

## Sp1 mediates phorbol ester (PMA)-induced expression of membrane-bound guanylyl cyclase GC-A in human monocytic THP-1 cells\*

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Cyclic guanosine monophosphate (cGMP) is synthesized by two types of enzymes: particulate (membrane-bound) guanylyl cyclases (pGCs) and soluble (cytosolic) guanylyl cyclases (sGCs). sGCs are primarily activated by binding of nitric oxide to their prosthetic heme group while pGCs are activated by binding of peptide ligands to their extracellular domains. One of them, pGC type A (GC-A) is activated by atrial and brain natriuretic peptides (ANP and BNP, respectively). Human monocytes isolated from peripheral blood mononuclear cells have been found to display sGC expression without concomitant expression of GC-A. However, GC-A activity appears in monocytes under certain conditions but a molecular mechanism of GC-A expression is still poorly understood. In this report we show that phorbol ester (PMA) induces transcription of a gene encoding GC-A in human monocytic THP-1 cells. Moreover, we find that PMA-treated THP-1 cells raise cGMP content following treatment with ANP. Studies using pharmacological inhibitors of protein kinases suggest involvement of protein kinase C (PKC), mitogen extracellular kinases (MEK1/2), and extracellular signal-regulated kinases (ERK1/2) in PMA-induced expression of the GC-A encoding gene in THP-1 cells. Finally, we show that PMA stimulates binding of Sp1 transcription factor to GC-rich DNA sequences and mithramycin A (a selective Sp1 inhibitor) inhibits expression of the GC-A mRNA in PMA-treated THP-1 cells. Taken together, our findings suggest that the PMA-stimulated PKC and MEK/ERK signaling pathways induce Sp1-mediated transcription of the GC-A encoding gene in human monocytic THP-1 cells.

**Key words:** membrane-bound guanylyl cyclase type A, phorbol 12-myristate 13-acetate, human monocytic cell line THP-1, protein kinases, Sp1 transcription factor

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**Abbreviations:** ANP, atrial natriuretic peptide; cGMP, cyclic guanosine monophosphate; ERK 1/2, extracellular signal-regulated kinases; GC-A, membrane-bound guanylyl cyclase type A; MEK 1/2, mitogen extracellular kinases; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; sGC, soluble guanylyl cyclase; Sp1, specificity protein 1

### INTRODUCTION

Guanylyl cyclase type A (GC-A) belongs to a family of particulate guanylyl cyclases (pGCs) which similarly to the soluble guanylyl cyclases (sGCs) catalyze synthesis of a common secondary messenger, namely cyclic GMP (cGMP), involved in many cellular processes (Pilz & Casteel, 2003; Potter *et al.*, 2006). Although both forms of GCs produce the same secondary messenger, activation of each of them triggers different signaling pathways leading to different cellular effects (Zolle *et al.*, 2000; Su *et al.*, 2005; Mitkiewicz *et al.*, 2011). This indicates that the final effect of cGMP depends on the site of its synthesis in the cell (cytosol or cell membrane). GC-A is a single membrane spanning receptor activated by binding of atrial natriuretic peptide (ANP) or brain natriuretic peptide (BNP) to the extracellular domain of the enzyme. The widespread expression of GC-A in different cell types and tissues (Potter *et al.*, 2006) suggests that this protein may regulate many cellular processes. The role of ANP and GC-A in the cardiovascular system is well documented. It has been shown that the ANP/GC-A system is implicated in reducing cardiac output, stimulation of natriuresis, diuresis, vasorelaxation and inhibition of the renin-angiotensin-aldosterone pathway (Pandey 2011; Potter *et al.*, 2006). The available data suggest that GC-A also regulates inflammatory reactions. Reports demonstrate anti-inflammatory action of this enzyme. It was found that the ANP-triggered GC-A/cGMP-signaling pathway decreases activity of AP-1 and NF- $\kappa$ B transcription factors in activated myeloid cells and thus suppresses release of inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF $\alpha$ ) and inhibits expression of inducible nitric oxide synthase (Kiemer *et al.*, 2000; Mitkiewicz *et al.*, 2011; Mezzasoma *et al.*, 2016). The anti-inflammatory action of ANP was shown to also occur in mice treated with LPS (Ladetzki-Baehs *et al.*, 2007; Nojiri *et al.*, 2014).

