; keratinocytes
1. Introduction

Besides its role as a neurotransmitter, acetylcholine (ACh) can be an important mediator of cell communication in many different tissues and organs. In this so-called “non-neuronal cholinergic system” various cell types express proteins and enzymes to synthesize, degrade and detect ACh (reviewed in [1–3]). There are two families of ACh receptors, the muscarinic ACh receptors (mAChRs) and the nicotinic ACh receptors (nAChRs). The mAChRs belong to the family of G protein-coupled receptors (GPCRs) and mediate their signaling via trimeric G proteins [4]. There are five mAChR subtypes (M1 to M5) and they either preferentially couple to inhibitory G proteins (M2 and M4) that lower the intracellular cyclic AMP levels or to stimulatory G proteins that mediate the release of second messengers like inositol 3,4,5-trisphosphate or diacylglycerol (M1, M3 and M5). These second messengers can activate different kinases and influence various target molecules like mitogen-activated protein (MAP) kinases (reviewed in [5]).

Non-neuronal ACh is of particular importance for the maintenance of the human skin [6,7], in which ACh regulates proliferation and differentiation of keratinocytes and supports e.g. wound healing [8,9]. We have earlier shown that in the human keratinocyte cell line HaCaT cholinergic signaling leads to activation of MAP kinases of the extracellular signal regulated kinase (ERK) family, namely ERK1 and ERK2, and the protein kinase B, also known as Akt [10]. This signaling pathway includes the transactivation of the epidermal growth factor receptor (EGFR), and was described in 1996 for the first time [11]. Interestingly, we were able to show that the mitogenic signaling and the Akt activation mediated by cholinergic signaling were fully dependent on the EGFR transactivation. Furthermore, we identified this pathway as the common triple-membrane-passing signaling, proceeding through the activation of a metalloproteinase and the release of an epidermal growth factor (EGF)-like ligand. However, the cholinergic MAP kinase activation does not involve a Src family kinase, protein kinase C or phosphatidylinositol 3-kinase or the ligands EGF, heparin-binding EGF (HB-EGF) or transforming growth factor α (TGFα) [10].

We here further analyzed the cholinergic signaling pathway leading to the activation of the MAP kinases ERK1/2 and the protein kinase Akt. We show that the matrix metalloproteinases MMP-2 and -9, as well as the members 10 and 17 of the ADAM (a disintegrin and metalloproteinase) family are not involved in the cholinergic signaling in HaCaT cells. Furthermore, the EGFR seems not to dimerize with its family members ErbB2 and ErbB3 under these conditions in HaCaT cells.

2. Results and Discussion

2.1. Results

2.1.1. The Involvement of Metalloproteinases in Cholinergic Signaling

MMP-2, MMP-9 and ADAM-17 are commonly known to take part in GPCR-mediated transactivation of the EGFR, in which they mediate the extracellular release of EGF-like ligands [12–15]. To analyze if one of these three proteases is involved in the ligand cleavage and release observed in HaCaT cells after CCh treatment, the inhibitor SB-3CT was employed. SB-3CT suppresses the function of MMP-2, MMP-9 and ADAM17 with different Ki values. The Ki value of SB-3CT for MMP-2 and MMP-9 is
within a nanomolar range, whereas its $K_i$ value for ADAM17 is $4 \mu M$. Therefore it is possible to discriminate between the effect of MMP-2/MMP-9 and that of ADAM17 if different concentrations of the inhibitor are used. Starved HaCaT cells were preincubated with 1 $\mu M$ and 5 $\mu M$ SB-3CT to allow differential observation of influences of MMP-2/MMP-9 and ADAM17 on the cholinergic signaling in HaCaT cells. Cells were then stimulated with 1 mM carbachol (CCh) or 16 nM EGF for 30 minutes, and the activation of Akt and ERK1/2 was analyzed with phospho-specific antibodies (Figure 1A). Quantification of three independent experiments revealed that there was no difference in Akt and ERK1/2 phosphorylation after CCh or EGF stimulation between SB-3CT (1 and 5 $\mu M$) and DMSO-treated control cells (Figure 1B, C).

