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Calcineurin mediates acetylcholinesterase expression during calcium ionophore A23187-induced HeLa cell apoptosis

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Abstract

We previously reported that acetylcholinesterase plays a critical role in apoptosis and its expression is regulated by Ca^{2+} mobilization. In the present study, we show that activated calpain, a cytosolic calcium-activated cysteine protease, and calcineurin, a calcium-dependent protein phosphatase, regulate acetylcholinesterase expression during A23187-induced apoptosis. The calpain inhibitor, calpeptin, and the calcineurin inhibitors, FK506 and cyclosporine A, inhibited acetylcholinesterase expression at both mRNA and protein levels and suppressed the activity of the human acetylcholinesterase promoter. In contrast, overexpression of constitutively active calcineurin significantly activated the acetylcholinesterase promoter. Furthermore, we identify a role for the transcription factor NFAT (nuclear factor of activated T cells), a calcineurin target, in regulating the acetylcholinesterase promoter during ionophore-induced apoptosis. Overexpression of human NFATc3 and NFATc4 greatly increased the acetylcholinesterase promoter activity in HeLa cells treated with A23187. Overexpression of constitutive nuclear NFATc4 activated the acetylcholinesterase promoter independent of A23187, whereas overexpression of dominant-negative NFAT blocked A23187-induced acetylcholinesterase promoter activation. These results indicate that calcineurin mediates acetylcholinesterase expression during apoptosis.
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Keywords: Acetylcholinesterase; Apoptosis; Calpain; Calcineurin; NFAT

1. Introduction

Acetylcholinesterase (AChE) is a type B carboxylesterase that rapidly hydrolyzes the neurotransmitter acetylcholine (ACh) at cholinergic synapses and neuromuscular junctions [1]. AChE is also involved in non-cholinergic processes such as cell adhesion, neuritogenesis, hematopoiesis, and tumorigenesis [2,3]. The molecular mechanisms regulating *ACHE* gene expression have been studied mostly in muscle [4–6], neurons and hematopoietic cells [2]. Although Stephenson and his colleagues predicted AChE's involvement with apoptosis based on its deletion from many leukemic lines in 1996 [7], our studies have firstly shown that AChE expression can be induced by various apoptosis stimuli in various cell types including those cell types derived from non-muscle, non-nervous and non-hematopoietic tissues, suggesting that AChE is a novel regulator of apoptosis [8]. We also showed that intracellular Ca^{2+} plays a critical role in regulating AChE expression,through modulating AChE mRNA stability and promoter activity, in HeLa cells during apoptosis triggered by the calcium ionophore A23187 [9]. However, the exact mechanisms by which Ca^{2+} regulates AChE expression during apoptosis are not fully understood.

Intracellular Ca^{2+} is an important second messenger, and modulates apoptosis by activating phospholipases, endonucleases,

Abbreviations: AChE, Acetylcholinesterase, acetylcholine acetyl hydrolase; CnA, calcineurin A; CnB, calcineurin B; CsA, cyclosporine A; DMSO, dimethyl sulphoxide; NFAT, nuclear factor of activated T cells

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and Ca^{2+}-dependent proteases [10]. One Ca^{2+}-dependent protease that is active during apoptosis is calpain [11–13]. Calpain is an intracellular cysteine protease that modulates Ca^{2+}-dependent apoptosis through a variety of mechanisms. Activated calpain cleaves and activates caspase-12 and cyclin-dependent kinase 5 (cdk5) to mediate calcium-triggered cell death [13,14]. Calpain also cleaves cain/cabin 1, an endogenous inhibitor of calcineurin, leading to its activation and calcium-triggered cell death [11]. The best-characterized calpains are two ubiquitously expressed isozymes, calpain I (μ-calpain) and calpain II (*m*-calpain), which are each composed of a large catalytic subunit (80 kDa) and a small regulatory subunit (30 kDa). Activation of calpain I and II requires micro- and millimolar concentrations of calcium, respectively [15].

Calcineurin is a calmodulin (CaM)-binding protein and a Ca^{2+}-dependent serine/threonine phosphatase that is directly regulated by Ca^{2+}/CaM [16]. Calcineurin can also be cleaved and activated by calpain [17–19]. Calcineurin consists of one catalytic (CnA, 60 kDa) and one regulatory (CnB, 18 kDa) subunit [20]. A growing number of studies have demonstrated the involvement of a calcineurin-dependent apoptotic cascade following calcium increase during apoptosis [21–25]. During myogenesis, the activation of calcineurin led to increased stability of AChE mRNA [26]. Moreover, we previously showed that Ca^{2+}-dependent signaling regulated AChE expression in HeLa cells during A23187-induced apoptosis [9]. Together, these studies suggest that the *ACHE* gene is a target of calcineurin. In this study, we investigate the relationship between calpain, calcineurin and AChE expression during HeLa cell apoptosis induced by A23187. We present evidence that calcineurin activates AChE expression, likely through the activation of the NFAT (nuclear factor of activated T cells) family of transcription factors.

