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EGF receptor transactivation is crucial for cholinergic MAP kinase signaling in human keratinocytes

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Non-Neuronale Cholinerge Systeme

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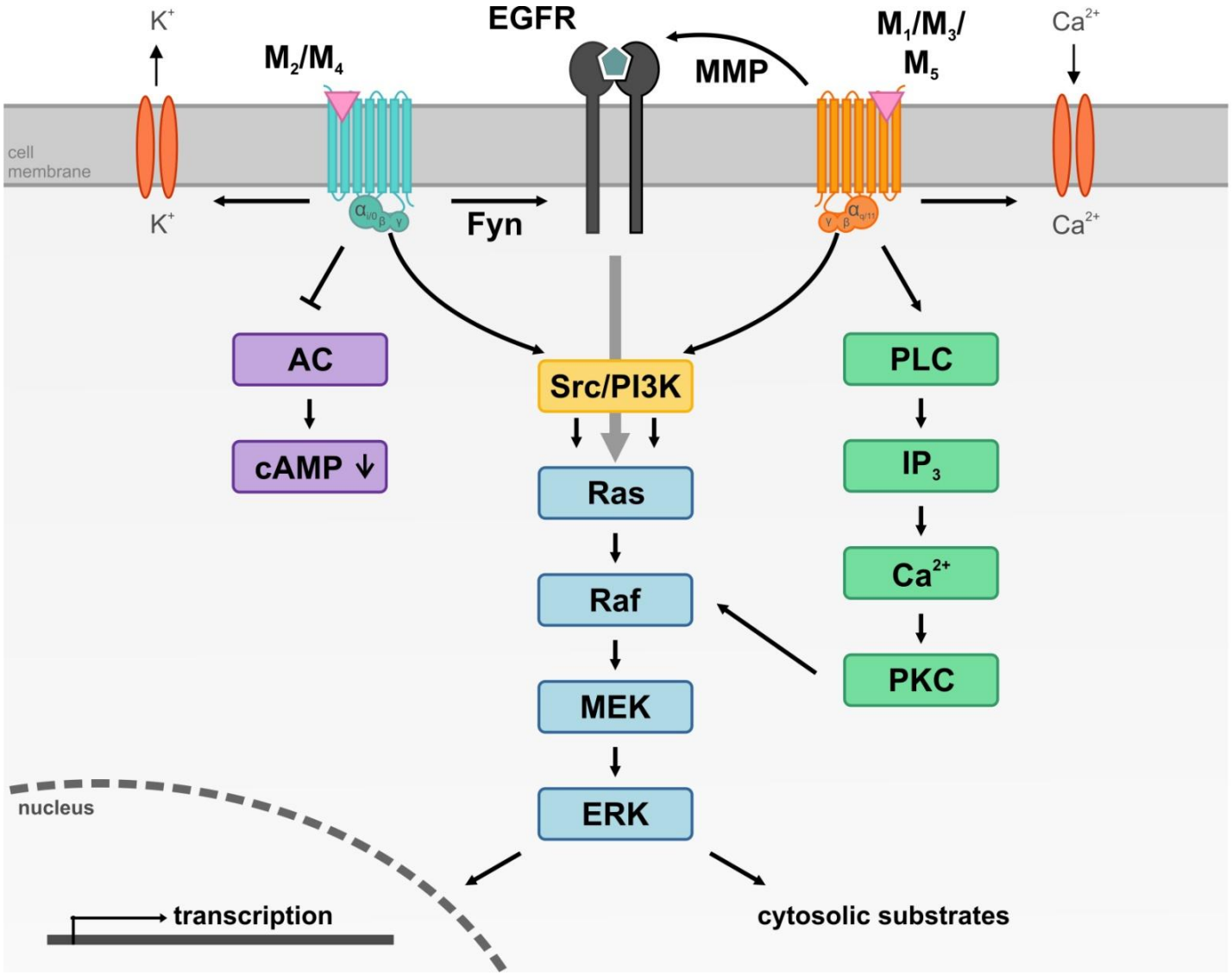
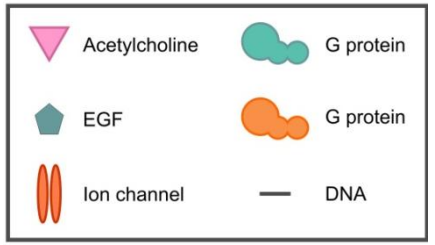
Canonical signaling of muscarinic receptors



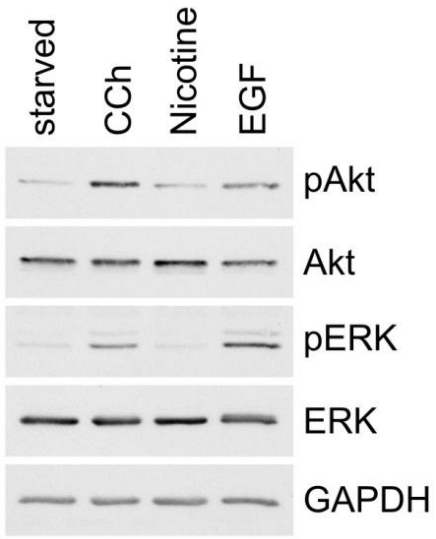


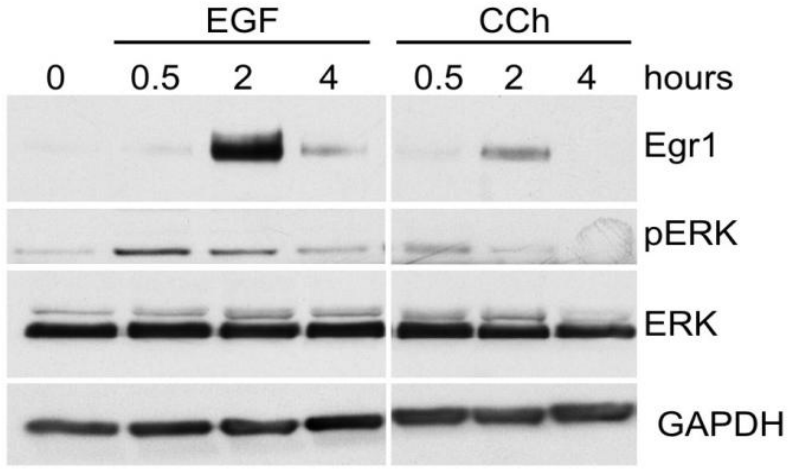
The non-neuronal cholinergic system is important in:

- skin
- urinary bladder
- immune system
- airways
- pancreas
- intestine
- blood circulation system
- heart

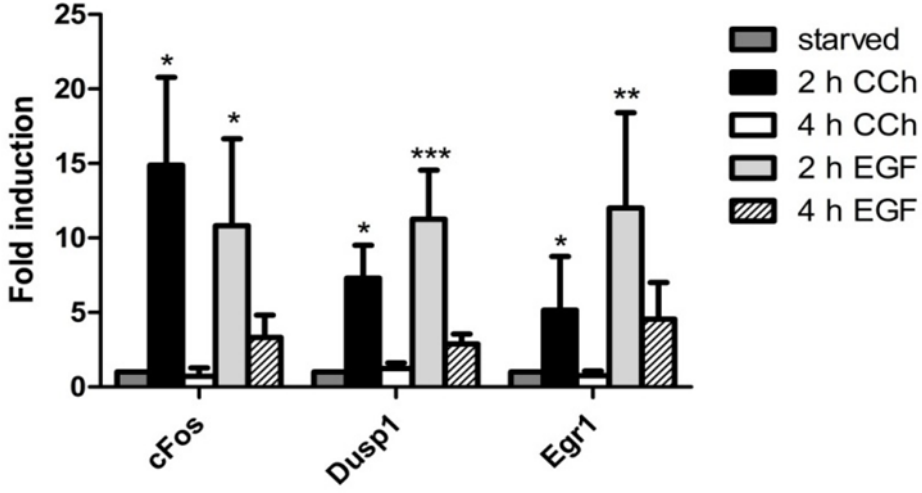
Ockenga et al., Non-Neuronal Functions of the M2 Muscarinic Acetylcholine Receptor, *Genes (Basel)* (2013), **4(2)**:171-197

ERK1/2 and Akt activation in HaCaT cells after cholinergic stimulation



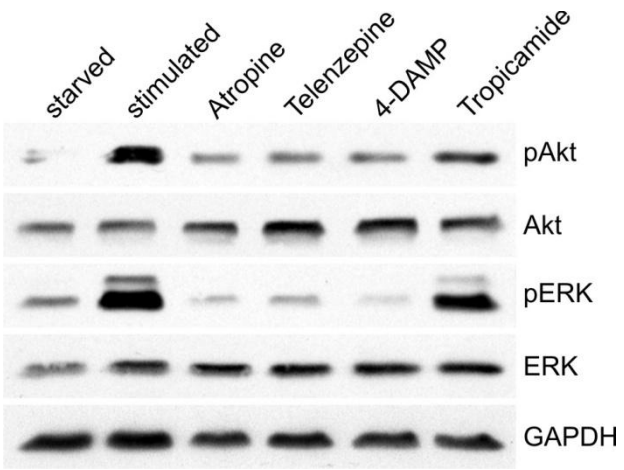


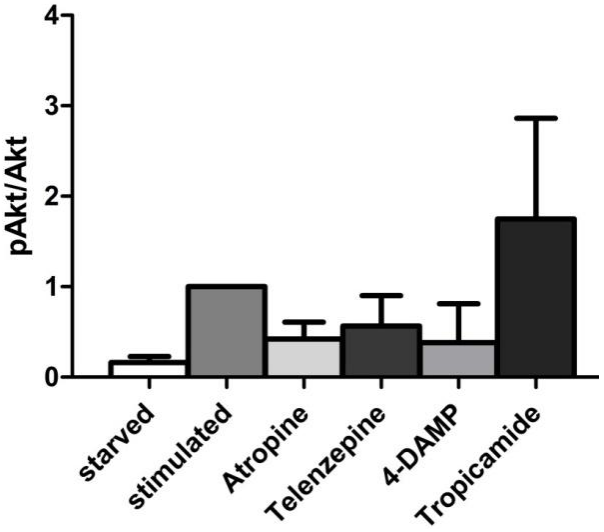
HaCaT cells were starved in serum-free medium over night and then stimulated with carbachol (CCh), nicotine or EGF (positive control). Only CCh but not nicotine induced the activation of ERK1/2 and Akt, which can be seen from the phosphorylation levels. The activation resulted in a transcriptional response increasing protein and mRNA levels of Egr1 and the mRNA levels of cFos and Dusp1.

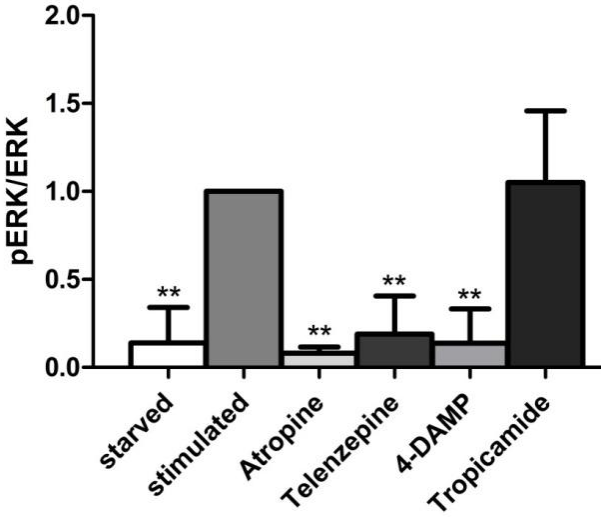


Ockenga et al., Epidermal Growth Factor Receptor Transactivation Is Required for Mitogen-Activated Protein Kinase Activation by Muscarinic Acetylcholine Receptors in HaCaT Keratinocytes, *Int J Mol Sci* (2014), **15**:21433-21454

Muscarinic receptors in ERK1/2 and Akt activation







Inhibitors: Atropine: all mAChRs; Telenzepine: M_1; 4-DAMP: M_3; Tropicamide: M_4

ERK1/2 and Akt signaling are mediated by mAChRs. The mAChR subtype-unspecific inhibitor atropine reduced their signaling. Various inhibitors, preferentially inhibiting certain subtypes revealed the involvement of M_1 and M_3, which is also the major receptor subtype in HaCaT cells (data not shown here).