In a human monocyte subset purified from peripheral blood mononuclear cells (PBMCs) the sGC activity was predominately detected (Bender *et al.*, 2004). It was shown that human monocytes do not express GC-A, but monocyte-derived dendritic cells do express functional GC-A (Morita *et al.*, 2003). Moreover, Bender and coworkers (Bender *et al.*, 2004) stimulated differentiation of human monocytes and found that GM-CSF differentiated macrophages, but not primary monocytes, express GC-A mRNA. However, the molecular mechanism of GC-A expression in monocytes/macrophages remains unclear.

Taking into account the fact that in some pathological conditions (i.e. in hypertension, heart failure, and cirrhosis) concentrations of GC-A ligands (ANP and BNP) in the blood plasma of patients were increased (Licata *et al.*, 2013; Szabó *et al.*, 2013), one can assume that concomitant increase in the GC-A expression may be significant in the development of these diseases. Therefore, it appears that a better understanding of the mechanisms responsible for the activation of the GC-A gene in monocytes/macrophages may be the basis for the development of a therapy for patients with chronically elevated levels of natriuretic peptides.

In this study, signaling pathway involved in the PMA-induced GC-A gene expression in the monocytic cell line was investigated and our findings show that the PMA-induced GC-A gene expression requires DNA-binding activity of the Sp1 transcription factor.

## MATERIALS AND METHODS

**Reagents.** RPMI 1640 and Dulbecco's PBS were from Cytogen (Sinn, Germany). Fetal calf serum was from Thermo Scientific HyClone (Cramlington, UK). ANP was obtained from Calbiochem (La Jolla, USA). Antibiotics, IBMX, PMA, U0126, FR180204, and mithramycin A were purchased from Sigma-Aldrich (St. Louis, USA). Ro 31-8220 was obtained from Merck Millipore (Darmstadt, Germany). Anti-GC-A antibody was from LifeSpan Biosciences (Seattle, USA). IRDye 800CW goat anti-rabbit IgG secondary antibody conjugate was from LI-COR Biosciences (Lincoln, USA).

**Cell culture.** Human monocytic cell line (THP-1) was obtained from European Collection of Authenticated Cell Cultures (ECACC). THP-1 cells were propagated in RPMI 1640 medium, supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, and with antibiotics (100 U/ml penicillin G, 0.25 µg/ml amphotericin B, 0.1 mg/ml streptomycin), at 37°C in 5% CO<sub>2</sub>. THP-1 monocytes were differentiated into macrophages by treatment with 500 nM PMA for 3 h at 37°C. Then, the cells were centrifuged at 400 × *g* for 5 min at 20°C, resuspended in culture medium, seeded in plates, and incubated at 37°C, 5% CO<sub>2</sub> for 72 h. In some experiments, THP-1 cells were treated with specific inhibitors (1 µM Ro 31-8220, 10 µM U0126, 50 µM FR180204 or 200 nM mithramycin A) for 45 min before stimulation with PMA. Cell viability in response to inhibitors was determined by Trypan Blue exclusion and was found not to be adversely affected.

**Induction and measurement of intracellular cGMP.** Control and PMA-treated cells (0.5 × 10<sup>6</sup> cells per well/24-well plate) were supplemented with a non-specific inhibitor of phosphodiesterases (0.5 mM IBMX) to prevent hydrolysis of the synthesized cGMP. Cells were incubated for 10 min at 37°C and then GC-A activator (0.1 µM ANP) was added. After 30 min incubation, the reaction was terminated, the cells were disintegrated and intracellular concentration of cGMP was determined using an immunoenzymatic assay (ELISA) based on rabbit polyclonal antibodies highly specific to cGMP (Kobialka *et al.*, 2003).

