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Calmodulin kinase II activation of mitogen-activated protein kinase in PC12 cell following *all-trans* retinoic acid treatment

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ABSTRACT

Previous studies have shown that apoptosis can be mediated by activation of either calmodulin kinase II (CaMKII) or mitogen-activated protein kinase (MAPK), ERK and p38. In the present study, we investigated whether CaMKII is involved in activation of ERK and p38 in response to *all-trans* retinoic acid (ATRA) treatment in PC12 cells. Results showed that ATRA-induced activation of ERK and p38 occurred later than that of CAMKII. Knockdown of CAMKII by siRNA significantly suppressed ATRA-induced activation of ERK and p38. These results demonstrated that activation of ERK and p38 following ATRA exposure is CAMKII-dependent. Treatment with ATRA also resulted in cell death characterized by apoptosis in PC12 cells. Results suggest that CaMKII-dependent activation of ERK and p38 is related to apoptotic cell death.

1. Introduction

All-trans retinoic acid (ATRA), the biologically active form of vitamin A, plays an important role in modulating the growth and differentiation of a variety of cell types during embryogenesis and in the development of organs and systems, especially the nervous system (Liou et al., 2005; Means and Gudas, 1995; Zile, 1998). In cultured keratinocytes, retinoids can induce apoptosis by activating either retinoic acid receptors or retinoid X receptors (Islam et al., 2000). In mouse embryonic palatal mesenchymal cells, ATRA treatment results in cell cycle block, growth inhibition, and apoptosis (Yu et al., 2005).

The ATRA-induced apoptosis is partly due to the activation of mitogen-activated protein kinase (MAPK) pathways (Yu and Xing, 2006). MAPK signaling is involved in many aspects of cellular functions, such as control of gene expression, proliferation, differentiation and apoptosis. There are three major MAPK pathways in mammalian cells: c-jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) (Chang and Karin, 2001; Cobb, 1999; Vaudry et al., 2002). In rat PC12 cells, the activation of JNK and p38 pathways is critical for induction of apoptosis (Xia et al., 1995). ERK is generally activated by growth factor receptors (Santos et al., 2007). However, ERK can also be activated by hypoxia, cadmium, or RA, and can be involved in the process of apoptosis (Ding and Templeton, 2000; Hou et al., 2003; Pettersson et al., 2004).

Calcium/calmodulin (CaM)/CaM Kinase II (CaMKII), a serine/threonine kinase is present throughout the body and highly concentrated in the brain. It is a general integrator of calcium signal transduction (Hudmon and Schulman, 2002). CaMKII has a broad range of substrates. Upon phosphorylation, many of these substrates are active in a wide variety of neurobiological processes such as neuronal and behavioral plasticity (Griffith, 2004; Griffith et al., 2003; Okamoto et al., 2007; Szabo et al., 2007). A role for CaMKII in mediating the process of apoptosis has been suggested by the observation that cadmium activates CaMKII and initiates CaMKII-dependent apoptosis in mesangial cells (Liu and Templeton, 2007).

The molecular mechanism by which MAPK signaling mediates the RA-induced apoptosis has yet to be determined. In endothelial cells, H_2O_2 causes a redox activation of p38 MAPK and ERK pathways. Such activation can be attenuated by specific inhibitors of CaMKII, suggesting that CaMKII is upstream of ERK and p38 pathways in endothelial cells (Nguyen et al., 2004). We hypothesized that RA-induced activation of ERK and p38 pathways and subsequent apoptosis requires CaMKII. Results showed that knockdown of CaMKII by small interference RNA (siRNA)

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depressed ATRA-induced activation of ERK and p38 in PC12 cells, indicating that CaMKII is involved in signal transduction mediated by the ERK and p38 pathways.

2. Materials and methods

2.1. Cell culture

Rat pheochromocytoma (PC12) cells (Peking Union Medical College cell culture center, Beijing, China) were grown in RPMI 1640 medium supplemented with 10% horse serum (Gibco USA), 5% fetal bovine serum (Evergreen Biological Products Co., Ltd., Hangzhou, China), and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin) (Brynczka et al., 2007; Fujita et al., 2006). The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Upon reaching 80–90% confluence in a 50 mL culture flask (25 cm^2), the culture medium was removed, and the cultures were treated with *all-trans* retinoic acid (ATRA, Sigma, St. Louis, USA) in serum-free RPMI 1640 medium plus nerve growth factor (NGF, Promega, USA) for a specified period of time, as stated in Section 3.