**Figure 1.** MMP-2, MMP-9 and ADAM17 are not responsible for cholinergic signaling. (A) Starved HaCaT cells were pretreated with the inhibitor SB-3CT (1 or 5 $\mu M$) or with DMSO as a control and then stimulated with 1 mM CCh or 16 nM EGF for 30 min. Cell lysates were separated by SDS-PAGE and the phosphorylation of ERK1/2 and Akt was analyzed with Western blot. (B,C) The amount of pAkt and pERK1/2 was determined by densitometric quantification and normalized to total Akt or ERK1/2. Data are shown relative to EGF-stimulated control cells. Bars represent the mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA.

As the used concentration of 5 $\mu M$ SB-3CT is just above its $K_i$ value for the inhibition of ADAM17, higher concentrations of SB-3CT were applied to rule out the possibility of an involvement of ADAM17. However, even 10 and 25 $\mu M$ SB-3CT did not influence ERK1/2 and Akt activation (Figure 2).
Figure 2. Higher concentrations of SB-3CT do not influence cholinergic ERK1/2 and Akt activation. Starved HaCaT cells were pretreated with the inhibitor SB-3CT (10 or 25 μM) or with DMSO as a control and then stimulated with 1 mM CCh or 16 nM EGF for 30 min. Cell lysates were separated by SDS-PAGE and the phosphorylation of ERK1/2 and Akt was analyzed with Western blot.

Another interesting member of the ADAM protein family is ADAM10, as it is also known for its function in GPCR-mediated EGFR transactivation [16]. ADAM10 can be inhibited with the inhibitor GI 254023X, which has an IC₅₀ value of 5.3 nM for ADAM10 [17]. However, preincubation with this inhibitor did not influence the cholinergic signaling in HaCaT cells towards ERK1/2 and Akt either (Figure 3).

Figure 3. ADAM10 is not involved in cholinergic signaling. (A) Starved HaCaT cells were pretreated with the inhibitor GI 254023X (1 μM) or with DMSO as a control and then stimulated with 1 mM CCh or 16 nM EGF for 30 min. Cell lysates were separated by SDS-PAGE and the phosphorylation of ERK1/2 and Akt was analyzed with Western blot. (B,C) The amount of pAkt and pERK1/2 was determined by densitometric quantification and normalized to total Akt or ERK1/2. Data are shown relative to EGF-stimulated control cells. Bars represent the mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA.
Even a higher concentration of GI 254023X, like 10 µM, which might also influence ADAM9 (IC50=280 nM) or ADAM17 (IC50=541 nM), did not reduce EKR1/2 or Akt signaling (Figure 4).

**Figure 4.** Not even higher concentrations of GI 254023X inhibit cholinergic ERK1/2 and Akt activation. (A) Starved HaCaT cells were pretreated with the inhibitor GI 254023X (10 µM) or with DMSO as a control and then stimulated with 1 mM CCh or 16 nM EGF for 30 min. Cell lysates were separated by SDS-PAGE and the phosphorylation of ERK1/2 and Akt was analyzed with Western blot. (B,C) The amount of pAkt and pERK1/2 was determined by densitometric quantification and normalized to total Akt or ERK1/2. Data are shown relative to EGF-stimulated control cells. Bars represent the mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA.

2.1.1. EGFR Dimerization upon Cholinergic Stimulation

Besides ubiquitination and phosphorylation of the EGFR, the fine tuning of cholinergic signaling in HaCaT cells is most likely regulated by the interaction and dimerization partners of the EGFR. Upon ligand binding, the EGFR can either homodimerize or form dimers with its family members ErbB2, ErbB3 and ErbB4. To study which member of the ErbB family is associated with the EGFR after cholinergic transactivation, a co-immunoprecipitation study was performed. Starved HaCaT cells were stimulated with CCh or EGF and the EGFR was immunoprecipitated from cell lysates. These samples were probed for the co-immunoprecipitation of ErbB2 and ErbB3 (Figure 5). However, ErbB3 was co-precipitated with the EGFR neither after CCh stimulation nor after EGF treatment nor in starved cells. For ErbB2, there was a weak interaction with the EGFR but no clear difference between starved, CCh- and EGF-stimulated cells was detectable.
Figure 5. Dimerization of the EGFR with ErbB2 and ErbB3 does not change after CCh and EGF stimulation. Serum-starved HaCaT cells were stimulated with 1 mM CCh or 16 nM EGF for 15 min. The cells were lysed and the EGFR was immunoprecipitated. The samples were separated by SDS-PAGE and immunoblotted for ErbB2, ErbB3 and EGFR. A control IP was performed from EGF-stimulated cells with a myc antibody to show specificity of the IP.

