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ORIGINAL ARTICLE/ÖZGÜN ARAŞTIRMA

Muscarinic agonist, antagonists and signaling pathway inhibitors change c-Fos and cyclin D_1 expression in K562 cells

K562 hücrelerinde muskarinik agonist, antagonist ve sinyal ileti yolağı inhibitörleri c-Fos ve siklin D_1 ekspresyonlarını değiştirir

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ABSTRACT

Objectives: Muscarinic acetylcholine receptors (mAChR) belong to a family of G protein coupled receptors (GPCRs). These mAChRs regulate several important physiological functions by activating a wide variety of cellular signaling pathways. We have previously shown that muscarinic acetylcholine (M_2, M_3 and M_4) receptors are expressed in K562 cells. In this study, we investigated the effect of muscarinic agonist, antagonists and different signaling pathway inhibitors on c-Fos and cyclin D_1 transcripts, using reverse transcriptase polymerase chain reaction (RT-PCR) that allows changes of very rare transcripts to be monitored.

Material and Methods: Total RNA was prepared from K562 cells challenged with muscarinic agonist, antagonists and inhibitors. c-Fos and cyclin D_1 expression were determined by RT-PCR.

Results: We showed that treatment with muscarinic agonist, antagonists and inhibitors leads to changes in c-Fos and cyclin D_1 expression in K562 cells.

Conclusions: Our results suggest that muscarinic receptors regulate expression of c-Fos and cyclin D_1 genes in K562 cells via different signaling pathways.

Key words: Atropine, Carbachol (CCh), c-Fos, Cyclin D_1

ÖZET

Amaç: Muskarinik asetilkolin reseptörleri (mAChR) G protein ile kenetli reseptör ailesinin üyesidirler. mAChR farklı sinyal ileti yolakları aracılığı ile bazı mühim fizyolojik fonksiyonları düzenler. K562 hücrelerinde M_2, M_3 ve M_4 reseptörlerinin eksprese olduğunu önceki çalışmalarımızda gösterdik. Bu çalışmada muskarinik agonist, antagonist ve farklı sinyal ileti yolağı inhibitörlerinin c-Fos ve siklin D_1 ekspresyonuna etkileri araştırılmıştır

Gereç ve Yöntem: Muskarinik reseptör agonist, antagonist ve sinyal yolağı inhibitörlerinin c-Fos ve siklin D_1 ekspresyonuna etkileri ters transkriptaz polimeraz zincir tepkimesi (TT-PZT) kullanılarak analiz edilmiştir.

Bulgular: Muskarinik agonist, antagonistler ve sinyal yolağı inhibitörleri K562 hücrelerinde c-Fos ve siklin D_1 transkriptlerinde değişime neden olmuştur.

Sonuç: Sonuçlarımız K562 hücrelerinde muskarinik reseptör aracılı c-Fos ve siklin D_1. mRNA ekspresyonlarının farklı sinyal ileti yolları ile düzenlendiğini düşündürmektedir.

Anahtar kelimeler: Atropin, c-Fos, Karbakol, Siklin D_1

Introduction

Acetylcholine is a neurotransmitter in the nervous system but it serves also as a paracrine or autocrine factor in different cell types, where it is linked to functions like proliferation and cell differentiation [1-3]. mAChRs mediate a wide array of cellular responses to acetylcholine in the central nervous system (CNS) and in non-nervous tissues innervated by the parasymphatic nervous system [4,5]. Muscarinic receptors are involved in diverse actions, including inhibition of adenylate cyclase, breakdown of phosphoinositide, regulation of nitric oxide (NO) synthesis, change of Ca^{2+} levels and modulation of K channels [6-8]. Muscarinic cholinergic receptors can also induce cell proliferation, differentiation and transformation. These effects are cell type dependent and receptor subtype specific. Many cells express a mixture of muscarinic receptor

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transcripts [9]. Some studies indicate that only the M_1 and M_3 receptor subtypes demonstrate growth modulation [10,11]. Different researchers have suggested that nonneuronal acetylcholine and cholinergic agonists alter cell growth and proliferation of lymphocytes [12]. Costa et al. demonstrated that acetylcholine released from T-lymphocytes acts via the M_3 acetylcholine muscarinic receptor (mAChR) to trigger nuclear signaling and up-regulation of gene expression in T- and B-lymphocytes [9]. Activation of mAChRs and nicotinic acetylcholine receptors (nAChRs) on lymphocytes increases the intracellular Ca^{2+} concentration, stimulates c-Fos gene expression and NO synthesis [9]. Fuji et al. showed that stimulation of mACh receptors induces Ca^{2+} oscillations and up-regulates c-Fos gene expression in T- and B-cell lines [13]. mAChR activation alters Ca^{2+}, c-Fos and c-jun mRNA and protein levels in the glial cell line 1321N1 [9-14]. Stimulation of muscarinic receptors induces expression of c-Fos in different cell lines including astrocytoma cells, neuroblastoma cells [15-18]. Muscarinic receptor expression has been recently observed in human mononuclear cells (MNL) and in some leukemic cell lines [8, 19-21]. We have previously demonstrated the presence of M_2, M_3 and M_4 mAChRs and M_3 subtype mediated NO signaling in K562 chronic myelogenous leukemic cells [8]. We also showed that collagenase clostridium histolyticum (CCh)-treatment leads to changes in muscarinic M_2, M_3 and M_4 receptor transcripts as well as M_2 and M_3 protein levels [3] and enhances cyclic adenosine monophosphate (cAMP) accumulation in these cells [21].

