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Transcriptional response to muscarinic acetylcholine receptor stimulation: Regulation of Egr-1 biosynthesis by ERK, Elk-1, MKP-1, and calcineurin in carbachol-stimulated human neuroblastoma cells

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Abstract

Carbachol-mediated activation of type M_3 muscarinic acetylcholine receptors induces the biosynthesis of the transcription factor Egr-1 in human SH-SY5Y neuroblastoma cells involving an activation of extracellular signal-regulated protein kinase. Carbachol triggered the phosphorylation of the ternary complex factor Elk-1, a key transcriptional regulator of serum response element-driven gene transcription, and strikingly enhanced the transcriptional activation potential of Elk-1. Chromatin immunoprecipitation experiments revealed that Elk-1 binds *in vivo* to the 5′-upstream region of the Egr-1 gene in carbachol-stimulated neuroblastoma cells. Together, these data indicate that Elk-1 connects the intracellular signaling cascade elicited by activation of M_3 muscarinic acetylcholine receptors with the transcription of the Egr-1 gene. Lentiviral-mediated expression of either MAP kinase phosphatase-1 (MKP-1) or a constitutively active mutant of calcineurin A inhibited Egr-1 biosynthesis following carbachol stimulation, indicating that these phosphatases function as shut-off devices of muscarinic acetylcholine receptor signaling. Additionally, carbachol stimulation increased transcription of a chromatin-embedded collagenase promoter/reporter gene, showing that AP-1 activity is enhanced in carbachol-stimulated neuroblastoma. Expression experiments revealed that both MKP-1 and a constitutively active mutant of calcineurin A impaired carbachol-induced upregulation of AP-1 activity. The fact that carbachol stimulation of neuroblastoma cells activates the transcription factors Egr-1 and AP-1 suggests that changes in the gene expression pattern are an integral part of muscarinic acetylcholine receptor signaling.

Keywords: AP-1; Calcineurin; Egr-1; Elk-1; MKP-1; Muscarinic acetylcholine receptor; Serum response element

In the central nervous system, the predominant receptors for the neurotransmitter acetylcholine are the muscarinic acetylcholine receptors. In the peripheral nervous system, cells stimulated by postganglionic parasympathic neurons use muscarinic acetylcholine receptors to perceive the neurotransmitter. The muscarinic acetylcholine receptors belong to the family of G-protein coupled receptors. The M_1, M_3, and M_5 receptor subtypes of muscarinic acetylcholine receptors preferentially couple with $G\alpha_{q/11}$ to activate an intracellular signaling cascade involving the activation of phospholipase C and the subsequent hydrolysis of phosphoinositides. The M_2 and M_4 subtypes are linked preferentially with G_i or G_o, leading to an inhibition of adenylate cyclase.

Many pharmacological studies have used SH-SY5Y neuroblastoma cells to study muscarinic receptor signaling. The cells express a relatively high density of pharmacologically homogenous M_3 muscarinic acetylcholine receptors [1–7]. The signal transduction cascade following M_3 muscarinic acetylcholine receptor stimulation has been elucidated. Together, activation of phospholipase C, generation of IP_3, elevation of the intracellular cellular

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Ca^{2+} concentration, activation of protein kinase C, transactivation of the EGF receptor, and phosphorylation and activation of extracellular signal-regulated protein kinase (ERK)[1] have been described as results of receptor stimulation [8–10].

Elevation of intracellular Ca^{2+} concentration and activation of ERK is frequently coupled with changes to the gene expression pattern. Stimulation of $P2X_7$ ionotropic receptors, for example, leads to the activation of the transcription factors NF-κB, NFAT, and Egr-1 [11–13]. Moreover, muscarinic receptor activation has been shown to trigger a mitogenic signal via activation of ERK [14]. Naturally, induction of proliferation is based on changes in the gene expression pattern of the cells. Accordingly, enhanced *in vitro* DNA-binding activity of AP-1 and induction of Egr-1 biosynthesis has been reported in muscarinic acetylcholine receptor expressing cells upon stimulation with carbachol [15–18]. Both Egr-1 and AP-1 connect cellular signaling cascades with changes in the gene expression pattern.

Here, we have analyzed the signaling cascade leading to enhanced Egr-1 expression in carbachol-stimulated SH-SY5Y neuroblastoma cells. Our studies were focused on nuclear events required for Egr-1 biosynthesis and on shut-off devices of the signaling chain. Stimulus-mediated transcription of the Egr-1 gene was impaired in cells overexpressing MKP-1 or a constitutively active mutant of calcineurin A. We further show that carbachol-induced stimulation of Egr-1 gene transcription requires the ternary complex factor Elk-1 via two clusters of serum response elements within the 5′-upstream region of the Egr-1 gene.