**Western blotting.** THP-1 cells (control and PMA-treated; 1 × 10<sup>6</sup> cells per well/6-well plate) were lysed in high salt lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.8 mM PMSF, protease inhibitor cocktail, 5% β-mercaptoethanol, and 1×Laemmli sample buffer) on ice for 20 min. Whole cell lysates were then incu-

bated at 99°C for 10 min and subjected to 12% SDS-PAGE. Next, the proteins were electrotransferred onto nitrocellulose membranes, blocked with 2% casein for 2 h, and then incubated overnight with anti-GC-A antibodies. After washing, the membranes were incubated with IRDye 800CW goat anti-rabbit IgG secondary antibody conjugate for 1 h. Immunoreactivities were determined using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

**Quantitative PCR (qPCR).** Total RNA was extracted from cells (1 × 10<sup>6</sup> cells per well/6-well plate) using ReliaPrep™ RNA Cell System, and cDNA was synthesized using M-MLV reverse transcriptase, according to the manufacturer's instructions (Promega, USA). cDNA was subjected to qPCR with GoTaq qPCR Master Mix with BRYT Green dye (Promega, USA) in a real-time PCR system (CFX Connect Real-time System, Bio-Rad, USA). For mRNA quantification, hypoxanthine phosphoribosyltransferase 1 (*HPRT*) housekeeping gene was used as a reference point. The sequences of primers used for qPCR were as follows: GC-A, 5'-ATGGTGCCTTC-GGGGTTGACTGA-3' (forward) and 5'-AAGGTGATCTGGTTGCATGCTGG-3' (reverse); *HPRT*, 5'-AGCTTGCTGGTGAAAAGGAC-3' (forward) and 5'-TTATAGTCAAGGGCATATCC-3' (reverse). qPCR data were analyzed using the 2<sup>-ΔΔC<sub>t</sub></sup> method (Livak & Schmittgen, 2001).

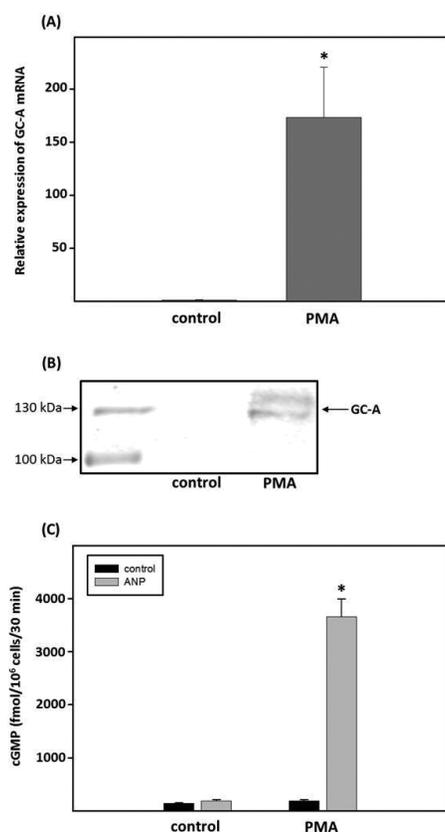
**Nuclear extract and electrophoretic mobility shift assay (EMSA).** Cells (control and PMA-treated, 1 × 10<sup>6</sup> cells per well/6-well plate) were washed with ice-cold PBS and disintegrated in ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate, 0.1% NP-40) on ice for 15 min. After centrifugation at 12000 × *g* for 1 min at 4°C, the supernatants were removed and the nuclear pellets were resuspended in 3× the packed nuclear volume of ice-cold high-salt buffer B (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM PMSF). The samples were gently vortexed at 4°C for 30 min, centrifuged at 12000 × *g* for 10 min at 4°C, and the supernatants (the nuclear extracts) were saved. Nuclear extracts (10 µg of protein) were incubated with 50 fmol of a double-stranded oligonucleotide 5'-ATTTCGATCGGGGCGGGGCGAGC-3' containing the Sp1 consensus-binding site and end-labeled with IRDye 700 infrared dye according to the manufacturer's protocol (Odyssey EMSA Buffer Kit, LI-COR Biosciences, USA). Electrophoretic mobility shift assay was performed in a 5% native polyacrylamide gel in TBE buffer (0.5×). The Sp1-DNA complexes were then analysed using Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

**Statistical analysis.** Data are presented as the means ± S.E. of the values obtained from at least three independent experiments. Statistical analysis was carried out using the unpaired Student's *t*-test assuming *p* < 0.05 to be statistically significant.