2.2. Western blotting for CaMKII, ERK, and p38 MAPK

Cells in serum-free medium were incubated with or without ATRA (10 μM) for a specified period of time as described in Section 3. At the end of treatment, the medium was removed and the cells were washed once with cold PBS. The cells were lysed in cell lysis buffer (Beyotime, Nantong, China) on ice for 30 min, after which the cell lysate was centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was transferred to a new test tube and stored at −20 °C until analysis. The protein concentration was determined using the Bradford protein assay (Beyotime, Nantong, China). An equal amount (60 μg) of protein from each sample was separated by 10% SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% DSM (defatted milk powder) in TBS (Tris base solution, pH 8.35) containing 0.02% Tween 20. The membranes were then blotted overnight at 4 °C with primary antibodies against CaMKII (#3362 Cell Signaling Technology, Boston, USA), phospho-Thr286-CaMKII (#3361 Cell Signaling Technology, Boston, USA), ERK (#9102 Cell Signaling Technology, Boston, USA), p38 (#9212 Cell Signaling Technology, Boston, USA), phosphorylated ERK (#9101 Cell Signaling Technology, Boston, USA) and phospho-p38 MAPK (#9211 Cell Signaling Technology, Boston, USA) (1:1000) or anti-actin (1:500) as previously described (Schworer et al., 1993). Signals were developed with horseradish peroxidase-conjugated secondary antibody at room temperature for 90 min. Blots were washed three times with TBS/Tween 20 and visualized with ECL Plus reagent (Beyotime, Nantong, China) according to the manufacturer's instructions.

2.3. Transient transfection with recombinant plasmid

The siRNA specific sequences targeting the rat CaMKII β-isoforms (GenBank accession # NM-001042356) were designed as described elsewhere (Okamoto et al., 2007). Target sequences were used to generate short hairpin RNA (shRNA) targeting the CaMKII β-subunit, the isoform that plays a primary role in signal transduction (Okamoto et al., 2007). The sequence used in the present study was 5′-GAG TAT GCA GCT AAG ATC A-3′, encoding siRNA hairpin transcript against CaMKII β, as shown previously (Okamoto et al., 2007).

To obtain high efficiency of silencing in these cells, plasmids expressing shRNA were produced using the pGenesil-1 (Genesil Biotechnology, Wuhan, China). The sense strand was 5′-GAT CCG AGT ATG CAG CTA AGA TCA ttcaagacg TGA TCT TAG CTG CAT ACT CTT TTT TGT CGA CA-3′, and the antisense strand was 5′-AGC TTG TCG ACA AAA AAG AGT ATG CAG CTA AGA TCA cgtcttgaa TGA TCT TAG CTG CAT ACT CG-3′. The underlined nucleotides represent the loop sequence. Annealed DNA was ligated into the linear vector and then transfected into PC12 cells using Lipofectamine 2000 kit (Invitrogen Beijing office, China) (Bain et al., 2004) when the PC12 cells were at 90–95% confluence according to the manufacturer's manual (Invitrogen Beijing office, China). A control scrambled sequence (plasmid) was used to ensure the specificity of siRNA transfection (Genesil Biotechnology, Wuhan, China). The transfection was performed in serum-free media, and the silencing efficiency was demonstrated by RT-PCR and Western blot. The transfection efficiency is between 60% and 70%, and inhibition efficiency is about 74.8%.

2.4. Detection of nuclear chromatin condensation by Hoechst 33258

The cells (5×10^4) were plated as previously described (Tong et al., 1997) on poly-D-lysine-coated glass coverslips in 24-well plates. After treatment with ATRA (10 μM) for 24 h, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C and stained with 0.5 μg/mL of Hoechst 33258 for 10 min at 4 °C. After cells were washed with distilled water, coverslips were mounted onto microscope slides and cell nuclear morphology was observed with a fluorescence microscope (Olympus IX70, Tokyo, Japan) using an excitation wavelength of 365 nm with an emission wavelength of 460 nm.