Materials and methods

Cell culture and transient transfections

Human SH-SY5Y neuroblastoma cells were cultured and transfected as previously described [19]. Cells were incubated for 24 h in medium containing 0.05% serum before stimulation. Stimulation with carbachol (Sigma #C-4382, dissolved in water and used at a concentration of 100 μM) or EGF (10 ng/ml, Promega, Mannheim, Germany, #G5021, dissolved in H_2O as a 100 μg/ml stock solution) was performed as indicated. The following muscarinic receptor antagonists were used: The M_1/M_3 antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP, Tocris Cat. #0482, dissolved in DMSO); the M_3 muscarinic acetylcholine receptor antagonist *p*-fluorohexahydro-sila-difenidol hydrochloride (pFHHSiD, Sigma #H127, dissolved in ethanol), and the M_2 receptor antagonist gallamine triethiodide (Sigma #G8134, dissolved in water).

Lentiviral gene transfer

The lentiviral transfer vector pFUW-MKP-1 has been described elsewhere [20,21]. A 1-kb EcoRI fragment containing the coding region of calcineurin A, truncated at amino acid residue 399, was cloned into the lentiviral transfer vector pFUW [22], generating the lentiviral transfer vector pFUWΔCnA. Recombinant lentiviruses produced with this transfer vector encode a constitutively active Ca^{2+}-independent enzyme. A lentiviral vector encoding luciferase (pFUWluc2) was constructed using plasmid pFUW. To generate plasmid pFWColl-luc, a lentiviral transfer vector containing the human collagenase promoter (sequence from −517 to +63) upstream of the luciferase open reading frame, we exchanged the ubiquitin C promoter with the collagenase promoter. The lentiviral transfer vectors pFWEgr-1.1luc and pFWEgr-1.2luc encode the luciferase reporter gene under the control of 239 or 490 nucleotides of the human Egr-1 5′-flanking region, respectively. The viral particles were produced as previously described [20] by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein and the transfer vector.

Expression vectors

The GAL4 expression plasmids pFA2CREB and pFA2Elk-1 were purchased from Stratagene. The GAL4-c-jun expression plasmid pGAL4-c-Jun was a gift of Michael Karin, University of California, San Diego. The GAL4 fusion proteins encoded by these plasmids contained the following transcriptional activation sequences: amino acids 1–281 of CREB (GAL4-CREB), amino acids 307–428 of Elk-1 (Gal4-Elk-1), and amino acids 1–246 of c-Jun (GAL4-c-jun), respectively. The GAL4-responsive reporter gene pUAS[5]luc has been described elsewhere [23].

RNase protection mapping

Cytoplasmic RNA was prepared from SH-SY5Y and 293T cells and analyzed by RNase protection mapping as described previously [24]. Plasmid T7mAChRIII, used to synthesize a riboprobe that specifically hybridizes with human M_3 receptor mRNA, was generated by cloning a filled-in NcoI/EcoRV-fragment (nucleotides 3530–3764, Accession No. U29589.1) of plasmid pT7T3D-PacI-CHRM3 (IMAGE #2048532, obtained from the RZPD, Deutsches Ressourcenzentrum für Genomforschung) into Ecl136II-cut pGEM4. The plasmid pSP6-G3PDH was used to synthesize a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cRNA probe (Ambion). Antisense ^{32}P-radiolabeled riboprobes were synthesized with T7 or SP6 polymerase, respectively. The riboprobe and the protected fragments were resolved on 5% acrylamide gels containing 8 M urea. Hybridization with M_3 receptor and GAPDH mRNA protected fragments of 233 and 199 nucleotides, respectively, from RNase digestion.

Western blots

Nuclear extracts were prepared as described [25]. Twenty-micrograms of nuclear proteins were separated by SDS–PAGE and the blots were incubated with antibodies directed against Egr-1 (Santa Cruz, Heidelberg, Germany, #sc-110), Sp1 (Santa Cruz, Heidelberg, Germany, #sc-59), MKP-1 (Santa Cruz, Heidelberg, Germany, #sc-1199), or phosphorylated Elk-1 (Santa Cruz, Heidelberg, Germany, #sc-8406). To detect ERK and Phospho-ERK, 20 μg of proteins derived from whole cell extract preparations were separated on SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with an antibody directed against ERK (Santa Cruz, Heidelberg, Germany, #sc-153) or the phosphorylated form of ERK (Promega, Mannheim, Germany, #V8031). Immunoreactive bands were detected using the ECL plus system (Amersham).

Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation experiments were performed as described elsewhere [24] with modifications. The chromatin was sonicated three times for 30 s. Immunocomplexes were eluted from the beads with 2 × 250 μl of elution buffer at room temperature. The cross-linking was reversed with 300 mM NaCl. The digestion with proteinase K was performed in a buffer containing 14 mM Tris–HCl, pH 7.5, 7 mM EDTA, 0.7% SDS, and 20 μg of proteinase K (Roche, Basel, Switzerland, #3115836). The PCR fragments were purified on Qiaquick columns

[1] *Abbreviations used:* CnA, calcineurin A; Egr, early growth response; ERK, extracellular signal-regulated protein kinase; MKP, MAP kinase phosphatase; SRE, serum response element.