## RESULTS

### PMA induces expression of the GC-A gene in THP-1 cells

The effect of PMA on GC-A expression was determined by qPCR. It was observed that GC-A mRNA expression was significantly increased in THP-1 cells treated with PMA (Fig. 1A). GC-A expression was confirmed



**Figure 1.** Expression of GC-A appears in PMA-treated THP-1 cells.

(A) Total RNA was isolated from the control and PMA-treated cells and the GC-A gene expression was determined using qPCR and was normalized to *HPRT*. (B) Cells were lysed and samples were subjected to SDS-PAGE; Western blotting was performed using an anti-GC-A antibody. (C) Cells were treated with 0.1  $\mu$ M ANP (GC-A activator) for 30 min in the presence of PDE inhibitor (0.5 mM IBMX). Then, intracellular cGMP was determined using ELISA. \* $p < 0.001$  versus control cells.

at the protein level using Western blotting. Anti-GC-A antibody recognized a 130-kDa band, corresponding to the molecular mass of GC-A, in extracts of PMA-treated cells but not in the control cells (Fig. 1B). Finally, an increased production of cGMP in PMA-treated cells was detected by ELISA. As shown in Fig. 1C, the level of cGMP produced by PMA-treated cells in response

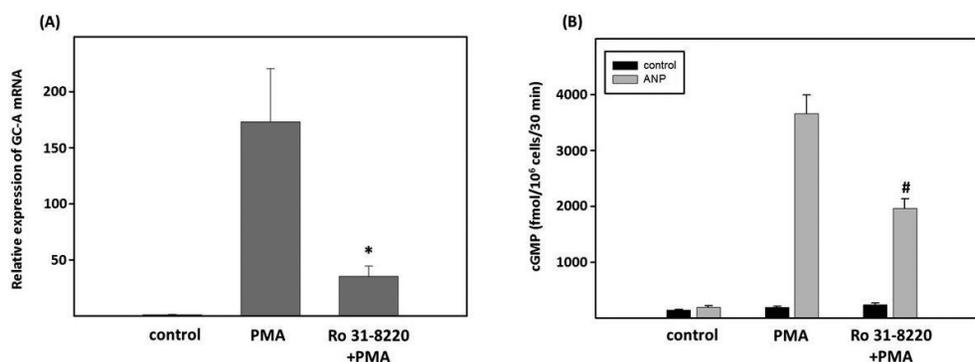
to ANP was about 20-fold higher ( $3660 \pm 332$  fmol/ $10^6$  cells) than in the control cells ( $190 \pm 27$  fmol/ $10^6$  cells).

### PKC is involved in activation of the GC-A gene

Because PMA is known to activate protein kinase C (PKC), PKC is thought to mediate PMA-induced effects. Therefore, we checked whether this protein kinase is involved in regulation of the GC-A gene expression in PMA-treated THP-1 cells. As shown by qPCR, the GC-A gene expression in PMA-treated cells was significantly reduced in the presence of Ro 31-8220, a selective PKC inhibitor (Fig. 2A). This result is consistent with the data obtained from an ELISA assay showing that Ro 31-8220 caused about 45% reduction in ANP-triggered cGMP accumulation in PMA-treated cells (Fig. 2B). The obtained results suggest that PKC mediates PMA-induced expression of the GC-A gene in THP-1 cells.

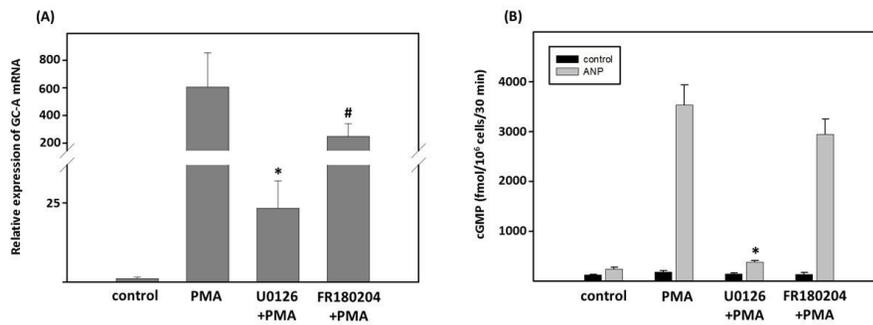
### MEK1/2 and ERK1/2 are engaged in PMA-induced expression of the GC-A gene

