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Muscarinic M2 receptors mediate transactivation of EGF receptor through Fyn kinase and without matrix metalloproteases

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Abstract

Transactivation of epidermal growth factor receptor (EGFR) by G protein-coupled receptors (GPCRs) has been attributed to the activation of matrix metalloproteases (MMPs) and the release of EGF family ligands such as HB-EGF. This mode of transactivation leads to signalling downstream of EGFR which is indistinguishable from that induced by the ligand. Here we provide evidence that in the COS-7 cell model EGFR transactivation via the muscarinic M2 receptor (M2R) is independent of MMPs and results in an incomplete EGFR signalling including ERK and Akt but not PLCγ1. Using dominant-negative mutants of c-Src and Fyn and Src-deficient SYF cells as well as by co-immunoprecipitation studies, we can demonstrate that the M2R-mediated transactivation of EGFR specifically involves Fyn but not c-Src or Yes. This specific role of Fyn can be verified in SH-SY5Y human neuroblastoma cells with endogenously expressed M2 receptors.

Keywords: M2 receptor; EGFR transactivation; Matrix metalloproteases; Fyn; COS-7 cells; SYF cells; SH-SY5Y cells

1. Introduction

In the last decade evidence has accumulated involving G protein-coupled receptors (GPCRs) into the regulation of mitogen-activated protein kinases (MAPKs) and cell growth [1]. Numerous studies have shown that GPCRs may utilize receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) as intermediate signalling protein thereby mimicking the signalling pathways downstream the EGFR [1–3]. Thus, stimulation of various GPCRs with their agonists results in autophosphorylation of the EGFR and, subsequently, to the generation of its signal transduction elements, e.g., activation of extracellular-regulated kinases 1/2 (ERKs), activation of PLCγ1, or activation of the phosphoinositide 3-kinase (PI 3-K)/Akt pathway [4,5]. Previously designated as ligand-independent transactivation [6], in 1999 a novel mechanistic concept of EGFR transactivation has been introduced. According to that ligand-mediated transactivation pathway, stimulation of GPCRs may result in the activation of transmembrane matrix metalloproteases (MMPs) and, subsequently, the proteolytic release of EGF-like peptides such as heparin-binding EGF (HB-EGF) at the cell surface. Then, these ligands may activate RTKs of the EGFR family in an autocrine or paracrine manner [3,7,8]. The mechanisms whereby a MMP may be activated through GPCR-induced signals are not yet well understood. They may include Gα as well as Gβγ subunits of Gi or Gq family proteins, the non-receptor tyrosine kinases Src or Pyk2, Ca^{2+}, or protein kinase C (PKC) [3,7,9].

A prototypic receptor which has been shown to induce EGFR transactivation via release of HB-EGF is the receptor for lysophosphatidic acid (LPAR) [7,10,11]. In the case of muscarinic acetylcholine receptors (MRs) consisting of five pharmacologically distinct subtypes, only the preferentially Gq protein/PLCβ/PKC coupled M1R/M3R subfamily has been clearly demonstrated to activate ERKs via EGFR transactivation

Abbreviations: GPCR, G protein-coupled receptor; M1R, M2R, M3R, muscarinic acetylcholine receptor subtypes; RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MMP, matrix metalloprotease; Iso, isoproterenol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PKC, protein kinase C; PLCβ, phospholipase Cβ; PLCγ1, phospholipase Cγ1; PI3-K, phosphoinositide 3-kinase; Akt, proteinkinase PKB/Akt; c-Src, Fyn, Yes, Src-family tyrosine kinases; MBP,, myelin basic protein; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-D]pyrimidine; AG1478, [4-(3-chloroanilino)-6,7-dimethoxyquinazoline].

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including MMPs. The M1R was shown to induce tyrosine phosphorylation of EGFR in different cells [12,13] as well as to transactivate the EGFR by the release of HB-EGF [7]. In various cells, stimulation of M3R was found to result in EGFR transactivation and activation of ERK [14–16] also including the proteolytic processing of EGF family members, e.g., TGFα in colonic epithelial cells [17]. In contrast, apart from the evidence that stimulation of the Gi protein-coupled M2 receptor leads to tyrosine phosphorylation of EGFR and thereby activation of ERKs [12], the mechanism whereby M2R signalling transactivates EGFR is not yet characterized. Furthermore, although it has been demonstrated previously that M2R-mediated activation of ERKs includes βγ-complexes as well as Src family member [18,19], it is not yet clear which type of Src kinase might be critically involved.

In the present study we examined the transactivation of EGFR and the subsequent downstream signalling by M2R. We demonstrate that in COS-7 cells transiently expressing M2R, the carbachol-induced EGFR signalling is independent of both activation of MMPs and the release of HB-EGF. Using various experimental approaches, we can further show that in our cellular model the transactivation of EGFR by M2R is specifically mediated by Fyn and does not involve c-Src or Yes. The critical role of Fyn in M2R signalling to the EGFR/ERK pathway was confirmed in SH-SY5Y neuroblastoma cells endogenously expressing M2R. Furthermore, compared with the EGF-induced activation of EGFR, the results also indicate that the ligand-independent transactivation of EGFR by M2R leads to incomplete EGFR downstream signalling.

