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Muscarinic receptor mediated cAMP response in human K562 chronic myelogenous leukemia cells

[İnsan K562 kronik myeloid lösemi hücrelerinde muskarinik reseptör aracılı cAMP cevabı]

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ABSTRACT

Objectives: Muscarinic acetylcholine receptors play key roles in regulating many diverse physiological processes. Recent studies suggest that muscarinic receptors mediate some cellular events in hematopoietic cells. Muscarinic receptors are expressed in different human cells. cAMP an intracellular signaling molecule, is involved in a wide variety of physiological functions and stimulation of muscarinic receptors leads to the alteration of intracellular cAMP levels. The present study investigated muscarinic receptors mediated cAMP level in K562 chronic myelogenous leukemic cells.

Methods: Muscarinic receptor mediated cAMP formation was measured by using the cAMP system colorimetric kit.

Results: Carbachol stimulated intracellular cAMP accumulation in K562 chronic myelogenous leukemic cells. The stimulatory effect of carbachol was abolished by atropine and tropicamide, while gallamine had little effect. The calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester inhibited effects of carbachol on the production of cAMP in K562 cells.

Conclusions: These results suggest that muscarinic receptor activation is linked to adenylate cyclase stimulation in K562 chronic myelogenous leukemic cells.

Key Words: Adenylate cyclase, signal transduction, carbachol

ÖZET

Amaç: Muskarinik asetilkolin reseptörleri farklı fizyolojik olayların düzenlenmesinde anahtar rol oynamaktadır. Son çalışmalarda muskarinik reseptörlerin hematopoietik hücrelerde bazı hücresel olayların düzenlenmesine aracılık ettiği önerilmektedir. Muskarinik reseptörlerin farklı insan hücrelerinde ekspresyonu olmaktadır. Hücre içi sinyal molekülü,cAMP çok sayıda fizyolojik fonksiyona katılmaktadır ve muskarinik reseptörlerin uyarılması hücreiçi cAMP düzeyinin değişimine neden olmaktadır.

Yöntem ve gereçler: Muskarinik reseptör aracılı cAMP oluşumu kolorimetrik kit kullanılarak ölçülmüştür.

Bulgular: Karbakol, K562 kronik myeloid lösemi hücrelerinde hücre içi cAMP oluşumunu uyardı. Karbakol'ün uyarıcı etkisi atropin ve tropikamid ile geri çevrilirken gallamin çok az etki gösterdi. Kalsiyum kelatörü 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraasetikasit Tetra (asetoksimetil) Ester (BAPTA/AM) K562 hücrelerinde cAMP üretiminde karbakol'ün etkisini inhibe etti.

Sonuç: Bu sonuçlara göre K562 hücrelerinde adenilat siklaz uyarılmasının muskarinik reseptör aktivasyonu ile bağlantılı olduğunu önermekteyiz.

Anahtar Kelimeler: Adenilat siklaz, sinyal iletimi, karbakol

Çıkar çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Cyclic AMP an intracellular signaling molecule, has been demonstrated in a variety of biological functions. Stimulation of muscarinic acetylcholine receptors (mAChR) cause the alteration of intracellular cAMP level by modulating the activity of adenylate cyclase [1,2]. Muscarinic acetylcholine receptors are members of the G-protein coupled receptor family (GPCR). Five muscarinic receptors have been identified, isolated and cloned in mammals [3-8]. Muscarinic receptors are coupled to a number of vital physiological functions such as smooth muscle contraction, neurotransmission, glandular secretion and cardiac contractility [2,9]. Muscarinic acetylcholine receptors regulate the activity of various effectors such as phospholipases C and D (PLC and PLD), adenylate cyclases , nitric oxide (NO) and ion channels [10,11]. The M_1, M_3 and M_5 receptor subtypes are coupled efficiently to the pertussis toxin-insensitive G_q family of G proteins, leading to activation of phospholipase C and D and subsequent hydrolysis of inositol 4,5-biphosphate (IP_3) [12,13]. M_2 and M_4 receptors inhibit adenylyl cyclase via the pertussis toxin (PTX)-sensitive G_i/G_o family of G proteins and cause only a modest stimulation of phosphatidylinositol (PI) hydrolysis when overexpressed [2,11,14]. M_1, M_3 and M_5 receptors were shown to stimulate adenylate cyclase in some cell lines [15,16].