NFAT is a family of transcription factors present in cells and tissues both inside and outside of the immune system [27] and is composed of at least four members: NFAT1, NFAT2, NFAT3, and NFAT4 (also called NFATp/NFATc2, NFATc/NFATc1, NFATc4, and NFATx/NFATc3, respectively) [28]. Upon calcium mobilization, activated calcineurin dephosphorylates NFATs, leading to their translocation to the nucleus [29,30]. NFATs are rapidly phosphorylated and exported to the cytoplasm upon termination of calcium signaling or by calcineurin inhibition with CsA and FK506 [31]. NFATs bind target gene promoters cooperatively with other nucleoproteins to induce transcription, thus providing a direct link between intracellular Ca^{2+} signaling and gene expression [28,31]. Recently, it was reported that in cultured rat muscle, NFATs could modulate transcription of the mouse AChE promoter [32]. In this study, we examine the regulation of the human AChE promoter by overexpressing wild type and variant human NFAT family proteins. Our data suggest that NFATs may be a transcriptional regulator of the human *ACHE* gene, and suggest for the first time that the calcineurin/NFAT pathway is involved in the regulation of *ACHE* gene expression during Ca^{2+}-induced apoptosis.

2. Materials and methods

2.1. Cell culture and treatments

HeLa cells were maintained in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (GIBCO-BRL). Cells were treated with DMSO (control) or A23187 (2 μM) (Calbiochem, Darmstadt, Germany) for different times. In some experiments, HeLa cells were pre-incubated with calpeptin, FK506, or CsA for 3 h, and then incubated with A23187 (2 μM).

2.2. Plasmid construction

A 2.2 kb fragment of the human AChE promoter [33] was subcloned into the *Bgl*II and *Hin*dIII sites of the pGL3-Basic vector (Promega, Madison, WI), which contains the firefly luciferase gene, to create pAChE-Luc [34]. A cDNA encoding human calcineurin A (a kind gift from Dr. Chi-Wing Chow, Albert Einstein College of Medicine, Bronx, New York) was amplified by PCR using the following primers: F, 5′-AATGAATTCACATGTCCGAGCCCAAGGC-3′; R, 5′-AATCTCGAGCTGAATATTGCTGCTATTACTGC-3′, and was then subcloned between the *Bam*HI and XhoII sites of the pcDNA4a vector (Invitrogen, Carlsbad, CA). A cDNA encoding human calcineurin B was obtained from HeLa cells by the reverse transcription of total RNA, followed by PCR using the following primers: F, 5′-AATGGATCCCGCCGAGCAAAAT-GGGA-3′; R, 5′-AATCTCGAGCACATCTACCACCATCTTTTTGTGGA-3′, and was cloned into the *Bam*HI/*Xho*I sites of pcDNA4a. Constitutively active calcineurin A (ΔCnA) was a generous gift of Dr. Michael Karin (University of California, San Diego, La Jolla, CA). Full-length human NFATc3 and NFATc4 were provided by Dr. Vrushank Dave (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). A cDNA encoding human NFATc4 was cloned between the *Bam*HI and *Xba*I sites of pEGFP-C1 (Clontech, Mountain View, CA), downstream of and in frame with the *EGFP* gene. Constitutive nuclear NFATc4 (cnNFATc4) and dominant-negative NFAT (dn NFAT) were supplied by Dr. Chi-Wing Chow (Albert Einstein College of Medicine, Bronx, New York).

2.3. DNA fragmentation analysis

Cells were harvested, washed with PBS, pelleted, and resuspended in 40 μl of 0.2 M Na_2HPO_4/4 mM citric acid. Cell preparations were then centrifuged at 1500 g for 15 min, supernatants removed and 3 μl of 0.25% NP-40 and 3 μl of RNase A (10 mg/ml) were added. After incubation at 37 °C for 60 min, 3 μl of proteinase K (10 mg/ml) was added and samples were incubated 30 min at 50° C. DNA was then visualized on 1.5% agarose gel by standard procedures.

2.4. Semiquantitative RT-PCR and two step real-time quantitative RT-PCR

Total RNA was extracted from HeLa cells using TRIzol Reagent (Invitrogen) and was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI). The resulting cDNAs were used as template to PCR-amplify NFATc1 (386 bp fragment; F, 5′-CGCCCGCACGCCTTCTACCA-3′; R, 5′-GACCATCTTCTTCCCGCCCACGAC-3′), NFATc2 (497 bp fragment; F, 5′-GCCGCCAGCCCGAGGTTCAC-3′; R, 5′-GGCGAGGCTGTTCAGGG-CATCCAT-3′), NFATc3 (311 bp fragment; F, 5′-CCAGCCCGGGAGACTT-CAATAGAT-3′; R, 5′-GCCCAGGAGCTTCACAACAGGAT-3′), NFATc4 (389 bp fragment; F, 5′-GGTTTCCCGGCCAGTCCAGGTCTA-3′; R, 5′-AAGGGCGGGGAAGGAAGGAAACT-3′), and GAPDH (588 bp fragment; 5′-CCACCCATGGCAAATTCCATGGCA-3′; 5′-TCTAGACGGCAGGT-CAGGTCC ACC-3′).

Real-time PCR was performed using the SYBR green I PCR kit (PE Biosystems, Foster City, CA) and PE Biosystems GeneAmp 5700 sequence detection system (PE Biosystems) as described previously [9]. AChE primers (F, 5′-AGCCGAGGCTGTGGTCCTGCATTACA-3′; R, 5′-CGCATCAGTCGCT-GGGCGAAGATTTT-3′) and GAPDH primers (see above) were used. Reactions were performed in triplicates, and AChE mRNA levels were calculated relative to the GAPDH levels using the following formula: $2^{-(CtAChE\text{-}CtGAPDH)TimeX}$,

where “Ct” is the cycle number at which fluorescence crossed the threshold, and “TimeX” is the corresponding time point.