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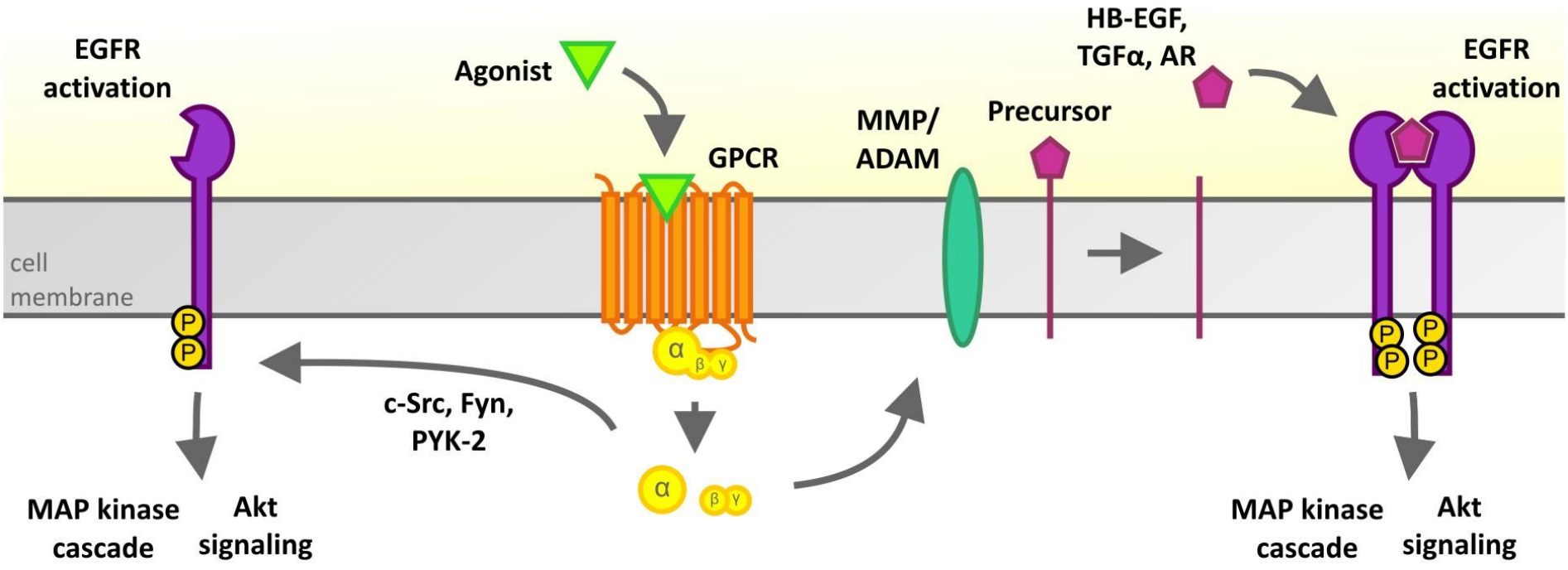
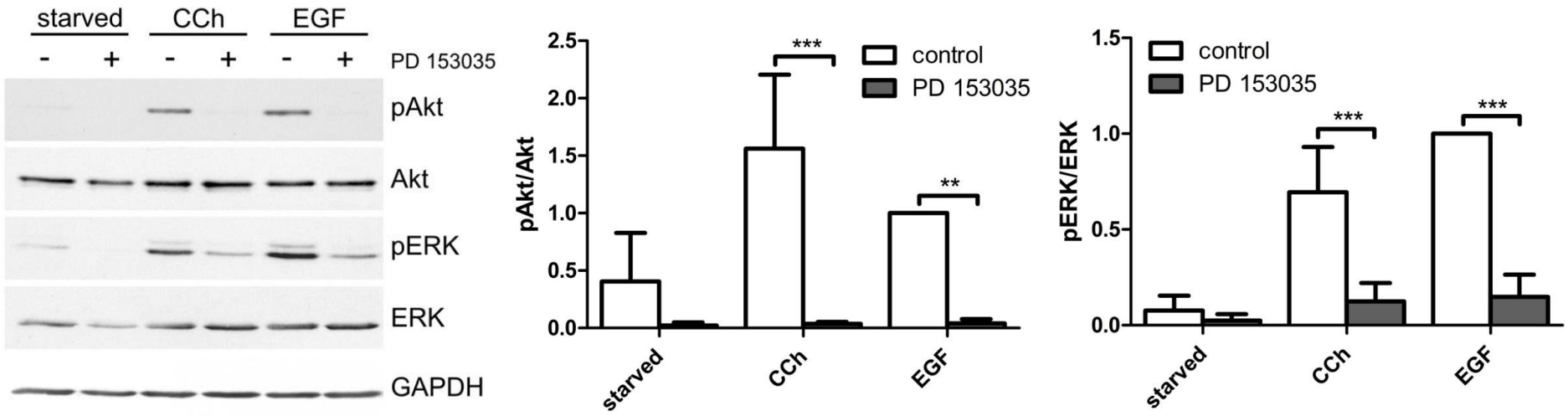
GPCR-mediated transactivation of the EGFR



- intracellular pathway
- protein kinases directly phosphory-late the EGFR
- activated EGFR mediates signaling

- triple-membrane-passing-signal
- MMPs or ADAMs are activated
- release of an extracellular ligand
- activated EGFR mediates signaling

Inhibition of EGFR kinase activity prevents cholinergic activation of Akt and ERK