2.5. Detection of DNA fragmentation by agarose gel electrophoresis

DNA fragmentation was examined by the procedure previously described (Ishikawa and Kitamura, 2000). Briefly, a total of 5×10^5 cells were harvested, washed with cold PBS, and lysed with 150 μL hypotonic lysis buffer (10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100 in 10 mM Tris–HCl, pH 7.4) for 15 min on ice and precipitated with 2.5% polyethylene glycol and 1 mM sodium chloride for 15 min at 4 °C. After centrifugation at 16,000 × *g* for 20 min at room temperature, the supernatant was incubated in the presence of proteinase K (300 μg/mL) at 37 °C for 1 h and precipitated with isopropanol at −20 °C before centrifugation. The DNA pellet (0.5 μg each) was dissolved in 10 μL of Tris–EDTA (pH 7.6), electrophoresed on a 1.5% agarose gel containing Goldview-I, and photographed by UV transillumination.

2.6. Flow cytometric assay for apoptotic cells

Apoptotic cells were quantified by measuring the externalized phosphatidylserine residues using an Annexin V-FITC/propidium iodide kit (BD Biosciences, San Diego, CA) following the manufacturer's instructions. We treated the cells with 16 μM ATRA (10 mM stock solution in DMSO) in serum-free medium supplemented with NGF (50 ng/mL) for 24 h. After treatment, cells were collected, washed with ice-cold PBS and suspended in a binding buffer provided in the kit at a concentration of 10^6 cells/mL. Then the cells were incubated for 15 min with fluorescein-conjugated Annexin V-FITC and propidium iodide (PI) before they were analyzed using the Epics XL flow cytometer (Beckman Coulter) and Expo32 software. Annexin V-FITC positive/propidium iodide negative cells (lower right quadrant) were considered to be early apoptotic, while the lower left quadrant contained the vital (double negative) cell population (Giuliano et al., 2009).

2.7. Statistical analysis

Differences in ERK1/2 or p38MAPK phosphorylation among cells treated with ATRA in the presence or absence of CaMKII

peptide inhibitor (siRNA) were compared in ANOVA followed by Duncan's post hoc test. Each experiment was repeated three times with triplicates for each time of experimentation. Statistical significance was set for $p < 0.05$. All grouped data were presented as means ± standard error (S.E.).

3. Results

3.1. ATRA activates CaMKII, ERK1/2 and p38 MAPK

Following treatment of the PC12 cells with 10 μM of ATRA, an increase in phosphorylation of CaMKII (phospho-CaMKII) was observed at 10 min, and the maximum increase was achieved at 20 min (Fig. 1A1 and A2). Treatment with ATRA also caused increases in phosphorylation of ERK and p38 MAPK. The phosphorylation of ERK and p38 MAPK was maximal at 1 h and 30 min, respectively following ATRA addition (Fig. 1B1 and B2).

3.2. Knockdown of CaMKII expression decreases ATRA-induced activation of ERK and p38 MAPK

To examine the possible role of CaMKII in ATRA-mediated activation of ERK1/2 or p38 MAPK, we used siRNA technology to knockdown CaMKII expression. Results from our previous studies indicated that transfection of PC12 cells with recombinant plasmid containing siRNA against CaMKII decreased expression of CaMKII in 24 h, and the minimum expression of CaMKII was reached by 72 h after the transfection. Therefore, the PC12 cells were pretreated with siRNA for 72 h prior to ATRA treatment for analysis of ERK1/2 and p38 MAPK phosphorylation. These experiments were repeated three times independently, and results from all three experiments were consistent and are presented. The cells were treated with ATRA (10 μM) for 1 h following 72 h siRNA transfection. Results showed that transient transfection of the PC12 cells with siRNA against CaMKII markedly reduced ATRA-induced phosphorylation of both ERK1/2 and p38 MAPK (Figs. 2 and 3).

3.3. ATRA induces apoptosis of PC12

The condensation of nuclear chromatin was demonstrated with Hoechst 33258 staining (Fig. 4A). Compared to the intensity of fluorescence of untreated control cells grown in complete medium (containing serum), the fluorescence in ATRA-treated cells in the presence of NGF was more intense, indicating ATRA-induced condensation of the chromatin material (Fig. 4A).