2.5. Western blotting

Immunoblotting was carried out as described previously [8]. The following primary antibodies were used: monoclonal anti-AChE antibody (BD Biosciences, San Jose, CA); monoclonal anti-actin antibody (Sigma, St. Louis, MO), polyclonal rabbit anti-calpain I large subunit (μ-type) antibody (Cell Signaling Technology, Beverly, MA) and polyclonal rabbit anti-cleaved caspase-3 (Cell Signaling Technology). The following secondary antibodies were used: HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were visualized using a chemiluminescence detection kit (ECL, Santa Cruz Biotechnology).

2.6. Transient transfections and luciferase assays

HeLa cells were transfected with the pAChE-Luc firefly luciferase reporter construct or the control renilla luciferase reporter plasmid pRL-SV40 (Promega, Madison, WI) using LipofectAMINE regent (Invitrogen). Each well of HeLa cells was co-transfected with pAChE-Luc (0.3 μg) and pRL-SV40 (0.03 μg). For co-transfection of the calcineurin and NFAT expression vectors, total DNA was normalized with corresponding empty vectors. Twenty-four hours after transfection, apoptosis was induced using 2 μM A23187. Luciferase activity assays were performed according the Dual-Luciferase Reporter Assay System (Promega), and activities were measured using a luminometerBGP (MGM). AChE promoter activity was analyzed by firefly luciferase activity normalized to renilla luciferase activity in each well.

2.7. Calcineurin phosphatase assay

After treatment, cells were collected, pelleted by centrifugation and lysed in a buffer (50 mM Tris–HCl pH 7.5, 10 mM EDTA, 100 mM NaCl, and 0.1% Triton X-100, 1 mM PMSF, 1 μg/ml Aprotinin, and 1.0 μM Pepstain A). Phosphate-reduced samples were prepared and calcineurin (also known as protein phosphatase 2B) activity was detected using the serine/threonine Phosphatase Assay System according to the manufacturer's instructions (Technical Bulletin no.218; Promega, Madison, WI). Upon addition of calmodulin, the amount of phosphate released from the phosphopeptide substrate was measured using molybdate dye solution.

2.8. Microscopic techniques

For NFAT localization studies, HeLa cells were transfected with pEGFP-NFATc4. Apoptosis was induced using 2 μM A23187 24 h later. After 90 min, treated and untreated cells were washed with PBS, plated on coverslips. The fluorescence signals were observed under a TCS NT laser confocal microscope (Leica Microsystems, Bensheim, Germany).

2.9. Statistical analyses

Each experiment was repeated at least three times and results were expressed as means ± S.E.M. $^{**}p<0.005$ and $^{*}p<0.05$ were calculated using Student's two-tailed t test.

3. Results

3.1. Induction of AChE expression and activation of calpain and calcineurin during apoptosis triggered by A23187

A23187 is a Ca^{2+} ionophore that equilibrates Ca^{2+} gradients across membranes and can cause a rapid rise in intracellular Ca^{2+} levels. A23187 induced DNA fragmentation, a hallmark of apoptosis, in HeLa cells and this was particularly evident after 48 h of A23187 treatment (Fig. 1A). Cleavage of caspase-3 is another hallmark of apoptosis. A23187 treatment led to increased caspase-3 cleavage over a 72 h period (Fig. 1B). AChE protein expression following A23187 treatment was also evident as detected by Western blot analysis (Fig. 1C). Interestingly, AChE protein levels were detectable as early as 12 h after A23187 treatment, in spite of a lack of significant morphological features of apoptosis.