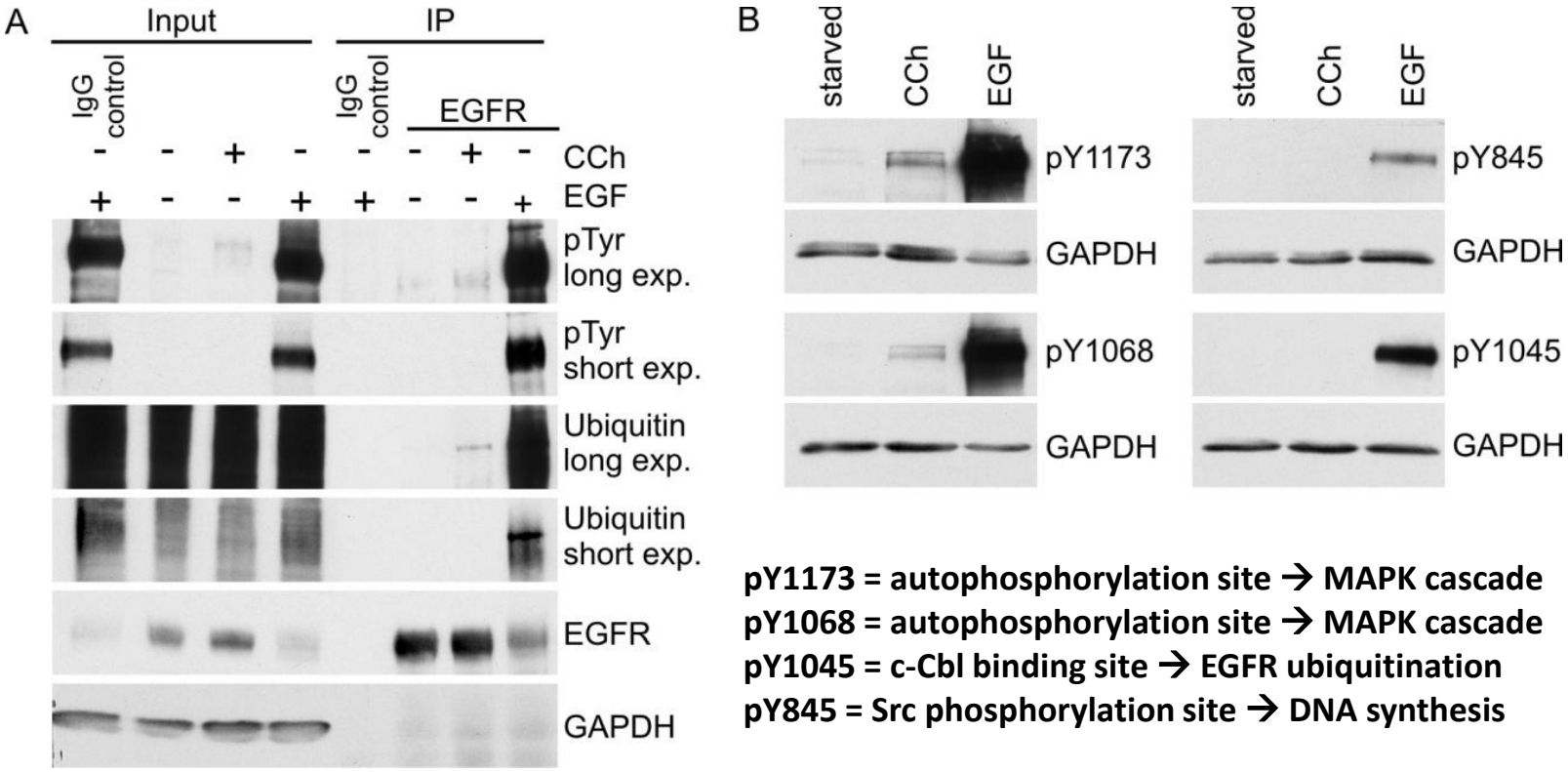


PD 153035 = specific inhibitor for the EGF receptor kinase

Inhibition of the EGFR kinase fully suppressed the activity of Akt (CCh-induced and basal one) and also significantly reduced ERK1/2 activation to the level of basal ERK phosphorylation (observed in starved cells). Thus, the activation of both Akt and ERK1/2 upon CCh stimulation in HaCaT cells is completely dependent on EGFR transactivation.

Ockenga et al., Epidermal Growth Factor Receptor Transactivation Is Required for Mitogen-Activated Protein Kinase Activation by Muscarinic Acetylcholine Receptors in HaCaT Keratinocytes, *Int J Mol Sci* (2014), **15**:21433-21454

Phosphorylation and ubiquitination of the EGFR.



pY1173 = autophosphorylation site → MAPK cascade
pY1068 = autophosphorylation site → MAPK cascade
pY1045 = c-Cbl binding site → EGFR ubiquitination
pY845 = Src phosphorylation site → DNA synthesis

CCh stimulation results only in weak phosphorylation or ubiquitination of the EGFR. Phosphorylation of Tyr-1173 and Tyr-1068 was detected after CCh stimulation, although it was much weaker than that induced by EGF. Consistent with the low degree of ubiquitination, CCh did not induce phosphorylation of Tyr-845, nor was any Tyr-P of Tyr-1045 observed.

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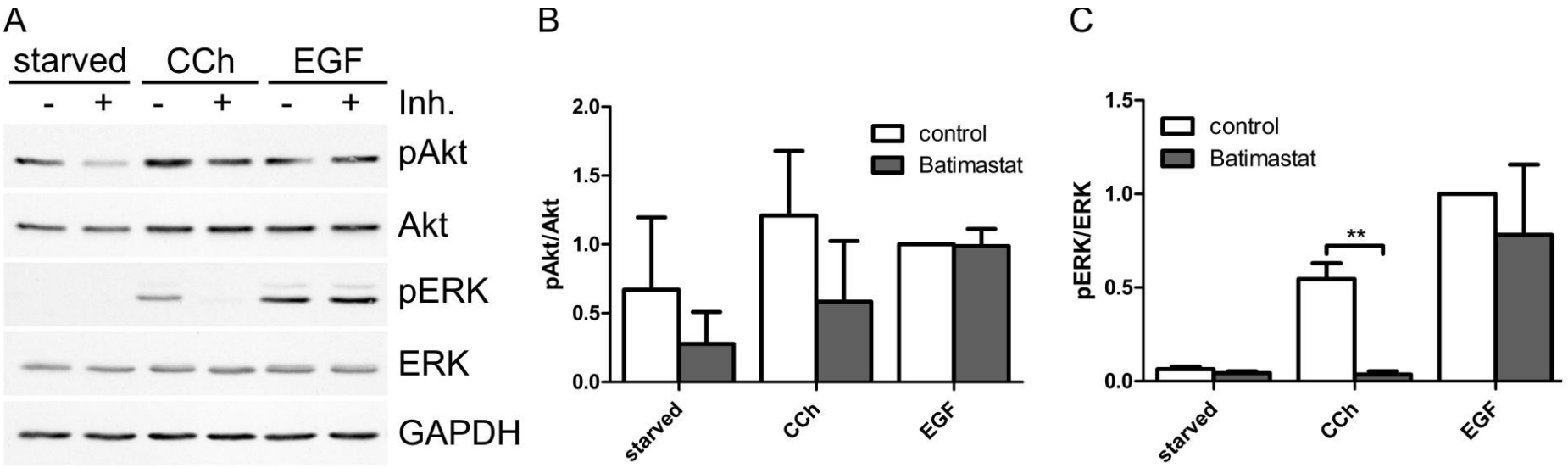
Cholinergic activation of ERK requires MMPs/ADAMs and extracellular ligand release.