Cyclin D_1 is an important regulator of G_1 to S-phase transition and an important cofactor for diverse transcription factors in different cell types [22]. Different studies showed that cyclin D_1 is a predominantly cytoplasmic protein in mammalian cancer cell lines [23]. The aim of this study was to examine muscarinic receptor mediated c-Fos and cyclin D_1 gene expression in K562 cells and to investigate the intracellular signaling pathways that couple receptor stimulation to these genes.

Materials and Methods

Carbamylcholine chloride (Carbachol), atropine, gallamine, tropicamide, 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; RPMI 1640 were from Sigma Chemical Co., St. Louis, MO, USA.; 2-aminoethoxydiphenyl borate (2APB), MEK1/2 inhibitor (UO126) were from Merck, Germany. Wortmannin was from Santa Cruz, CA.

Cell culture

K562 cells (American Type Culture Collection, VA, USA) seeded at $1X10^6$ cells/ml were maintained at 37°C in culture flasks in RPMI 1640 supplemented with 10% fetal calf serum, in a humidified atmosphere of 5% CO_2 and 95% air, with one half of the medium being replaced every 3-4 days. The cells were counted on a hemocytometer after dilution with 0.4% trypan blue. The experiments were performed in cell suspensions adjusted to reach a concentration of $1X10^5$ cells/ml. Cells were placed into medium with 10% serum added. The effects of muscarinic receptor agonist and antagonists were investigated on the levels of c-Fos and cyclin D_1 expression in K562 cells. Cells were pre-treated for 30 min with antagonists followed by CCh (100 µM) stimulation for 30 min. Cells grown on flask in serum-free medium were deprived of growth factors for 24 h before challenge with 100 µM CCh or its vehicle for 30 min. Inhibitors 2APB; inhibitor of $Ins(1,4,5)P_3$-induced Ca^{2+} release from ER, Wortmannin; inhibitor of phoshoinositide 3-kinase, UO126; selective inhibitor of both MEK1 and MEK2) were added 30 min prior to CCh. The cells were washed twice, resuspended in phosphate buffered saline (PBS) and centrifuged at 700 g for 5 min at room temperature.

RNA extraction: Total RNA was isolated by the guanidium thiocyanatephenol-chloroform extraction method, as previously described by Chomczynski and Sacchi [24]. Purity and quantitation were assessed by A_{260}/A_{280} ratios.

Reverse transcription: Reverse transcription of total RNA was carried out in a volume of 50 µl containing 0.2 mM deoxynucleoside triphosphates, 1.5-3.0 mM $MgCl_2$, 2-3 µg RNA, 50 pmol of primers and 10 ml 5XRT-PCR buffer (Roche One step RT-PCR kit). The following primers were used to amplify cyclin D_1(726bp), c-Fos (431bp) and β actin (660bp). Cyclin D_1, upstream 5′- CCC TCG GTG TCC TAC TTC AAA -3′ and downstream 5′- CAC CTC CTC CTC CTC CTC TTC-3; c-Fos, upstream, 5- CCT CAC CCT TTC GGA GTC CC-3′ and downstream 5′- CTC CTT CAG CAG GTT GGC AAT CT -3′. β actin, upstream 5′ - GAC GGG GTC ACC CAC ACT GTG CCC ATC TA-3′ and downstream 5′- CTA GAA GCA TTT GCG GTG GAC GAT GGA GG-3′[19, 25-27]. Amplification was carried out for a total of 30 cycles. After an initial denaturation step of 2 min at 94°C, amplification was carried out for a total of 30 cycles according to the following parameters: 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s, 30 cycles, 72°C for 4 min,1 cycle. As a negative control, the reverse transcriptase (RT) enzyme was omitted from the reaction mixture. Samples were electrophoresed on 2% agarose gels, containing 1 µg/ml ethidium bromide and were viewed under UV light. Gels were photographed and analyzed on Biodoc Analyze software (Whatman Biometra).