The apoptotic cell death following ATRA treatment in PC12 cells was also confirmed by DNA gel electrophoretic assay. Gel electrophoresis showed no DNA ladders in cells cultured with complete serum-containing medium (Fig. 4B lane 2), while clear DNA ladders were observed in cells from either serum-free culture (Fig. 4B lane 3). ATRA treatment resulted in a concentration dependent increase in DNA fragmentation in NGF + ATRA-treated cells grown in serum-free medium (Fig. 4B lanes 4 and 5).

The ATRA-induced cellular apoptosis was further analyzed by flow cytometry. As shown in Fig. 5, treatment with 16 μM ATRA resulted in a decrease in vital cells from 96% in the control to 75%, and an increase in Annexin V positive cells (early apoptotic cells) from 3% in the control to 19%. Meanwhile, the PI positive cells were also increased following ATRA treatment.

4. Discussion

The PC12 cell line was cloned from rat pheochromocytoma cells. These cells can be induced to differentiate and to acquire a neuronal-like phenotype (Greene and Tischler, 1976) and have been widely used as a model to study neuronal differentiation and apoptosis (Brynczka et al., 2007; Canon et al., 2004; Rausch et al.,

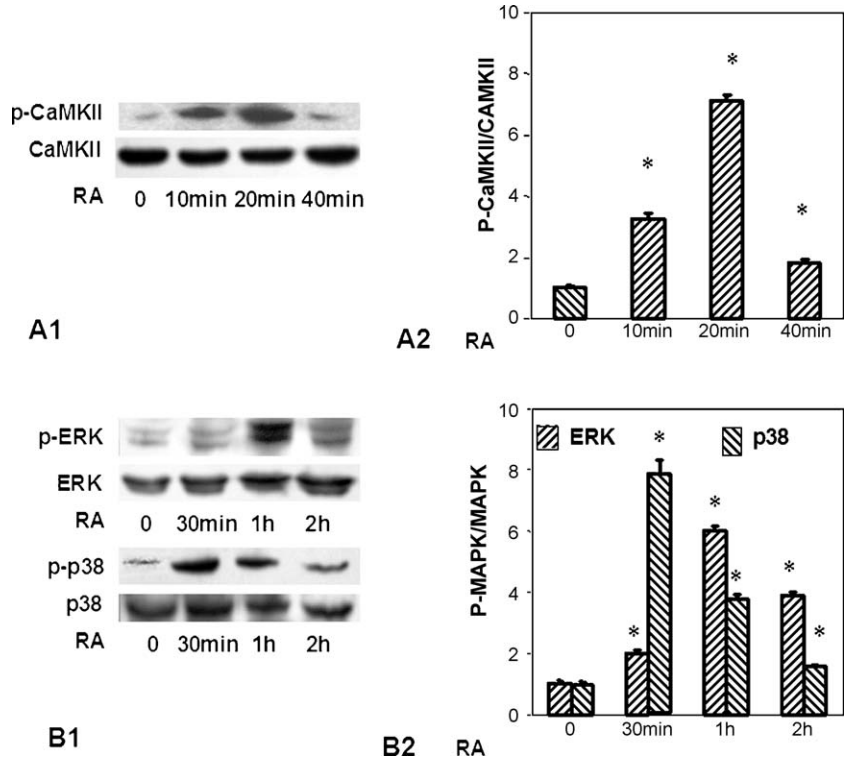

Fig. 1. RA increases phosphorylation of CaMKII, ERK and p38 MAPK in PC12 cells. Western blots of CaMKII phosphorylated on Thr286 (A), phosphorylated p42/p44 ERK (pERK) and p38 MAPK (B) after treatment of PC12 cells with 10 μM ATRA for the period of time indicated in the figure. The blots are representative of three independent experiments in each case. Quantitative data were from three independent experiments with triplicates each (A2 and B2). Data are mean ± S.E. ($n = 3$). $^{*}p < 0.05$ compared to time point "0" (no ATRA treatment).