Batimastat = broad spectrum inhibitor of MMPs and ADAMs

Inhibition of MMPs and ADAMs with the broad range inhibitor Batimastat resulted in almost complete inhibition of ERK1/2 activation upon CCh stimulation and a reduction of Akt activation by approx. 50%. This implicates that MMPs/ADAMs are involved in the transactivation of EGFR upon cholinergic stimulus in HaCaT cells. However, ERK1/2 activation is completely dependent on MMPs/ADAMs, whereas Akt is only partially dependent on these.

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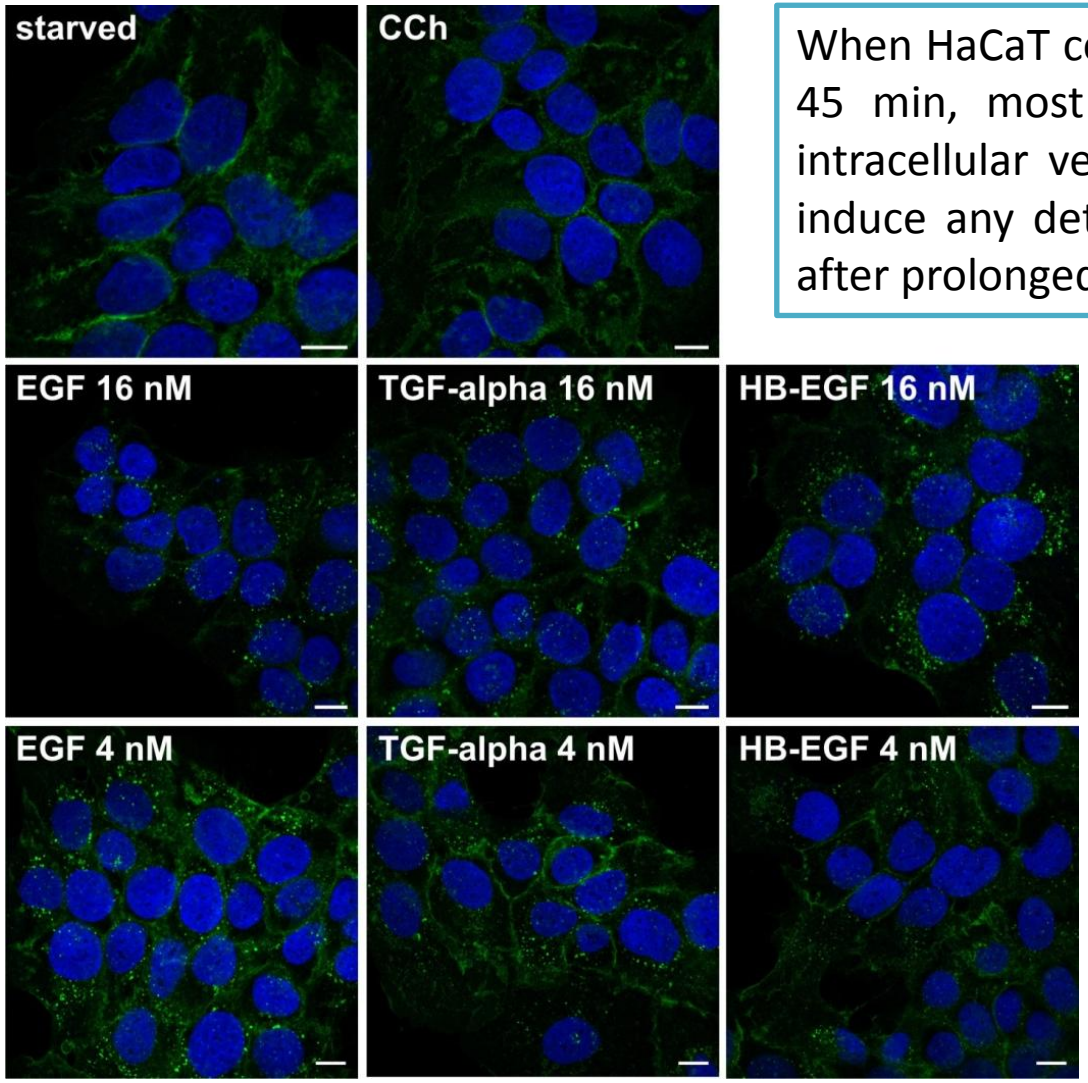
EGFR is internalized after EGF, TGF-α and HB-EGF but not after CCh treatment.