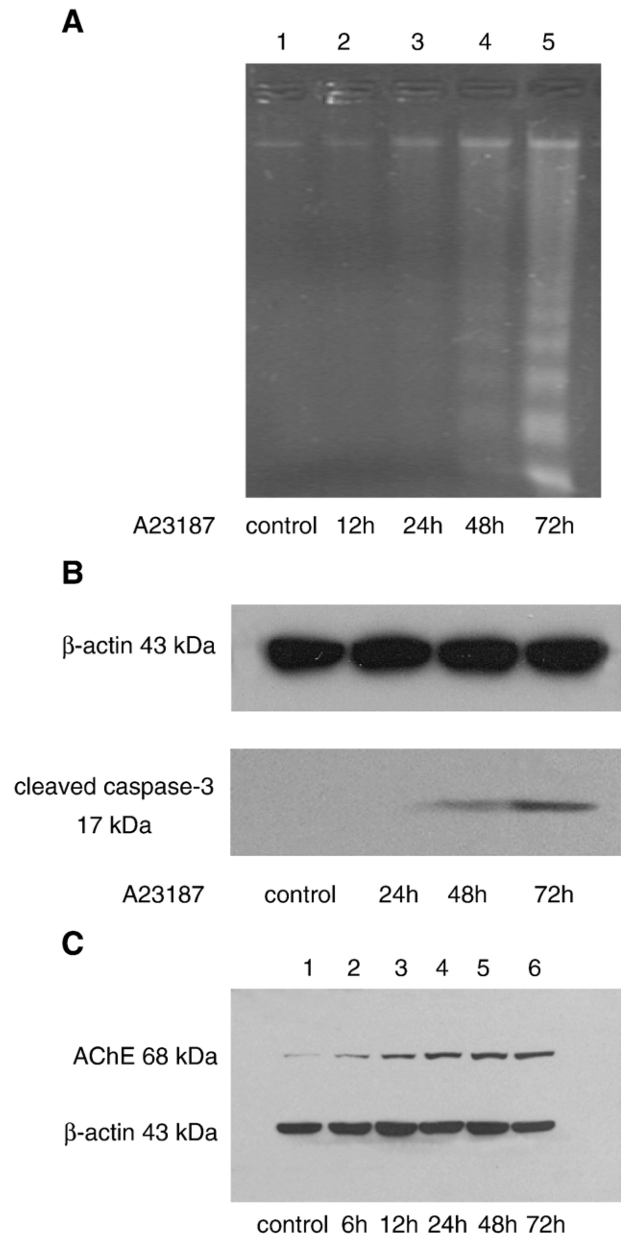

Fig. 1. A23187 induced apoptosis and increase in AChE expression in HeLa cells. (A) DNA fragmentation analysis. (B) A23187-induced caspase-3 activation in HeLa cells. Western blot analysis of extract of HeLa cells treated with DMSO (control) or A23187 (2 μM) for indicated time. (C) AChE protein level was analyzed by Western blot after HeLa cells were treated with DMSO (control) or A23187 (2 μM) for indicated time.

Calpain is frequently activated during calcium-induced apoptosis [12,13,35], and A23187 treatment can lead to calpain activation [11], as evidenced by autoproteolytic cleavage of the

80 kDa subunit to a 75 kDa fragment [36]. After exposure of HeLa cells to A23187, the 75 kDa fragment of calpain I increased to peak levels by 24 h and then decreased thereafter (Fig. 2A). Interestingly, the 80 kDa fragment of calpain I did not decrease until 48 h of ionophore treatment, suggesting that A23187 may also increase calpain I expression. Notably, when HeLa cells were preincubated for 3 h with calpeptin, a calpain inhibitor, then treated with A23187, calpain I autoproteolysis was inhibited in a concentration dependent manner (Fig. 2B), confirming A23187 increases the activity of calpain in HeLa cells.

It is known that calcineurin could be activated in a calpain-dependent manner and mediated calcium-triggered cell death [11]. We then measured calcineurin activity during A23187-induced HeLa cell apoptosis. Fig. 3A shows that A23187 produced a time-dependent increase in calcineurin activity in HeLa cells. The increased activity was inhibited by FK506 and cyclosporin (CsA), two inhibitors of calcineurin, in a dose-dependent manner (Fig. 3B). When HeLa cells were preincubated with calpeptin, calcineurin activation was also suppressed in A23187 treated cells, indicating that calcineurin activation requires calpain (Fig. 3B).

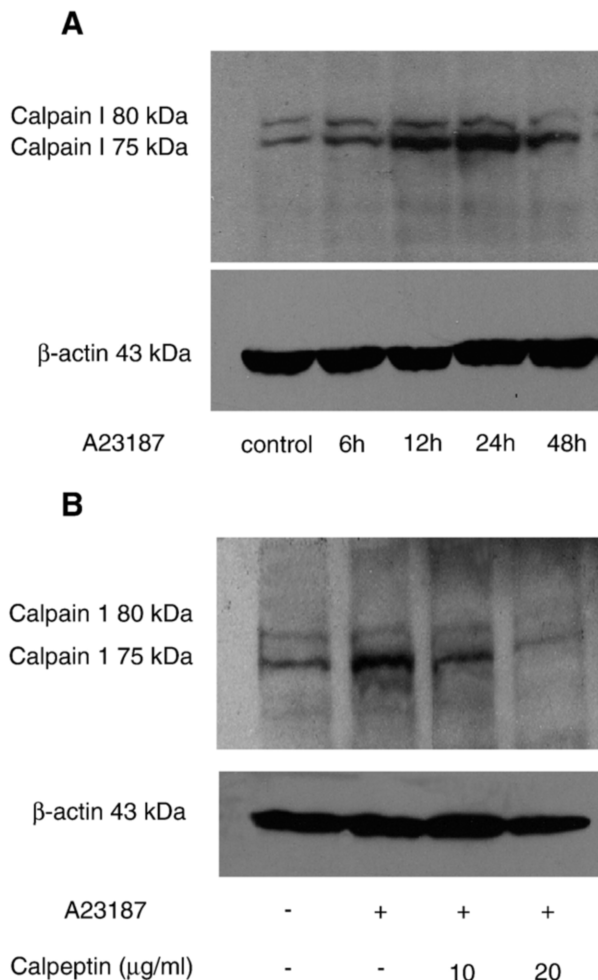

Fig. 2. Calpain was activated during HeLa cell apoptosis induced by A23187. (A) A23187 increased the 75 kDa fragment of calpain I in HeLa cells. (B) The calpain inhibitor, calpeptin, inhibited calpain expression. HeLa cells were treated with DMSO or A23187 (2 μM) for 24 h in the absence or presence of calpeptin pretreatment for 3 h. Calpain I protein levels were analyzed by Western blot.