2.1. Discussion

MMPs or ADAMs can be activated upon muscarinic stimulation and mediate the extracellular release of an EGF-like ligand into the surrounding medium [18]. As shown earlier by us, the ERK1/2 activation mediated by cholinergic EGFR transactivation is fully dependent on metalloproteinases in HaCaT cells. The signaling is completely abolished after treatment with a broad spectrum inhibitor Batimastat for MMPs and ADAMs [10]. Three members of these protease families are widely described to be mediators of EGFR transactivation, namely MMP-2, MMP-9 and ADAM17 [12–15]. However, none of these is likely to be the mediator of EGFR transactivation after CCh stimulation in HaCaT cells. Even concentrations of the specific inhibitor SB-3CT far higher than the relevant Ki values are not able to reduce cholinergic signaling towards ERK1/2 and Akt. Therefore, another MMP or ADAM must be the mediator of cholinergic signaling in HaCaT cells.

ADAM10 is also known for its function in GPCR-mediated EGFR transactivation, which results in mitogenic signaling towards ERK1/2 [16,19]. The potent inhibitor GI 254023X can specifically block its enzymatic activity and has already been demonstrated to function in HaCaT cells [17,20]. However, also ADAM10 does not seem to be involved in the EGFR transactivation induced by a cholinergic stimulus in HaCaT cells. Even though two different concentrations of the inhibitor were tested, with one clearly above the respective IC50 value, no reduction in ERK1/2 or Akt activity was observable. Our group recently published data showing that MMP-3 is under strong transcriptional regulation by cholinergic stimulants [21]. Therefore, it is plausible that MMP-3 is also directly involved in the transactivation process.

As we showed in our previous publication, stimulation of HaCaT cells with CCh does not induce considerable endocytosis of the EGFR, which is different from the direct receptor stimulation with EGF [10]. The endocytosis behavior of the EGFR might be influenced by the respective ligand...
released and its dimerization partner. The EGFR dimerizes after ligand binding with either another EGFR monomer or with its family members ErbB2, ErbB3 or ErbB4. All receptors have different binding affinities for the different ligands and differ in their downstream signaling. ErbB2 is unable to directly interact with a ligand, whereas ErbB3 is defective in its kinase activity [22,23]. No dimerization of the EGFR with ErbB3 could be observed in HaCaT cells after cholinergic stimulation, whereas a weak interaction with ErbB2 was detectable. However, this interaction showed no difference between starved and stimulated cells. Therefore, the EGFR might form mainly homodimers upon CCh treatment.

An overview of the extended model of EGFR transactivation in cholinergically stimulated HaCaT cells is given in Figure 6.

Figure 6. Cholinergic transactivation of the EGFR in HaCaT cells and the subsequent activation of ERK1/2 and Akt. Stimulation of mAChRs results in MMP or ADAM activation and the release of an EGF-like ligand, which then activates the EGFR. The protease involved in this process is neither MMP-2 nor MMP-9 nor ADAM10 nor ADAM17. Upon ligand binding, the EGFR most likely homodimerizes. Downstream thereof, MAP kinases ERK1/2 and the Akt kinase are activated.

3. Experimental Section

3.1. Reagents

Human EGF and CCh were purchased from Sigma-Aldrich (Taufkirchen, Germany). SB-3CT was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GI 254023X was a kind gift from Andreas Ludwig (Institute of Pharmacology and Toxicology, RWTH Aachen University, Germany).