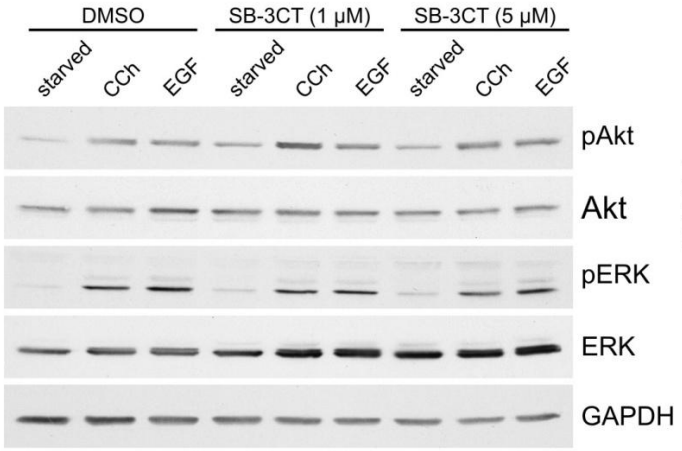


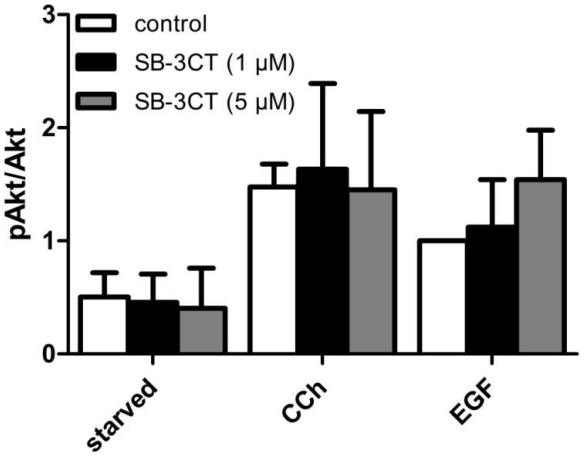
When HaCaT cells were stimulated with EGF for 15–45 min, most of the EGFR became localized in intracellular vesicles. However, CCh was unable to induce any detectable degree of endocytosis even after prolonged stimulation for 45 min.

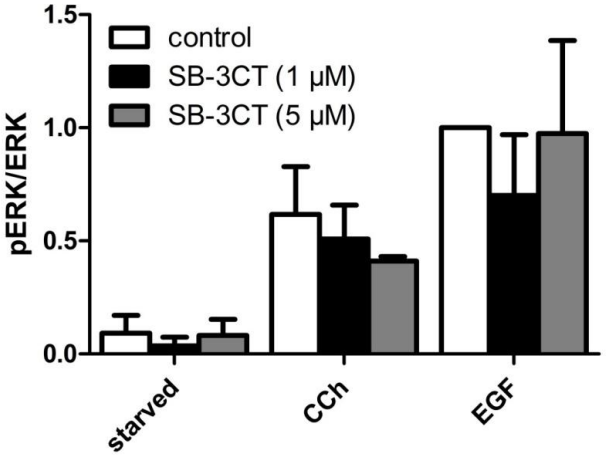
It is therefore likely that transactivation of EGFR in HaCaT is mediated by another ligand of the EGF-family. HB-EGF and TGF-α are typical ligands that bind to EGFR and have been shown to be involved in EGFR transactivation by other GPCRs. However, both TGF-α and HB-EGF induced EGFR endocytosis, implicating that they are unlikely to be responsible for the cholinergic EGFR transactivation.

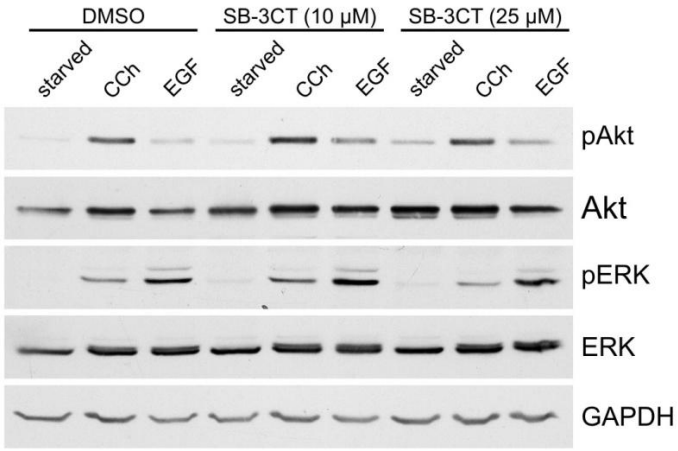
Ockenga et al., Epidermal Growth Factor Receptor Transactivation Is Required for Mitogen-Activated Protein Kinase Activation by Muscarinic Acetylcholine Receptors in HaCaT Keratinocytes, *Int J Mol Sci* (2014), **15**:21433-21454

MMP-2, MMP-9 and ADAM17 in transactivation





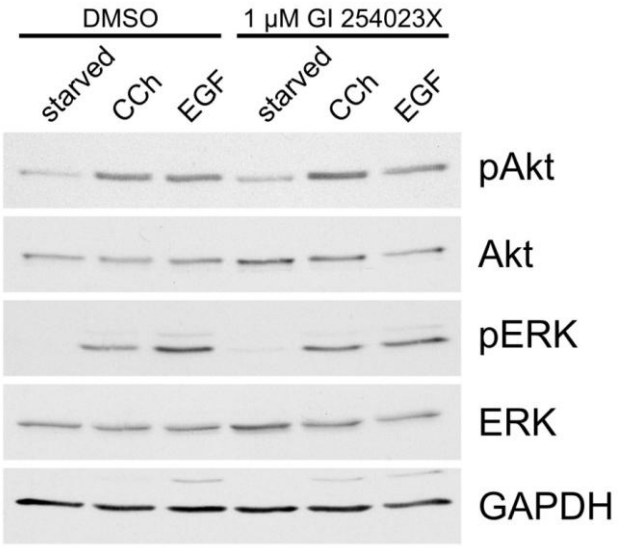




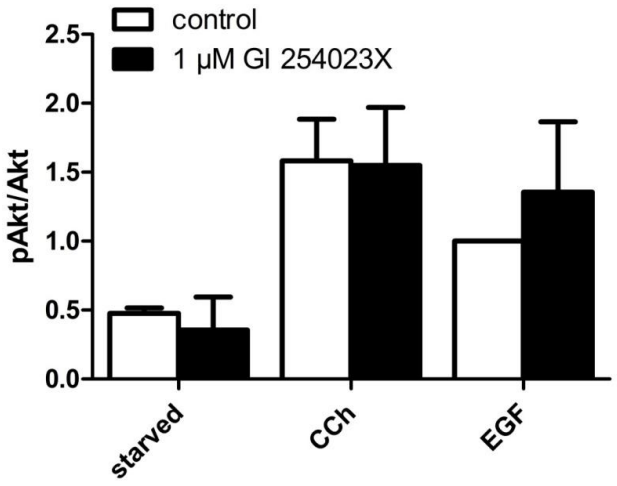
MMP-2, MMP-9 and ADAM17 are commonly known to take part in GPCR-mediated transactivation of the EGFR, in which they cause the extracellular release of EGF-like ligands. However, their inhibition does not affect the cholinergic transactivation of the EGFR and the downstream signaling in HaCaT cells.