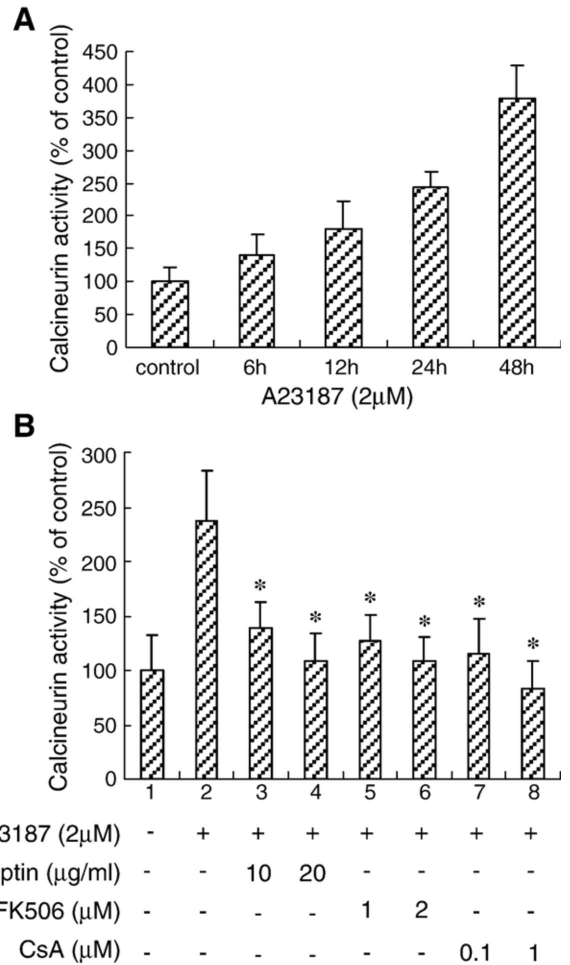

Fig. 3. Calcineurin was activated during HeLa cell apoptosis induced by A23187. (A) HeLa cells were treated with DMSO (control) or A23187 (2 μM). Cellular calcineurin activity was determined. (B) Cellular calcineurin activity was measured after HeLa cells were treated with DMSO or A23187 (2 μM) for 24 h in the absence or presence of inhibitors pretreatment for 3 h. *$p<0.05$ values are significantly different from cells incubated with A23187.

3.2. Induction of AChE expression during A23187-induced apoptosis is regulated by calpain and calcineurin

To determine whether the induction of AChE expression and activation of calpain and calcineurin are causally related, we investigated the effects of calpain and calcineurin inhibitors on A23187-induced AChE expression. HeLa cells were pretreated with the calpain inhibitor calpeptin or calcineurin inhibitors FK506 and CsA for 3 h, followed by A23187 treatment for 24 h. Whole cell extracts were then prepared for analyzing AChE mRNA and protein levels. We have previously reported that the 482 bp tailed AChE-T fragment, but not the AChE-E and AChE-R fragments, was induced during HeLa cell apoptosis induced by A23187

through semiquantitative RT-PCR analysis [9]. In the present study, AChE-T mRNA fragment levels were measured by real-time RT-PCR, and the housekeeping gene *GAPDH* was used as an internal control. Calpeptin, FK506, or CsA treatment led to a decrease in AChE mRNA levels in dose-dependent manners (Fig. 4A, C, and E). Western blot analysis revealed that AChE protein levels were also decreased by these inhibitors in dose-dependent manners, whereas control actin levels were unchanged (Fig. 4B, D, and F). These results suggest that A23187-induced AChE expression requires both calpain and calcineurin.

3.3. Calcineurin is required for activation of the AChE promoter during A23187-induced apoptosis

To determine whether calpain and calcineurin are required for A23187-induced AChE promoter activity, we transiently co-transfected HeLa cells with a reporter construct containing a 2.2-kb fragment of the human AChE promoter fused to the firefly luciferase reporter gene (pAChE-Luc) and an internal control renilla luciferase reporter plasmid (pRL-SV40). Cells were then treated with and without A23187 (2 μM) in the presence or absence of calpeptin, FK506, or CsA. As shown in