It is well known that PKC activates the MAP kinase pathway. Therefore, we examined whether MEK1/2 (mitogen extracellular kinases) and ERK1/2 (extracellular signal-regulated kinases) are required for the PMA-induced expression of the GC-A gene in THP-1 cells. Involvement of MEK1/2 and ERK1/2 in this process was tested using specific protein kinase inhibitors: U0126 in the case of MEK1/2, and FR180204 in the case of ERK1/2. As shown in the Fig. 3A, the PMA-induced GC-A expression was markedly decreased in the presence of MEK1/2 inhibitor. U0126 also caused an almost complete inhibition of the GC-A activity. It was observed that the detected level of ANP-triggered total cellular cGMP accumulation was reduced from  $3534 \pm 396$  fmol/ $10^6$  cells in PMA-treated cells to  $379 \pm 29$  fmol/ $10^6$  cells in cells preincubated with U0126 (Fig. 3B). The stimulatory effect of PMA was also reduced by FR180204. However, the use of this inhibitor resulted in a significantly weaker inhibition of the GC-A gene expression (Fig. 3A) and did not affect accumulation of cGMP in PMA-treated cells (Fig. 3B). Because U0126 inhibits activation of ERK1/2 by inhibiting the kinase activity of MEK1/2, the obtained results suggest that activation of MEK/ERK-signaling cassette is essential for the PMA-induced GC-A expression.



**Figure 2.** PKC mediates the PMA-induced expression of the gene encoding GC-A.

THP-1 cells were preincubated with 1  $\mu$ M Ro-318220 for 45 min and then treated with PMA for 72 h. (A) Total RNA was isolated from the cells and the GC-A gene expression was determined using qPCR and was normalized to *HPRT*. (B) Cells were treated with 0.1  $\mu$ M ANP for 30 min in the presence of 0.5 mM IBMX. Then, intracellular cGMP was determined using ELISA. \* $p < 0.001$  versus PMA-treated cells, # $p < 0.01$  versus PMA-treated cells.

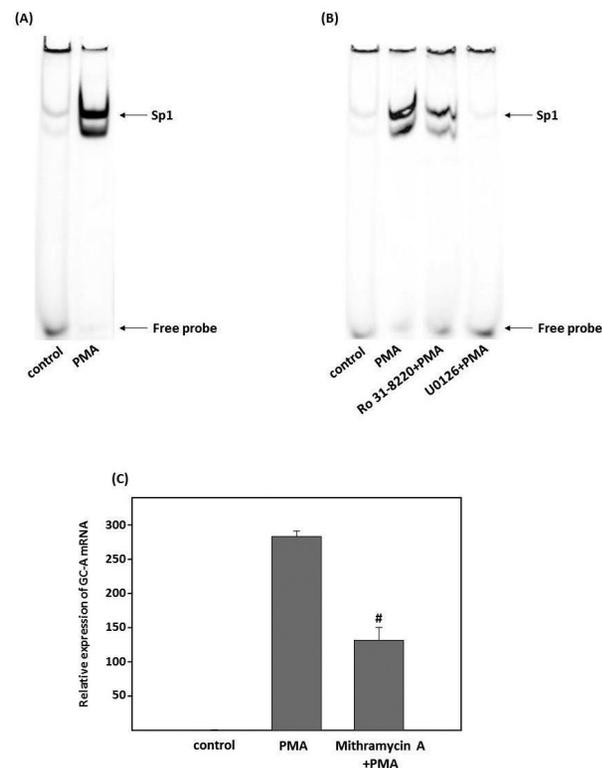


**Figure 3. MEK1/2 and ERK1/2 are involved in the PMA-induced expression of GC-A.**

THP-1 cells were preincubated with 10  $\mu$ M U0126 or 50  $\mu$ M FR180204 for 45 min and then treated with PMA for 72 h. **(A)** Total RNA was isolated from the cells and the GC-A gene expression was determined using qPCR and was normalized to *HPRT*. **(B)** Cells were treated with the 0.1  $\mu$ M ANP for 30 min in the presence of 0.5 mM IBMX. Then, intracellular cGMP was determined using ELISA. \* $p < 0.001$  versus PMA-treated cells, # $p < 0.01$  versus PMA-treated cells.