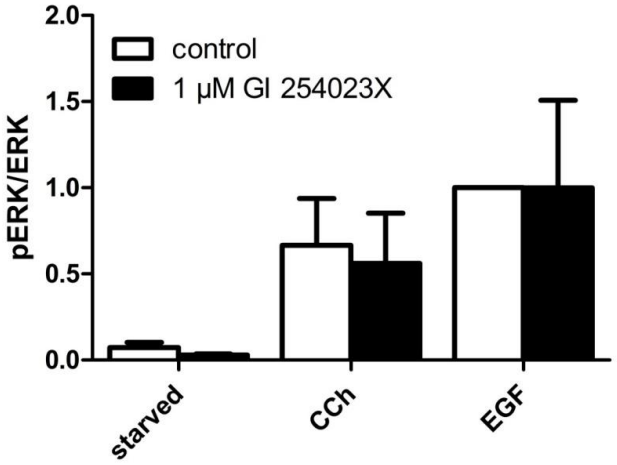
SB-3CT = Inhibitor for MMP-2/-9 and ADAM17 (with different K_i values: MMP-2/MMP-9 in nanomolar range, ADAM17: 4 µM)

ADAM10 in cholinergic transactivation



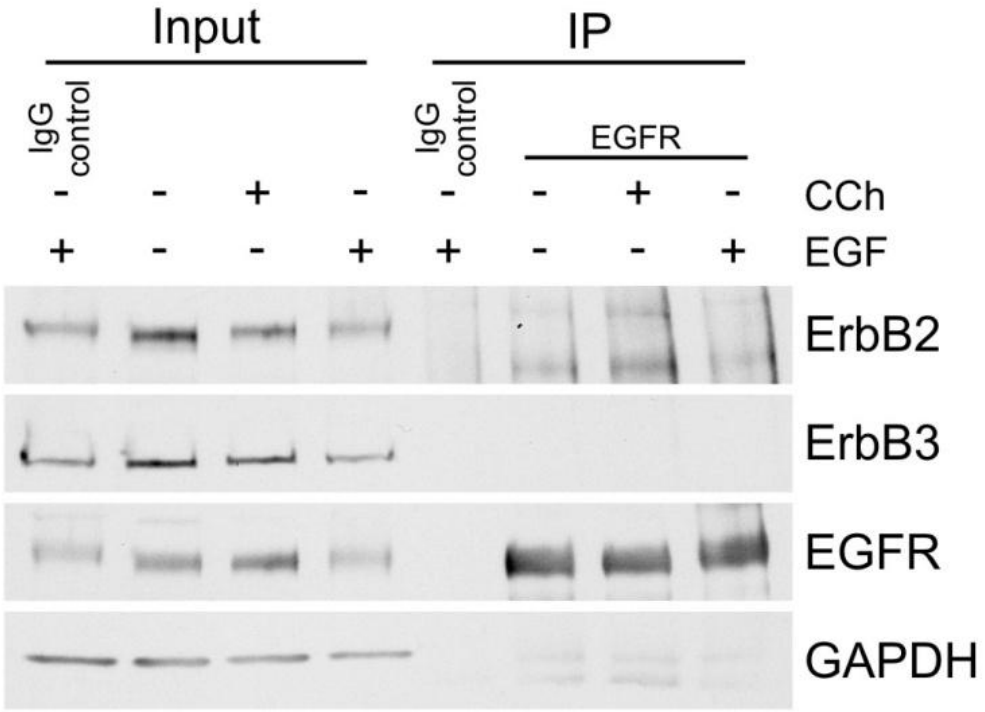
ADAM10 is also known for its function in GPCR-mediated EGFR transactivation. However, also preincubation with this inhibitor did not influence cholinergic signaling in HaCaT cells towards ERK1/2 and Akt.