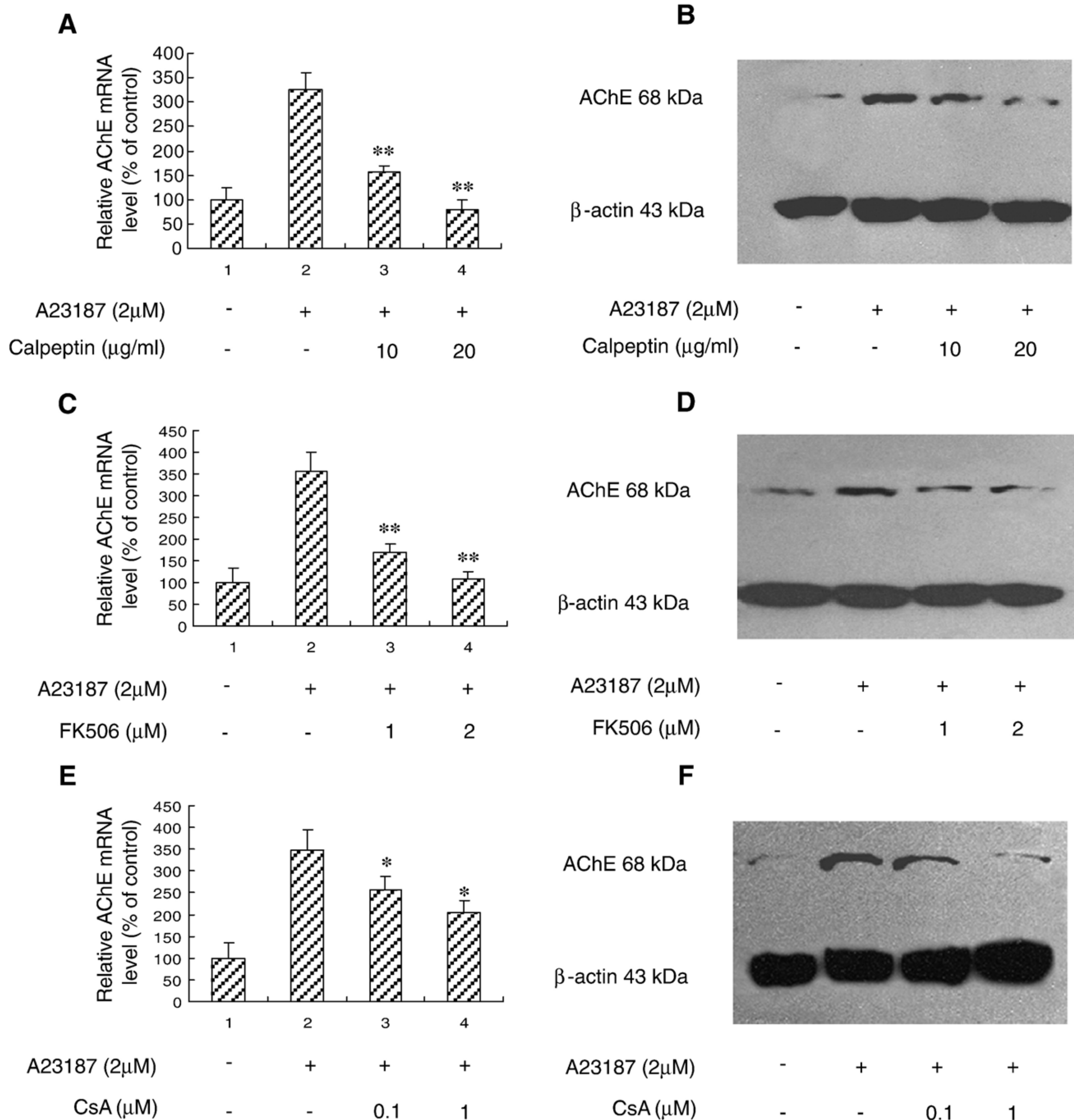

Fig. 4. Increases in AChE mRNA and protein expression during A23187-induced apoptosis were blocked by calpain and calcineurin inhibitors. AChE mRNA levels were analyzed by real-time quantitative RT-PCR and AChE protein levels were analyzed by Western blot after HeLa cells were treated with DMSO or A23187 (2 μM) for 24 h in the absence or presence of inhibitors pretreatment for 3 h. $^{**}p<0.005$ and $^{*}p<0.05$ values are significantly different from cells incubated with A23187.

Fig. 5A, all three inhibitors decreased A23187-induced AChE promoter activation in dose-dependent manners. These results suggest that calpain and calcineurin play a role in modulating AChE promoter activity.

To test whether the *ACHE* gene responds to a calcineurin-stimulated signaling pathway, we co-transfected pAChE-Luc and pRL-SV40, with expression vectors encoding the calcineurin catalytic A (CnA), and regulatory (CnB) subunits. Co-transfection of full-length CnA and CnB did not obviously activate the AChE promoter in HeLa cells without treatment of A23187. However, in the presence of A23187, overexpression of CnA and CnB caused a significant increase in the AChE promoter activity (Fig. 5B). To test whether calcineurin is directly involved in AChE promoter activation, we co-transfected cells with a C-terminal deleted form of calcineurin (ΔCnA) which is constitutively active even in the absence of elevated calcium [37]. Compared with cells expressing full-length CnA and CnB, those cells co-transfected with ΔCnA and CnB exhibited high AChE promoter activity in the absence of A23187 (Fig. 5B). In the presence of A23187, AChE promoter activity was further upregulated, indicating activation of endogenous calcineurin. FK506 and CsA can also block the effects of ΔCnA on AChE expression (Fig. 5B). Fig. 5C shows that ΔCnA activated the AChE promoter in a dose-dependent manner. These data indicated that calcineurin is both necessary and sufficient to increase AChE promoter activity in HeLa cells in a calcium-dependent manner.

3.4. The NFAT family of transcription factors is involved in the activation of the AChE promoter in A23187-treated cells

One target of calcineurin is NFAT (nuclear factor of activated T cells). NFAT transcription factors translocate from the cytoplasm to the nucleus following dephosphorylation by calcineurin in some systems [29,30]. Thus NFAT factors are good candidates for mediating calcineurin-dependent regulation of the AChE promoter. To investigate this possibility, we first examined the expression of *NFAT* genes in HeLa cells by RT-PCR. Using primers that specifically recognize *NFATc1*, *NFATc2*, *NFATc3*, and *NFATc4*, we were able to PCR-amplify products of expected sizes (Fig. 6), suggesting that these four *NFAT* genes are expressed in HeLa cells. Based on band intensity, NFATc3 is present at high levels (Fig. 6, lane 4), NFATc1 and NFATc4 at intermediate levels, (Fig. 6 lanes 2 and 5), and NFATc2 is present at low levels (Fig. 6, lane 3).