Kurzen and colleagues have previously reported that the non-neuronal cholinergic system exists in a number of cell types, including the cells of the immune system [17]. Previous studies suggested that acetylcholine in blood has a role in the regulation of the immune system [18,19]. M_1 to M_5 muscarinic receptor subtypes have been shown to be present in human mononuclear leukocytes and in some leukemic cell lines [20-22]. K562 cells derived from human with chronic myelogenous leukaemia in blast crisis behave as pluripotent hematopoietic stem cells and are commonly used as model systems to study hematopoietic cell growth and differentiation [23]. The human leukaemic cell line K562 is a pluripotent stem cell with the potential to mature along a megakaryocytic or erythroid line [23]. Recent studies suggest that muscarinic receptors mediate some cellular events in hematopoietic cells [24].

Various studies support that muscarinic receptors are involved in the transformation of cells. On the other hand, muscarinic receptor mediated signaling pathway in hematopoietic cells are not clear. It is therefore necessary to further investigate and understand signal transduction pathways of chronic myelogenous leukemia cells. We previously demonstrated that the transcripts for M_2, M_3 and M_4 were present, whereas that for M_1 was absent in K562 cells [24]. We also demonstrated the muscarinic receptor M_3 subtype may mediate NO signaling in K562 erythroleukaemic cells [24]. The aim of this study was to investigate changes in intracellular cAMP levels when a K562 erythroleukaemia cell line is stimulated by the muscarinic agonist carbachol.

Materials and Methods

Materials

Carbamylcholine chloride (Carbachol), atropine, gallamine, tropicamide, were from Sigma Chemical Co.,St. Louis, MO, U.S.A.; RPMI 1640 and DMEM were from Sigma Chemical Co., St Louis, MO, USA.; cAMP kit assay system and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) were from Calbiochem, Germany.

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 $1X10^4$ cells/ml.

cAMP Assays

K562 cells were cultured in 96 well plates; 24 h before the cAMP experiments, the medium was changed to DMEM without supplements, and cell treatments were performed in triplicates. The cells were washed with serum free medium. The cells were incubated for 10 min with carbachol (10^{-8} to 10^{-3}M). To investigate the effects of the mAChR agonist and antagonists on cAMP basal levels, cells were treated 10 min with carbachol (10^{-4}M) in the absence and presence of one of the following mAChR antagonists: atropine, gallamine, or tropicamide, (10^{-5}M). These antagonists were added 5 min prior the incubation of cells with carbachol. We also used BAPTA/AM, as an intracellular Ca^{2+} mobilization blocker. K562 cells were treated with BAPTA/AM (50µM) (30 min) and then treated with carbachol (10 min). The cAMP level was measured with the colorimetric cAMP system kit (Calbiochem Germany) according to the manufacturer's instructions.

Statistical analysis

The results were expressed as mean values ± the standard error of the mean (S.E.M) of determinations from at least three independent experiments. Differences between means were analyzed by Student's *t*-test. All statistical tests were performed with the Prism program (Graphpad Software) and $P<0.05$ was considered significant.

Results

M_1, M_3 and M_5 subtypes are known to stimulate adenylate cyclase while M_2 and M_4 subtypes inhibit the enzyme. K562 cells were stimulated with agonist for various periods of time. The treatment of K562 cells with carbachol (10^{-4}M) caused increases in cAMP and the maximum response occurred within 10 min (data not shown).

Treatment of K562 cells with different concentrations of carbachol for 10 min increased the amount of cAMP. The maximum stimulatory effect was observed with the concentration of (10^{-4}M) with EC_{50} value of 7.709 X10^{-8} (n=3) (Figure 1).

Muscarinic antagonists were tested for their ability to antagonize the effect of carbachol (10^{-4}M) on cAMP accumulation. As shown in Figure 2, the basal cAMP content was increased by 51% after direct activation of adenylate cyclase with carbachol (10^{-4}M). The antagonists atropine (non-selective), gallamine (M_2/M_4–selective) and tropicamide (M_4-selective) at 10^{-5}M concentration reversed the stimulatory effect of carbachol on cAMP formation but these effects did not reach statistical significance (P>0.05).

Among these antagonists, atropine displayed the strongest effect, while gallamine was the least effective. Since stronger inhibition was obtained with the nonselective antagonist than the M_2/M_4–selective antagonists, we suggest that cAMP production is mediated by M_3 as well as M_2, and M_4 mAChRs.

To test the role of Ca^{2+} in carbachol mediated cAMP stimulation, we used BAPTA/AM, an intracellular calcium chelator. When K562 cells were treated with BAPTA/AM 50 µM for 30 min prior to the addition of carbachol (10^{-4}M, 10 min), the cAMP level was significantly reduced (Figure 3). These results show that intracellular Ca^{2+} is involved in muscarinic receptor mediated cAMP formation in K562 cells.