### Sp1 transcription factor participates in regulation of the GC-A gene expression

Sp1 is a ubiquitously expressed DNA-binding protein that can regulate transcription in response to physiological and pathophysiological stimuli. It has been shown that the Sp1 protein is implicated in regulation of various genes associated with different cellular processes. Therefore, as a first step, it was tested whether treat-



**Figure 4. Sp1 mediates PMA-induced expression of the GC-A gene in THP-1 cells.**

**(A, B)** THP-1 cells were treated with PMA in the absence or presence of specific inhibitors (1  $\mu$ M Ro 31-8220 or 10  $\mu$ M U0126) and afterwards nuclear extracts were obtained, normalized for protein concentration and subjected to EMSA using oligonucleotides containing the Sp1 consensus binding site. **(C)** THP-1 cells were stimulated with PMA for 72 h in the absence or presence of Sp1 inhibitor (200 nM mithramycin A). Subsequently, total RNA was isolated from the cells and the GC-A gene expression was determined using qPCR and was normalized to *HPRT*. # $p < 0.01$  versus PMA-treated cells.

ment of THP-1 cells with PMA resulted in an increase in DNA-binding activity to a synthetic double-stranded oligonucleotide containing the Sp1 consensus binding site. As shown by an EMSA method, the DNA-binding activity of Sp1 was barely detectable in control cells, but it increased markedly following the PMA treatment (Fig. 4A). The Sp1 protein is phosphorylated by various protein kinases, including PKC isoforms, ERK1/2 etc., at various sites within the protein (Chu, 2012). On the basis of our earlier results suggesting involvement of PKC and MEK1/2 in regulation of the GC-A expression, we examined the influence of PKC and MEK1/2 activities on DNA-binding activity of Sp1 in the PMA-treated cells. As shown by the EMSA method (Fig. 4B), the DNA-binding activity of Sp1 in PMA-treated cells was decreased in the presence of the PKC inhibitor, and was completely abolished in the presence of the MEK1/2 inhibitor. These findings support the hypothesis that in THP-1 cells both, PKC and MEK/ERK-signaling cassette, are involved in triggering the DNA-binding activity of Sp1 protein in response to PMA.

In the next step, in order to determine whether the PMA-induced GC-A gene expression requires DNA-bound Sp1 molecules, the THP-1 cells were pretreated with mithramycin A, a known Sp1 inhibitor, before treatment with PMA. qPCR analysis revealed that mithramycin A considerably suppressed expression of GC-A mRNA in the PMA-treated cells (Fig. 4C). Compared to level of the GC-A gene expression in PMA-treated cells in the absence of an inhibitor, mithramycin A reduced the PMA-induced expression of the GC-A gene by about 50%. Because mithramycin A binds to GC-rich DNA sequences, this result suggests that binding of Sp1 molecules to the GC boxes in the promoter region of the GC-A encoding gene is necessary for the PMA-induced GC-A gene expression in THP-1 cells.

### DISCUSSION

Predominantly sGC was detected in human monocytes, but a large increase in the GC-A expression was observed in monocyte-derived macrophages (Bender *et al.*, 2004). However, there is no data on factors and mechanisms that induce the GC-A expression in monocytes/macrophages. This raises questions about molecular mechanisms of activation of the GC-A gene in human monocytes/macrophages. Human monocytic cell line THP-1 was used as a model system for studying gene expression changes in PMA-induced differentiation