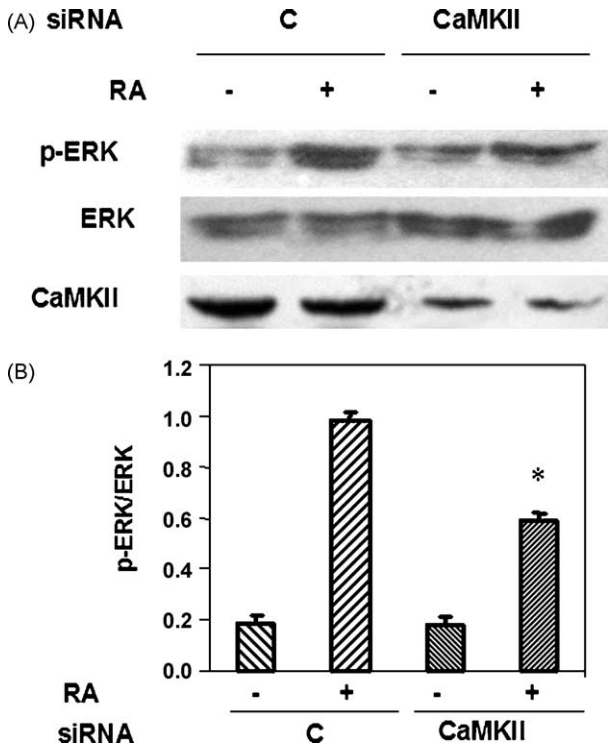

Fig. 2. ATRA activation of ERK MAPK requires CaMKII. Confluent PC12 cells were pretreated with a selective CaMKII siRNA for 72 h before treatment with ATRA (10 μM) for 1 h. Blots are representative of three independent experiments (A). Data are mean ± S.E. ($n = 3$). Each treatment or time point was repeated in three experiments with three samples taken independently. All the data from the three independent experiments are combined for analysis. Significant difference following ATRA treatment between control (C) and siRNA transfected cells is indicated by (*) at $p < 0.05$.

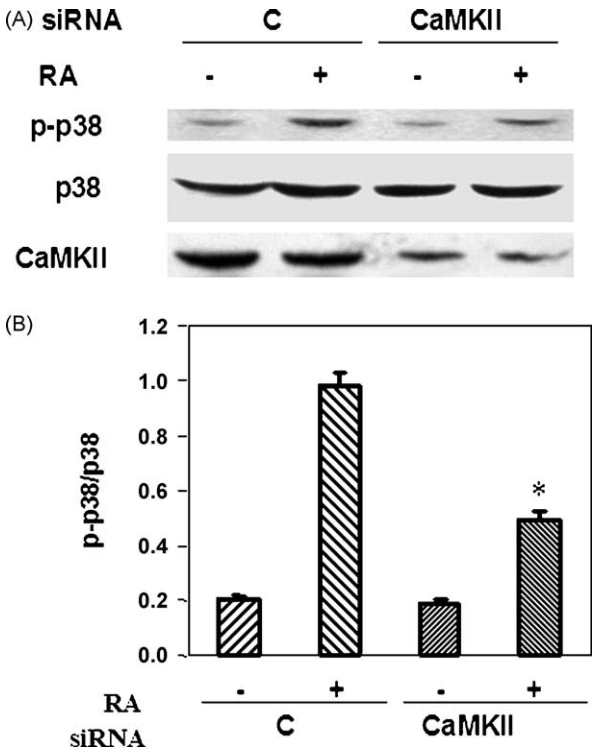

Fig. 3. ATRA activation of p39 MAPK requires CaMKII. PC12 cells were pretreated with a selective CaMKII siRNA for 72 h then treated with ATRA (10 μM) for 1 h. Phosphorylation of p38 MAPK was analyzed by Western blot (A). Each experiment was repeated three times with three samples each and data are mean ± S.E. ($n = 3$) in (B). Significant difference following ATRA treatment between control (C) and siRNA transfected cells is indicated by (*) at $p < 0.05$.