Discussion

Muscarinic receptors regulate multiple signaling pathways by activating G proteins. GPCRs are known to modulate adenylate cyclase activity via different mechanisms [25]. mAChRs are known to regulate the intracellular level of cAMP. Mammalian mAChRs stimulate

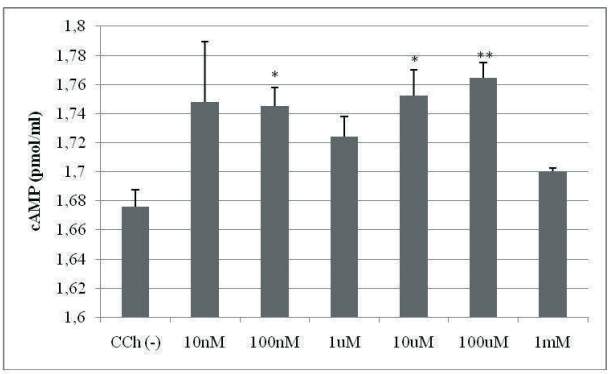

Figure 1: Agonist dose-response relationships for stimulating cyclic AMP accumulation in K562 cells. Cultures were incubated with varying concentrations of carbachol under the conditions described under Materials and Methods. The data are expressed as percent stimulation of basal cyclic AMP formation. The basal cyclic AMP concentration was increased by muscarinic agonist. Each bar and vertical line represent the mean ± SEM of three independent experiments. Significantly different from control (P<0.05, t test).

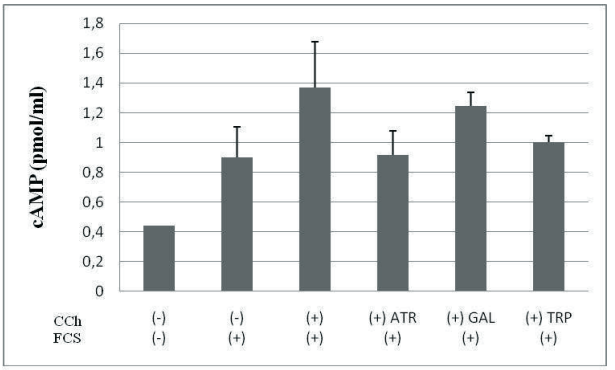

Figure 2: Effect of carbachol on intracellular cAMP levels in K562 cells. Cells were treated with carbachol (10^{-4}M 10min), in the absence and presence of muscarinic antagonists (10^{-5}M) Antagonists were added 5 min before the incubation of cells with carbachol. cAMP accumulation was measured as described in Materials and Methods. Each bar represents the mean ± SEM of three independent experiments. Compared with control (untreated cells).

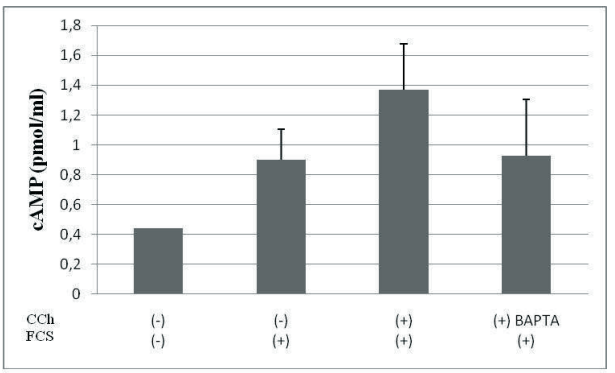

Figure 3: Involvement of Ca^{2+} in muscarinic receptor mediated cAMP production. K562 cells were treated with Ca^{2+}-chelating agent BAPTA/AM 50 µM for 30min before the addition of carbachol (100 µM 10 min). Intracellular cAMP levels were measured in intact cells. Data represent mean ± SEM of three independent experiments. (P<0.05)compared with carbachole alone using the t test

or inhibit cAMP formation in different cell types [26]. Several studies show that activation of M_1, M_3, and M_5 mAChRs can also stimulate adenylyl cyclase and inhibit cell proliferation in a number of cell types [27]. mAChR subtypes are widely expressed in central and peripheral tissues [12,28].