Data were presented as a sample experiment. The experiments were repeated twice (duplicated), yielding essentially identical results. Data were analyzed and graphics were drawn on GraphPad Prism 5 software. Data were presented as a percentage of the control.

Results

1. The effect of carbachol treatment on cyclin D_1 expression in K562 cells

Muscarinic agonist CCh treatment decreased cyclin D_1 expression in K562 cells. Preincubation for 30 min with the muscarinic antagonists atropine, gallamine and tropicamide reversed CCh effects on cyclin D_1 expression, in contrast to 4-DAMP, which had no effect (Figure 1 A,B).

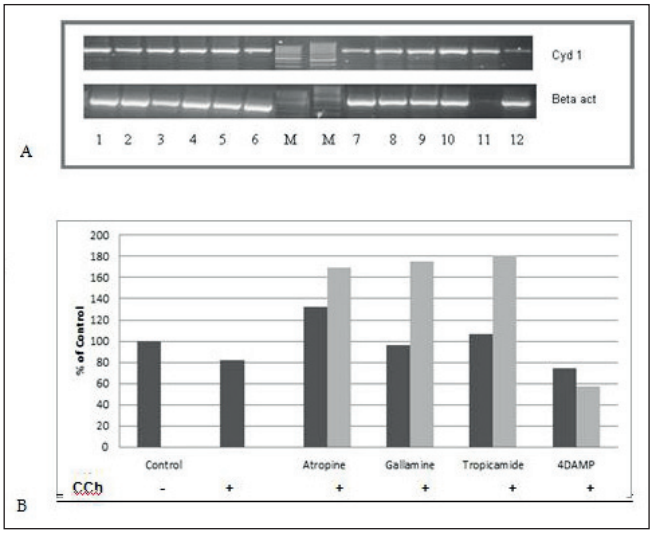

Figure 1 A, B. RT-PCR identified cDNA for cyclin D_1 mRNA levels in K562 cells. **A)** K562 cells treated with 100 µM CCh for 30 min. Antagonists were added 30 min prior to CCh. Data are presented as a sample experiment. 1) Control, 2) CCh, 3) Atropine, 4) Gallamine, 5) Tropicamide, 6) 4DAMP, 7) Control, 8) CCh, 9) (Atropine+CCh), 10) (Gallamine+CCh), 11) (Tropicamide+CCh), 12) (4DAMP+CCh). **B)** mRNA levels were normalized to β-actin mRNA levels. The experiments were repeated twice(duplicated), yielding essentially identical results. Data were presented as a percentage of control. PCR products of the expected sizes were as follows: Cyclin D_1 (726bp), and β-actin (660bp).

2. The effect of carbachol treatment on c-Fos expression in K562 cells

Carbachol led to a small decrease in c-Fos mRNA levels. The decrease induced by CCh on c-Fos mRNA levels was prevented by the muscarinic antagonist, tropicamide (Figure 2).

3. Effect of CCh and inhibitors on cyclin D_1 and c-Fos expression in K562 cells

To investigate the role of signaling pathways in CCh-mediated effects on cyclin D_1 and c-Fos expression, we challenged K562 cells with the calcium chelator 2APB, Phosphoinositide (PI) 3-kinase inhibitor, wortmannin, and MEK 1-2 inhibitor UO126. The inhibitory effect of CCh on c-Fos expression was fully reversed by UO126, whereas the

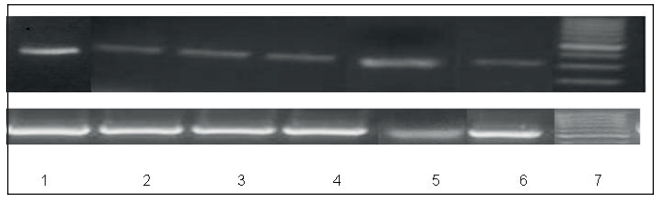

Figure 2. RT-PCR identified cDNA for c-Fos mRNA levels in K562 cells. K562 cells were treated with 100 µM CCh for 30 min. Antagonists were added 30 min prior to CCh. Gene transcript quantity was measured by relative RT-PCR 1) Control, 2) CCh, 3) (Atropine+CCh), 4) (Gallamine+CCh), 5) (Tropicamide+CCh), 6) (4DAMP+CCh), 7) Marker (M). Data are presented as a sample experiment. β-actin was used as the loading control. The experiments were repeated twice (duplicated), yielding essentially identical results. PCR products of the expected sizes were as follows: c-Fos (431bp) and β-actin (660bp).