1989; Tong et al., 1997). In the current study, we demonstrate that CaMKII is involved in ATRA-induced ERK and p38 phosphorylation in PC12 cells. Treatment with 10 μM ATRA for 10 min caused an increase in phosphorylation of CaMKII. There were also increases in phosphorylation of p38 MAPK and ERK 30 min and 60 min after ATRA treatment, respectively. The time course of the ATRA-induced phosphorylation of CaMKII, ERK and p38 is much more rapid than the previously reported transcriptional effects of retinoic acid action (Chen and Kelly, 1996), indicating that these might be mediated through the non-genomic pathway. Results from the follow-up experiments showed that knockdown of CaMKII expression depressed ATRA-induced activation of ERK and p38 MAPK, indicating that CaMKII is upstream of ERK and p38 MAPK pathways in ATRA-treated PC12 cells.

Our data provide insight into the ATRA-induced signaling mechanisms in PC12 cells. These finding are consistent with earlier reports of the CaMKII/MAPK pathways in other *in vitro* models. In thyroid TAD-2 cells, fibronectin induces activation of CaMKII and ERK. Inhibition of CaMKII by a specific inhibitory peptide blocks fibronectin-dependent ERK phosphorylation (Illario et al., 2003). In vascular smooth muscle cells, activation of ERK by ionomycin, angiotensin II and thrombin is CaMKII-dependent. Pretreatment with selective CaMKII inhibitors results in attenuation of ERK phosphorylation (Ginnan and Singer, 2002). In L929 fibroblasts, stimulation of cell proliferation by neokyotorphin requires MAPK and CaMKII. A specific CaMKII inhibitor completely suppresses the proliferative effect of neokyotorphin (Sazonova et al., 2007). Similarly, addition of H_2O_2 to bovine aortic endothelial cells increases activation and phosphorylation of ERK and p38 MAPK, while a specific inhibitor or a CaMKII-specific inhibitory peptide reduces such effects (Nguyen et al., 2004). All data indicate that CaMKII is responsible for ATRA-mediated activation of ERK and p38 MAPK.

Retinoic acid has been shown to induce apoptosis of various cells *in vivo* and *in vitro* (Altucci et al., 2001; Islam et al., 2000; Rodriguez-Leon et al., 1999; Yu et al., 2005; Yu and Xing, 2006). In the present study, PC12 cells underwent apoptosis when grown in the serum-deprived culture medium, whereas treatment with NGF could protect the cells from apoptotic process. Conversely, cells underwent substantial death when the cells were co-treated with ATRA and NGF in serum-deprived culture medium, suggesting that ATRA abolishes the anti-apoptotic effect of NGF. The mechanisms by which ATRA induces cell death in NGF-supported undifferentiated PC12 cells are unknown. A similar observation has been reported in PC12 cells cultured under serum-deprived condition, where ATRA again abolishes the anti-apoptotic effect of NGF, dibutyryl cAMP or insulin (Tong et al., 1997).

Current study suggests that ATRA induces apoptotic cell death through CaMKII-activated ERK and p38 MAPK pathways. Involvement of MAPK in apoptosis of PC12 cells has been reported. The activation of JNK and p38 pathways is critical for induction of apoptosis (Xia et al., 1995). Activation of ERK may lead to apoptosis in PC12 cells. In hypoxia-induced PC12 cell death, there is an activation of ERK, p38 and JNK. Inhibition of MAPKs by sesamin and sesamolin correlates well with the reduction of apoptotic cells (Hou et al., 2003). H_2O_2 stimulates phosphorylation of ERK, JNK and p38 in PC12 cells, with resultant PC12 cell death (Fujita et al., 2006). Our data showed that following ATRA treatment there were increases in both the phosphorylation of ERK and p38, as well as in the number of apoptotic cells demonstrated by DNA ladder formation and increases in Annexin V positive cells. It appears that PC12 cells respond to excess ATRA treatment in a manner analogous to the way they respond to the stress imposed by hypoxia or H_2O_2, regarding the involvement of MAPK.