into macrophage-like cells (Dieter & Schwende, 2000). Here, by applying qPCR, Western blotting, and ELISA (Fig. 1), we found that the active form of GC-A receptor was produced in PMA-differentiated macrophage-like cells, but not in undifferentiated monocytic THP-1 cells, although few literature reports show the presence of GC-A in these cells (Glezeva *et al.*, 2013; Mezzasoma *et al.*, 2016). It is well known that activation of PKC by PMA triggers activation of the MEK/ERK-signaling cassette involved in transduction of extracellular stimuli to molecular networks responsible for differentiation and other cellular processes (Dieter & Schwende, 2000; Miranda *et al.*, 2002). Therefore, in order to determine whether these signaling molecules are involved in the observed induction of the GCA expression in THP-1 cells, the specific inhibitors of PKC (Ro 31-8220), MEK1/2 (U0126), and ERK 1/2 (FR180204) were used. Results of our experiments showed that both, Ro 31-8220 and U0126, significantly reduced the PMA-induced up-regulation of the GCA gene expression (Figs. 2 and 3). The stimulatory effect of PMA on the GC-A mRNA expression was also inhibited by FR180204 (Fig. 3A). However, this reduction was weaker, although still statistically significant ( $p < 0.01$ ). These results suggest that in THP-1 cells, both PKC and the MEK/ERK-signaling cassette are required for the PMA-induced GC-A expression. Presumably, PMA-activated PKC *via* phosphorylation of the Raf-1 protein kinase triggers the MEK/ERK-dependent signaling cascade, leading to the increase in the GC-A gene expression.

Promoter region of the GC-A gene contains three potential Sp1-binding sites and their involvement in gene activation has been demonstrated (Liang *et al.*, 1999; Garg *et al.*, 2002). A few reports have shown that the Sp1 protein is involved in activation of the GC-A gene (Liang *et al.*, 2001; Kumar *et al.*, 2014). However, mechanisms of Sp1-mediated activation of the GC-A gene in different cell types remain unresolved. Based on these findings, we tested whether the observed up-regulation of the GC-A gene expression in PMA-treated THP-1 cells was also mediated by Sp1 protein. And indeed, our results show that Sp1 protein is involved in this process. Firstly, as shown by the EMSA method, the DNA-binding activity of Sp1 protein was markedly increased in the PMA-treated THP-1 cells (Fig. 4A). Moreover, the elevated activity of Sp1 protein in PMA-treated cells was markedly diminished in the presence of the PKC and MEK1/2 inhibitors (Fig. 4B), which also significantly reduced the PMA-mediated expression of GC-A (Figs. 2 and 3). Secondly, the inhibition of the DNA-binding activity of Sp1 protein had considerably decreased the GC-A expression in the PMA-treated cells (Fig. 4C). From these results we conclude that PMA stimulates the PKC/MAPK-dependent signaling cascade in THP-1 cells, which then triggers the DNA-binding activity of the Sp1 protein and subsequently leads to the up-regulation of the GC-A gene expression.

Taking into account that mithramycin A did not completely, but by about 50%, inhibit the PMA-induced GC-A gene expression (Fig. 4C), it is possible that other transcription factors are involved in the PMA-triggered GC-A expression. It was shown that the GC-A gene promoter region contains a CCAAT motif (Yamaguchi *et al.*, 1990), which is recognized by the NF-Y transcription factor (Dolfini *et al.*, 2012). Liang and co-workers (Liang *et al.*, 2001) noted that in the rat aortic smooth muscle cells NF-Y is important for the GC-A gene transcription, because mutation of the CCAAT element significantly reduced activity of the GC-A promoter. Other

investigators had shown that the GC-A gene was activated by retinoic acid-triggered transcriptional activator complex consisting of retinoic acid receptors, recruited by Sp1 and Ets-1 transcription factors to the promoter region of the GC-A gene (Kumar *et al.*, 2010). Thus, it is possible that some other transcription factors cooperate with Sp1 protein in regulation of the PMA-triggered GC-A expression in THP-1 cells. Our unpublished data suggest that activity of NF-Y transcription factor is elevated in the PMA-treated THP-1 cells. However, more data are needed to confirm involvement of the NF-Y protein in the PMA-induced GC-A expression.

In summary, the data presented here support the report's conclusion that PMA-triggered activation of PKC followed by activation of the MEK/ERK-signaling cassette leads to Sp1-mediated expression of the GC-A encoding gene in human monocytic THP-1 cells.

### Conflict of interest

The authors declare that they have no conflict of interest.

### Acknowledgements

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