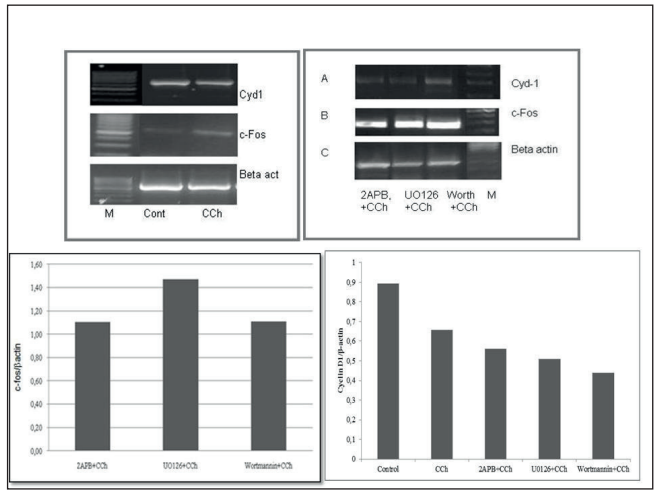

Figure 3 A, B. RT-PCR identified cDNA for Cyclin D_1 and c-Fos in cultured K562 cells. K562 cells were treated with 100 µM CCh for 30 min. Inhibitors were added 30 min prior to CCh. Gene transcript quantity was measured by relative RT-PCR using the internal standard β-actin RT-PCR signal. PCR products of cyclin D_1 and c-Fos were resolved on 2% agarose gels and visualized by ethidium bromide staining. **A)** Cyclin D_1 and c-Fos expression. Data are presented as a sample experiment. Inhibitors: Marker (M), Control (Cont), CCh; (2APB+CCh), (UO126+CCh) (Wortmannin+CCh), M. **B)** mRNA levels were normalized to β-actin mRNA levels. Data were presented as a percentage of control. The experiments were repeated twice (duplicated). PCR products of the expected sizes were as follows: Cyclin D_1 (726bp), c-Fos (431bp) and β-actin (660bp).

effect on cyclin D_1 expression was potentiated by all three inhibitors (Figure 3 A,B).

Discussion

Muscarinic receptors regulate multiple signaling pathways by activating G proteins. mAChR subtypes are widely expressed in the central and peripheral nervous systems [4, 28]. Previous different studies postulated that muscarinic acetylcholine receptors are functional in some hematopoietic cells [8, 21-29]. Our previous RT-PCR studies have shown

that M_2, M_3 and M_4 mAChR subtypes are expressed in K562 cells and suggested that they are involved in regulation of NO signaling and adenylate cyclase activity [8, 29]. This study was designed to test whether muscarinic agonist carbachol regulates cyclin D_1 and c-Fos expression in K562 cells.

We found that exposure of K562 cells supplemented with 10% serum to CCh led to an inhibition of DNA synthesis [3] and a decrease in cyclin D_1 expression. Inhibition of cyclin D_1 expression was fully reversed by the muscarinic antagonists atropine (non-selective), gallamine (M_2/M_4 selective) and tropicamide (M_4 selective) but not by 4-DAMP (M_3 selective). In order to explore the underlying intracellular signaling pathways involved in CCh-induced inhibition of cyclin D_1, K562 cells were treated with pathway specific inhibitors. Neither the calcium chelator 2APB, PI3 Kinase inhibitor, wortmannin, nor MEK 1-2 inhibitor UO126 reversed the inhibiton. On the contrary, inhibition was enhanced with all of the inhibitors. In a previous study by Nicke et al. carbachol-induced inhibition of DNA synthesis was detected in 3T3 cells and this effect was found to be accompanied with an increase in $p21^{cip1}$, a decrease in cyclin D_1 and E levels, and Rb hypophosphorylation [30]. This study also showed that the time course of cyclin D_1 degradation closely paralleled the observed inhibition of DNA synthesis.

Stimulation of muscarinic receptors are known to induce c-Fos expression in neuronal cell lines and brain regions [15]. Trejo et al. suggested that the mobilization of intracellular Ca^{2+} and activation of protein kinase C within the first minute of mAChR occupation are sufficient for induction of c-Fos and c-jun in a glial cell line [29, 31]. Muscarinic receptors have been shown couple to both growth-stimulatory and inhibitory signaling pathways depending on the cell model and the assay conditions [30]. Under conditions of DNA inhibition, CCh led to a slight decrease in c-Fos expression in K562 cells. CCh-induced inhibition of c-Fos was abrogated by the M_4 muscarinic antagonist, tropicamide. UO126 reversed the inhibition induced by CCh, suggesting that the muscarinic receptor induced effect on c-Fos gene may be regulated via the MEK 1-2 pathway.

In conclusion, our data imply that muscarinic receptors are involved in regulation of cyclin D_1 and c-Fos expression through different muscarinic receptor subtypes and signal transduction pathways in K562 cells.

Acknowledgments

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Declaration of interest: The authors report no conflicts of interest.

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