Four muscarinic receptors transcripts (M_2, M_3, M_4 and M_5) were identified in human erythroid progenitor cells cultured for 7 days [29]. Several studies show that both β-2 and muscarinic receptors of the immune cells suppress cellular immune responses [22]. Ricci *et al* reported that peripheral blood lymphocytes expressed muscarinic cholinergic receptors [30]. Their findings suggested the existence of autocrine/paracrine pathways in T-cell-dependent immune responses [30]

Previous studies postulated that muscarinic acetylcholine receptors are functional in some hematopoietic cells [21]. Our previous RT-PCR studies have shown that M_2, M_3 and M_4 mAChR subtypes are expressed in K562

cells [24]. This study showed that the presence of muscarinic receptors may be linked to adenylate cyclase in K562 cells. Carbachol induced an increase in intracellular cAMP accumulation in K562 cells, an effect reversed with the nonselective mAChR antagonist atropine, and the M_2/M_4-selective mAChR antagonists tropicamide and gallamine. The participation of Ca^{2+} in the activation of adenylate cyclase has been shown in different cell types [31-33]. We observed that the Ca^{2+}-chelating agent BAPTA/AM reversed the stimulatory effect of carbachol on cAMP formation in K562 cells. Therefore Ca^{2+} may be involved in the activation of adenylate cyclase by mAChRs in K562 cells.

Carbachol has been shown to inhibit adenylate cyclase activity in NG108-15 hybrid cells [34]. Meeker and Harden report that activation of muscarinic cholinergic receptors of 1321N1 human astrocytoma cells caused attenuation of cyclic AMP accumulation [35,36]. Tian *et al*. (2001) suggest that CCh caused concentration dependent stimulation of cAMP formation in pancreatic islets in the presence of low stimulatory concentration of glucose. The presence of BAPTA/AM decreased CCh-stimulated cAMP level by nearly 50% in pancreatic islets [37]. M_4 muscarinic receptors are coupled to inhibition of adenylyl cyclase activity in most neuronal tissues and cells, but M_4 receptors activation stimulated cAMP formation in the olfactory bulb [38]. On the other hand in M_4-transfected CHO cells, M_4 mAChR induced increases in cAMP with CCh. M_1, M_3, and M_5 receptors stimulated cyclic AMP formation in some cells [39,40]. Some researchers suggested that M_1, M_3, and M_5 receptor mediated cyclic AMP response may be downstream from the phosphoinositide response, resulting from calcium or protein kinase C activation of adenylate cyclase [26,34]. Carbachol can reduce the adenylyl cyclase activity in dog thyroid cells. Michal *et al* have presented evidence for direct coupling of muscarinic M_2 receptors to the subunits of G_s and $G_{11/q}$, resulting in stimulation of cAMP synthesis and IP_3 accumulation, respectively in M_2 CHO cell [41]. mAChR expressed in FRT cells. Montiel *et al.* suggested that cross talk between cAMP and PLC pathways is involved in the regulation of Ca^{2+} mobilization in FRT cells [42]. Watson *et al* demonstrated that an increase in intracellular Ca^{2+} stimulated cAMP accumulation via adenylyl cyclase 8 [43]. Muscarinic receptor activation results in increased intracellular calcium and upregulation of c-fos. Stimulation of T and B cells via M_3 and /or M_5 mAChRs induces intracellular Ca^{2+} signaling that triggers nuclear signaling and upregulates gene expression [44,45]. Fujii *et al.* suggested that these effects modulate leukemic cell function [22,44,45,46].

These contradictory cAMP responses of muscarinic receptor activation have been attributed to a number of factors such as differences in cell models, receptor level, the growth context of the cells.

Muscarinic receptors and ligands are important in different types of cancer, and muscarinic receptor mediated signaling plays an important role in cancer progression [46]. Muscarinic signaling is indicated in different types of malignancies. Expression of muscarinic receptors, ChAT, and CrAT, as well as ACh production, is reported in human lukemia cell lines [22,46].

In conclusion, our previous data demonstrated the expression of M_2, M_3 and M_4 mAChRs in K562 cells and that the M_3 subtype may mediate NO signaling in K562 chronic myelogenous leukemic cells [24]. Carbachol-induced intracellular Ca^{2+} release was maximal when 10^{-4}M concentration of agonist were applied to K562 cells and this effect was reversed by 4-DAMP (10^{-5}M) (unpublished data). Here, we suggest that M_2, M_3, M_4 receptors may be functionally important in adenylate cyclase activation in K562 cells and that Ca^{2+} is involved in this effect. These experiments will give insights for further understanding the role of muscarinic receptor mediated signal transduction in chronic myelogenous leukemia cells and possible therapeutic roles for muscarinic agonists and antagonists. Further investigations are needed to explain muscarinic receptor mediated signaling pathways, including the role of cAMP and other second messengers in the pathophysiology of diseases of hematopoetic cells.

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Declaration of interest:

The authors report no conflicts of interest.

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