GI 254023X = specific inhibitor for ADAM10

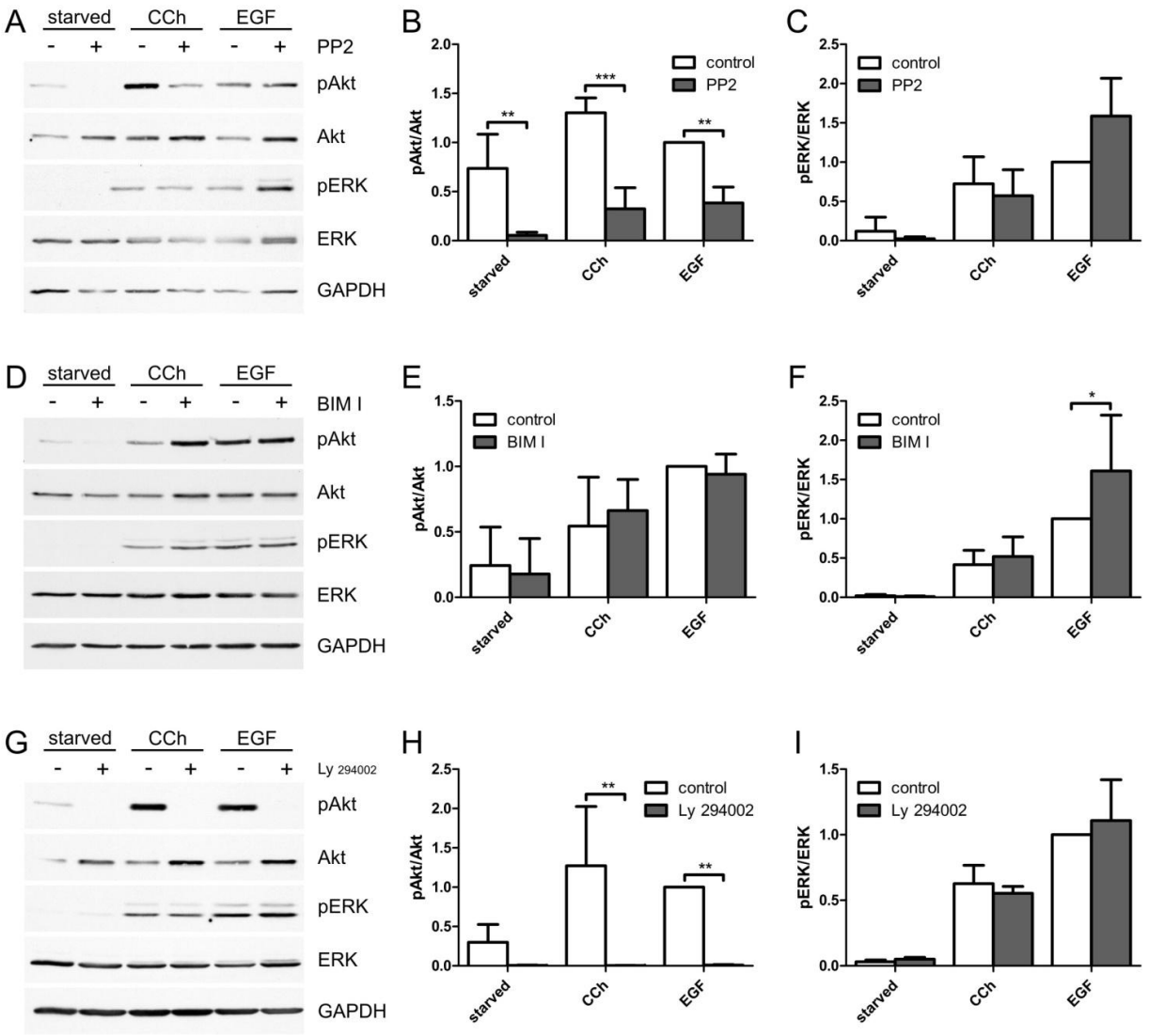
EGFR dimerization after CCh stimulation



Besides the ubiquitination and phosphorylation of the EGFR, the fine tuning of cholinergic signaling in HaCaT cells is most likely regulated by the interaction and the 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. 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.

Involment of protein kinases



Although inhibition of Src and PI3K reduced Akt activation, it showed no inhibitory effect on ERK1/2 activation. The PKC inhibitor affected neither Akt nor ERK activity. Thus, although both ERK1/2 and Akt activation by CCh are dependent on EGFR transactivation, the signaling pathways downstream from the EGFR differ.

PP2 = Src family kinase inhibitor
BIM I = PKC inhibitor
Ly 294002 = PI3K inhibitor

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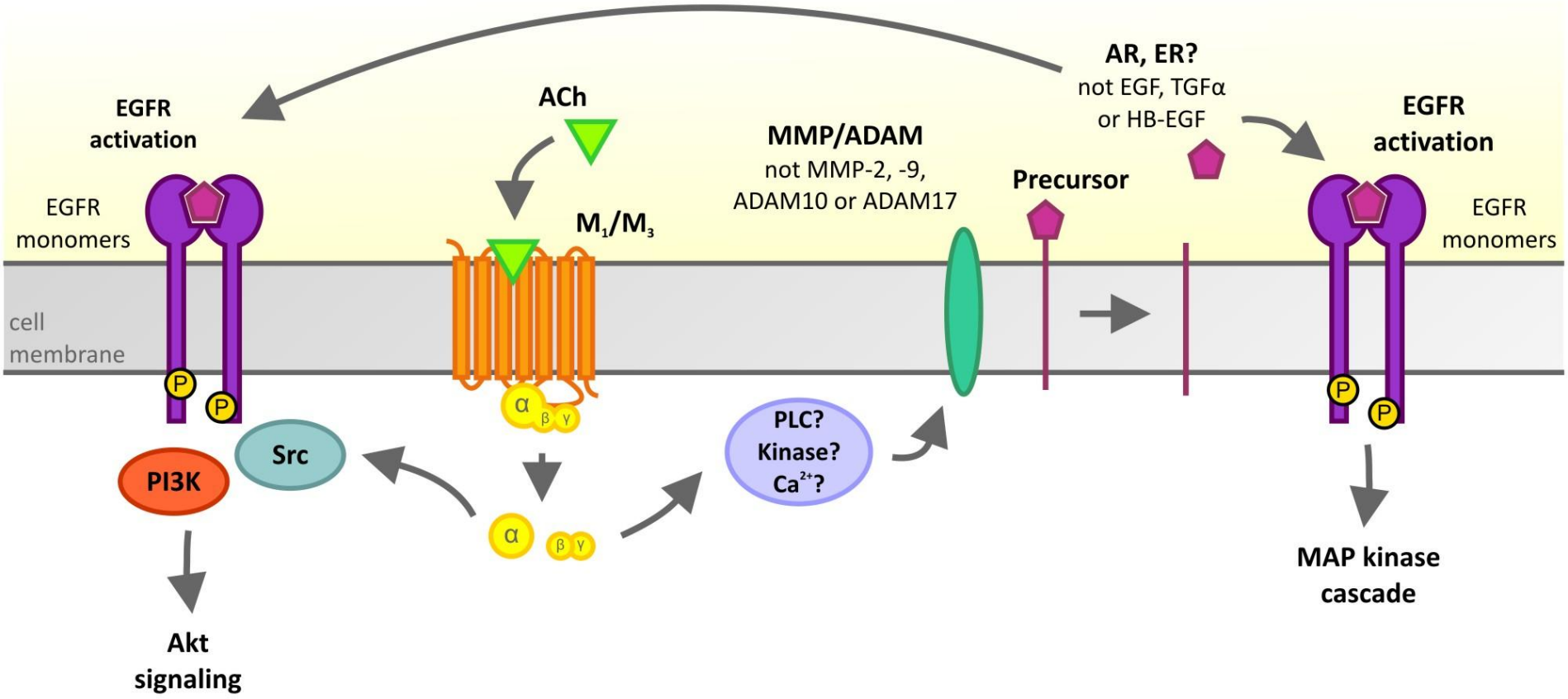
Extended model of EGFR transactivation in CCh-stimulated HaCaT cells



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 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.