We next examined whether NFAT factors translocated to the nucleus in response to A23187 treatment. We transfected an expression construct encoding an EGFP–NFATc4 fusion protein into HeLa cells, and treated with and without A23187. Ninety minutes after treatment, treated and untreated cells were examined by fluorescence microscopy to determine the cellular localization of green fluorescence. In control cultures, green fluorescence was mainly distributed in cytoplasm and was excluded from nuclei (Fig. 7A). In contrast, green fluorescence was detectable in nuclei of A23187-treated cells (Fig. 7B).

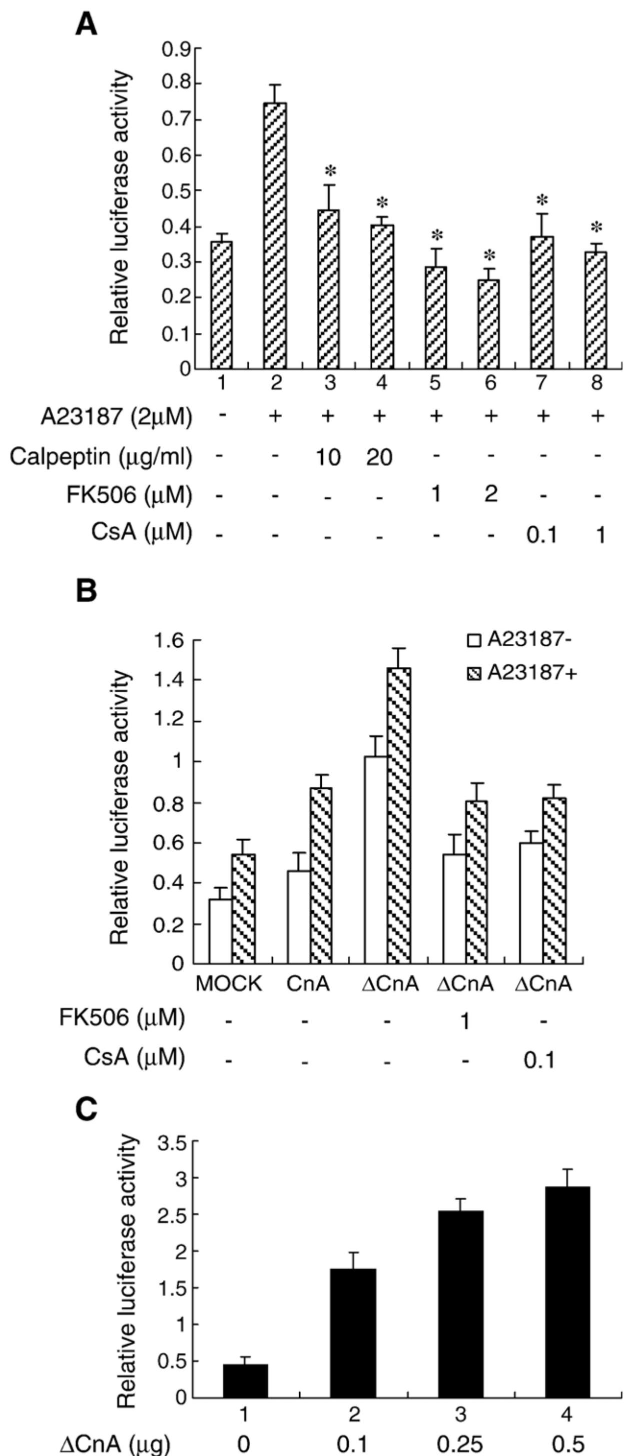

Fig. 5. The human AChE promoter activities were mediated by calcineurin. (A) Each well of HeLa cells was co-transfected with pAChE-Luc and pRL-SV40. (B) Each well of HeLa cells was co-transfected with pAChE-Luc, pRL-SV40, along with expression vectors for CnA (0.1 μg), or ΔCnA (0.1 μg). Each well was also co-transfected with CnB (0.1 μg). Empty vectors appear as MOCK. AChE promoter activity was analyzed after transfected HeLa cells were treated with DMSO or A23187 (2 μM) for 24 h in the absence or presence of inhibitors pretreatment for 3 h (A, B). *$p<0.05$ values are significantly different from cells incubated with A23187. (C) Each well of cells was co-transfected with pAChE-Luc, pRL-SV40, along with different amounts of ΔCnA and relative empty vectors. Each well was also co-transfected with CnB (0.5 μg). After 24 h, AChE promoter activity was analyzed in each well.

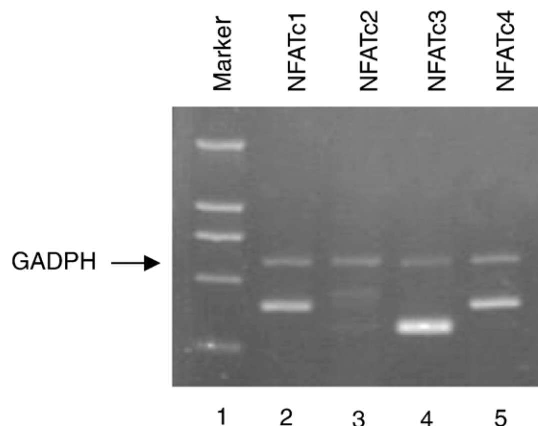

Fig. 6. Expression of NFAT mRNA in HeLa cells. Semi-quantitative RT-PCR was performed on total RNA isolated from HeLa cells. Unique sets of primer pairs were for each gene. GADPH mRNA was amplified as internal control.