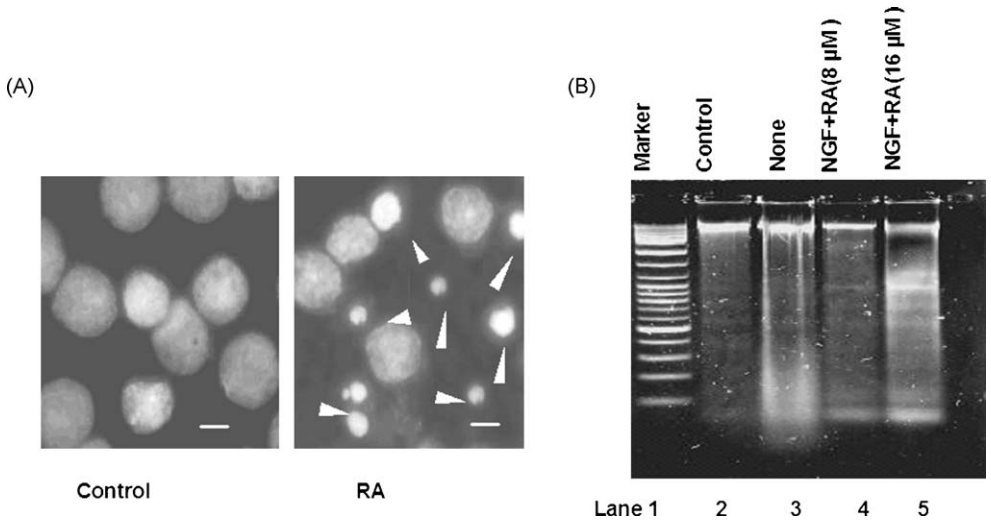

Fig. 4. ATRA induces DNA condensation and DNA ladder in PC12 cells. (A) ATRA-induced nuclear chromatin condensation in PC12 cells. PC12 cells were cultured in complete medium (control) or NGF plus ATRA (10 μM in serum-free medium) (NGF + RA) for 24 h, then stained with Hoechst 33258, and visualized by fluorescence microscopy (400×; the scale bar indicates 25 μm). Arrowheads indicate condensed or fragmented nuclei. (B) DNA fragmentation was examined by agarose gel electrophoresis and Goldview staining. Cells were grown in complete serum medium (lane 2), serum-free RPMI 1640 medium without any treatment (None, lane 3), or NGF plus ATRA (8 μM, lane 4, and 16 μM, lane 5) for 12 h in serum-free medium.

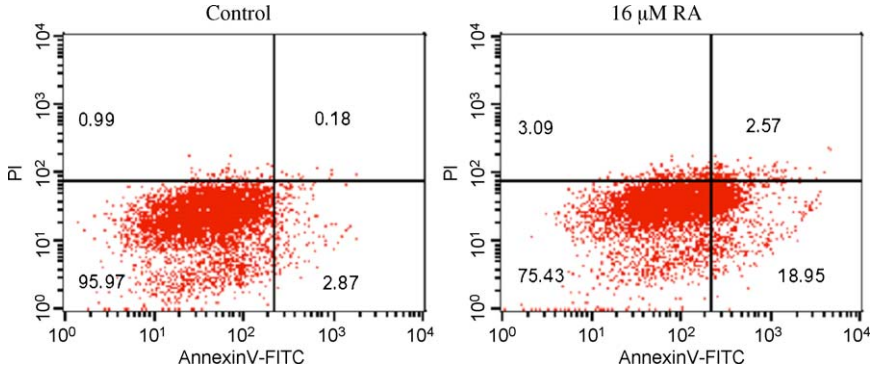

Fig. 5. Flow cytometric analysis of Annexin V-FITC/propidium iodide (PI) labelled PC12 cells. The cells were treated for 24 h with 16 μM ATRA in the presence of NGF (50 ng/mL). Then Annexin V-FITC and PI were added as described in the manufacturer's instructions. A flow cytometric analysis was performed to quantify the percentage of live and early apoptotic cells. The percentage of cells that were live (lower left quadrant), Annexin V positive (lower right quadrant), PI positive (upper left quadrant), and both PI and Annexin V positive (upper right quadrant) is indicated in each quadrant in the figures.

In conclusion, we have demonstrated that CaMKII activates ERK and p38 MAPK in PC12 cells following ATRA treatment. Activation of ERK and p38 MAPK by CaMKII could result in apoptotic death.

Conflict of interest

None.

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