3.2. Cell Culture

HaCaT cells (obtained from Dr. Boukamp, German Cancer Research Center, Heidelberg, Germany) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies, Carlsbad, CA)
high glucose, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% non-essential amino acids (all from Life Technologies). The cells were grown in a humidified incubator at 37 °C and 8% CO₂.

3.3. Cell Stimulation and Inhibitor Treatment

HaCaT cells were grown until they reached 80%-100% confluency, and were then starved in serum-free medium for 24 h. The cells were stimulated with CCh (1 mM) or EGF (16 nM) for 30 or 15 min in serum-free medium. Control cells were treated with serum-free medium without any stimulant. Pharmacological inhibitors were added 30 min before stimulation. SB-3CT was used in 1, 5, 10 or 25 µM concentration, whereas GI 254023X was used as 1 or 10 µM.

3.4. Antibodies

Monoclonal mouse antibodies against pAkt (Ser473) and myc, as well as polyclonal rabbit antibodies against Akt and monoclonal rabbit antibodies against ErbB2 and ErbB3 were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies against pERK1/2 and EGFR, as well as rabbit polyclonal antibodies against ERK2 were from Santa Cruz Biotechnology. A mouse monoclonal antibody against GAPDH was obtained from Abcam (Cambridge, UK). Secondary antibodies goat anti-mouse and goat anti-rabbit coupled to horseradish peroxidase were obtained from Dako (Glostrup, Denmark).

3.5. Cell lysis, Gel Electrophoresis and Western Blot

Cells were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA and 1% Nonidet P-40 supplemented with protease inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate and 1 mM sodium fluoride. Protein concentration was measured with the protein assay reagent of Bio-Rad (Munich, Germany). Equal protein amounts were analyzed by SDS-PAGE and Western blot.

3.6. Co-immunoprecipitation

Cells were lysed in immunoprecipitation buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and 60 mM n-octyl-β-D-glucopyranoside supplemented with protease inhibitors. EGFR was precipitated with its specific antibody. A myc antibody was used as an IgG control. The antibodies (2 µg) were precoupled to 20 µl of magnetic Dynabeads Protein G (Life Technologies). Cell lysates were incubated with the antibody-coupled Dynabeads for 16 h at 4 °C.

3.6. Statistical Analysis

All experiments were performed three times, all independent from each other. For the statistical analysis, Western blot bands of phosphorylated proteins were quantified by densitometric analysis using Quantity One software (Bio-Rad) and normalized to the total amount of the respective protein. Data are shown as the mean ± SD. For statistical comparison, two-way analysis of variance (ANOVA) was employed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA).
4. Conclusions  

Cholinergic stimulation of HaCaT cells results in the transactivation of their EGFR. The EGFR in turn is necessary for the downstream activation of ERK1/2 and Akt. The employed signaling pathway includes a triple-membrane-passing signal accompanied by the activation of an MMP or ADAM [10]. We here show that the protease involved herein is not MMP-2, MMP-9, ADAM10 or ADAM17, even though these are commonly activated in GPCR-mediated transactivation processes. A recent publication from our group makes the involvement of MMP-3 very likely [21]. However, further experiments with specific MMP-3 inhibitors or an MMP-3 knockdown are necessary to clarify this possibility.

The dimerization partner of the EGFR has an influence on the fine tuning of the receptor’s signaling. Additionally, differential dimerization of ErbBs gives rise to a complex signaling network (reviewed in [24]). Using a co-immunoprecipitation approach, we could not detect the formation of heterodimers between EGFR and ErbB2 or ErbB3 upon cholinergic stimulation. However, further experiments should include ErbB4 to analyze if this EGFR family member might be the dimerization partner of the EGFR during cholinergic transactivation. To verify the formation of homodimers other experimental set-ups that allow kinetic measurements need to be chosen, e.g., bioluminescence resonance energy transfer (BRET).

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Author Contributions

WO performed the experiments, assembled the figures and wrote the manuscript draft. RT finalized the text. WO & RT were responsible for the design of the study and interpretation of the data. Both authors read and agreed with the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References and Notes


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