2. Materials and methods

2.1. Cell culture and transfection

COS-7 cells, SH-SY5Y cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and SYF (c-Src−/−Yes−/Fyn−/−) as well as SYF+ (SYF cells, in which c-Src had been reintroduced) (from ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Subconfluent cells were transiently transfected with the cDNAs expression vectors coding for EGFR-WT (from Dr. F.-D. Boehmer, University of Jena, Germany), the dominant negative mutant c-SrcRF (K295R/Y527F) (from Dr. Joan Brugge, Harvard Medical School, USA), and/or the cDNA of Fyn-wt, the dominant-negative mutant FynK299M, and the inactive mutants FynΔSH3 and FynΔSH2 (from Dr. Filippo Giancotti, Sloan-Kettering Institute for Cancer Research, New York, USA). EGFR-ΔCR1 (Δ242–259) was kindly provided by Dr. A.W. Burgess, Ludwig Institute for Cancer Research, Melbourne, Australia. As indicated, cells were transfected with cDNA encoding muscarinic M2 or M3 receptors (Guthrie Resource Center, Sayre, PA, USA). For some experiments, cells were co-transfected with HA-tagged ERK2. Transfections were performed by the DEAE-dextran technique or using Lipofectamine 2000 as well as POLYPLUS as indicated.

2.2. Cell stimulation and preparation of lysates

For preparation of lysates, cells (transfected cells 2 days after transfection) were exposed to serum-free medium for 20 h and then left untreated or stimulated with the various agents as indicated. For some experiments, cells were pre-treated with the inhibitors AG1478, PP2, GM6001 or CRM197 for 30 min. Then, cells were washed in cold phosphate-buffered saline (PBS) and lysed at 4 °C in a buffer containing 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), 20 μg/ml aprotinin, and 20 μg/ml leupeptin. The lysates were centrifuged at 14,000×*g* for 5 min at 4 °C. Equivalent expression levels of cDNA constructs were verified by Western blotting with the appropriate antibodies.

2.3. Immunoprecipitation and Immunoblotting

Lysates from treated and untreated COS-7 were immunoprecipitated with the respective antibodies and protein A-sepharose (3 mg/tube) for 2 h at 4 °C. Immunoprecipitates were washed three times with PBS buffer supplemented with 1% Triton X-100, subjected to SDS-PAGE and blotted onto PVDF membranes. For Western blotting, the ECL detection system from Amersham was used. Immunoblotting, stripping and reprobing were performed according to the respective manufacturers' instructions.

2.4. MAP kinase assay

For MAPK assay, endogenous ERK1 was immunoprecipitated with polyclonal anti-ERK1 antibody. For some experiments, epitope-tagged HA-ERK2 was immunoprecipitated using 12CA5 antibody. Bound proteins were washed three times with PBS supplemented with 1% Triton X-100 and 2 mM sodium vanadate, once with 0.5 M LiCl in 100 mM Tris–HCl, pH 7.5, and once with kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, and 0.5 mM sodium vanadate). Reactions were performed in 30 μl kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM unlabeled ATP, 3.3 μM DTT, and 1.5 mg/ml myelin basic protein (MBP) at 30 °C for 20 min. Reactions were terminated by addition of 1 vol. Laemmli buffer. Samples were boiled and proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (13% gels). Phosphorylated MBP was visualized by autoradiography.

2.5. Materials

[γ-^{32}P] ATP (3000 Ci/mmol) was obtained from Perkin Elmer Life Science, Germany. Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, antibiotic mixture, and the transfection reagent Lipofectamine 2000 were purchased from Invitrogen (Karlsruhe, Germany). POLYPLUS was obtained from Biomol, Hamburg, Germany. Enhanced chemiluminescence (ECL) detection reagent, Protein G-Sepharose, Protein A-Sepharose and Hybond polyvinylidene difluoride (PVDF) membrane were purchased from Amersham Biosciences. Phospho-ERK1/2, and phospho-Akt (Ser473) antibodies were from Cell Signaling Technology. The antibodies anti-PLCγ1, anti-ERK1, anti-ERK2, anti-Src, anti-Fyn, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, USA. Anti-Akt was from BD Biosciences, Heidelberg, Germany. EGFR antibody (clone 13G8) and anti-EGFR-pY845 were from NanoTools. Antikoerpertechnik GmbH, Teningen, Germany. The monoclonal EGFR-antibody mab425 was a generous gift of Dr. A. Sutter, Merck KGaA, Darmstadt, Germany. Anti-HA.11 (12CA5) was obtained from BAbCO, Berkeley, CA, USA, Myelin basic protein (MBP), EGF, isoproterenol, carbachol, lysophosphatidic acid, and standard chemicals were purchased from Sigma. The inhibitors AG1478, PP2, GM6001 and CRM197 were obtained from Calbiochem, Germany. Pirenzepine and AQ-RA741 were generous gifts from Dr. Karl Thomae Chemisch-Pharmazeutische Fabrik (Biberach, Germany). 4-DAMP was kindly provided by Dr. R.B. Barlow (Bristol, UK).

3. Results

3.1. Transactivation of EGFR by M2R leads to activation of ERK and Akt but not of PLCγ1 and is independent of MMPs

First, we addressed the EGFR pathway in COS-7 cells transiently expressing M2R by stimulation with carbachol. The results in Fig. 1 show that M2R mediates tyrosine phosphorylation (transactivation) of EGFR (Fig. 1A) and, subsequently, EGFR-dependent (because inhibited by AG1478) activation of