To test the involvement of NFAT factors in A23187-induced activation of the AChE promoter, we co-transfected the pAChE-Luc and pRL-SV40 reporter construct into HeLa cells along with expression constructs encoding wild type human *NFATc3* or *NFATc4* gene. In the absence of A23187 treatment, the NFATc3 and NFATc4 did not significantly activate the AChE reporter construct, whereas increased promoter activity was observed after treatment with A23187 in the presence of NFATc3 or NFATc4 than without these factors (Fig. 8A and C). Activation of the AChE reporter construct by overexpression of constitutively active calcineurin (ΔCnA) was further increased by co-expression of NFAT factors (Fig. 8A and C). The increase in AChE promoter activity in cells treated with A23187 and overexpressing NFATc3 and NFATc4 was blocked by co-transfection of a dominant-negative NFAT (dnNFAT) expression vector [38] (Fig. 8B and D). The dnNFAT (NFAT3 amino acids 1–130) has been shown to specifically inhibit NFAT-mediated gene expression by preventing nuclear translocation [39]. We also co-transfected of pAChE-Luc and pRL-SV40 along with an expression vector encoding a constitutive nuclear NFATc4 (cnNFATc4), in which conserved serine residues have been replaced with alanines to promote nuclear localization and increased transcriptional activity [38,40]. Remarkably, co-transfection cnNFATc4 with the AChE reporter construct activated the AChE promoter in a dose-dependent manner independently of A23187 treatment (Fig. 8E). These results suggest that NFAT family members could be potent transcriptional regulators of the *ACHE* gene. In addition, Fig. 8F shows that A23187-dependent AChE promoter activity was blocked by co-expression of the dnNFAT, indicating inhibition of the endogenous NFAT by dnNFAT. Together these results suggest that NFAT factors are involved in the A23187-induced increase of the AChE promoter activity in HeLa cells.

4. Discussion

Previous studies suggested that AChE could be a novel regulator of apoptosis [8,41]. Elucidating the regulatory mechanisms of AChE expression during apoptosis may lead to better therapies that involve apoptosis modulation, such as cancers Alzheimer's and Parkinson's disease [42]. Ca^{2+} is one of the most important signaling agents in mammalian cells, acting as a messenger to regulate growth, differentiation, and apoptosis. Coordination of all these signaling functions requires precise regulation of intracellular Ca^{2+} levels. We have shown that intracellular Ca^{2+} plays a critical role in regulating AChE expression during apoptosis [9]. Calpain and calcineurin are two important mediators of the effects of increased Ca^{2+} levels. In this study, we choose HeLa cells, a cell line that does not express AChE strongly in normal states as previously reported [9]. We demonstrated that AChE up-regulation during HeLa apoptosis induced by A23187 was mediated by calpain/calcineurin pathway.

Calpain and calcineurin may regulate apoptosis through a variety of mechanisms. Calpain can cleave pro-apoptotic proteins such as caspase-12, Bax, and Bid, leading to an increase in their activity [13,43,44]. Our results show that one of calpain inhibitor, calpeptin, blocked A23187-induced AChE expression at both mRNA and protein levels, suggesting that calpain may mediate AChE up-regulation indirectly without cleaving it. We also found that calpeptin suppressed the increase in calcineurin activity in A23187-treated HeLa cells, indicating that calcineurin activation was calpain-dependent. It has been reported that calpain, which is activated by increased cytoplasmic Ca^{2+}, can cleave the endogenous calcineurin inhibitor cain/cabin1 leading to activation of calcineurin [11], suggesting a mechanism for how calcineurin is activated by a rise in the cytoplasmic Ca^{2+} concentration. Calcineurin seems to have a dual function and could exert its effects on apoptosis either by the activation of specific transcriptional pathways or by direct de-phosphorylation of proteins including BAD and caspase-9 involved in the apoptotic pathway [24,45,46]. Using the calcineurin inhibitors FK506 and CsA, we have found evidence that calcineurin is required for A23187-induced AChE expression. Overexpression of calcineurin greatly increased the activity of the human AChE promoter in A23187-treated cells, whereas calcineurin inhibitors lowered the increase in

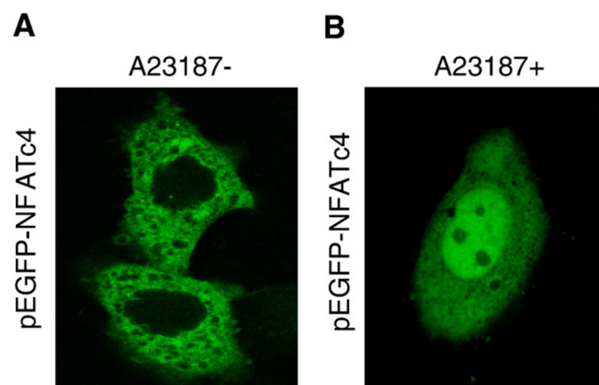

Fig. 7. NFATc4 translocated to the nucleus in HeLa cells induced by A23187. HeLa cells were transfected with an expression construct (pEGFP-NFATc4) encoding an EGFP-NFATc4 fusion protein. Twenty-four hours later, transfected cells were left untreated (A) or treated with A23187 (2 μM) (B). After 90 min, treated and untreated cells were washed with PBS and viewed by fluorescence microscopy to determine the cellular localization of green fluorescence.

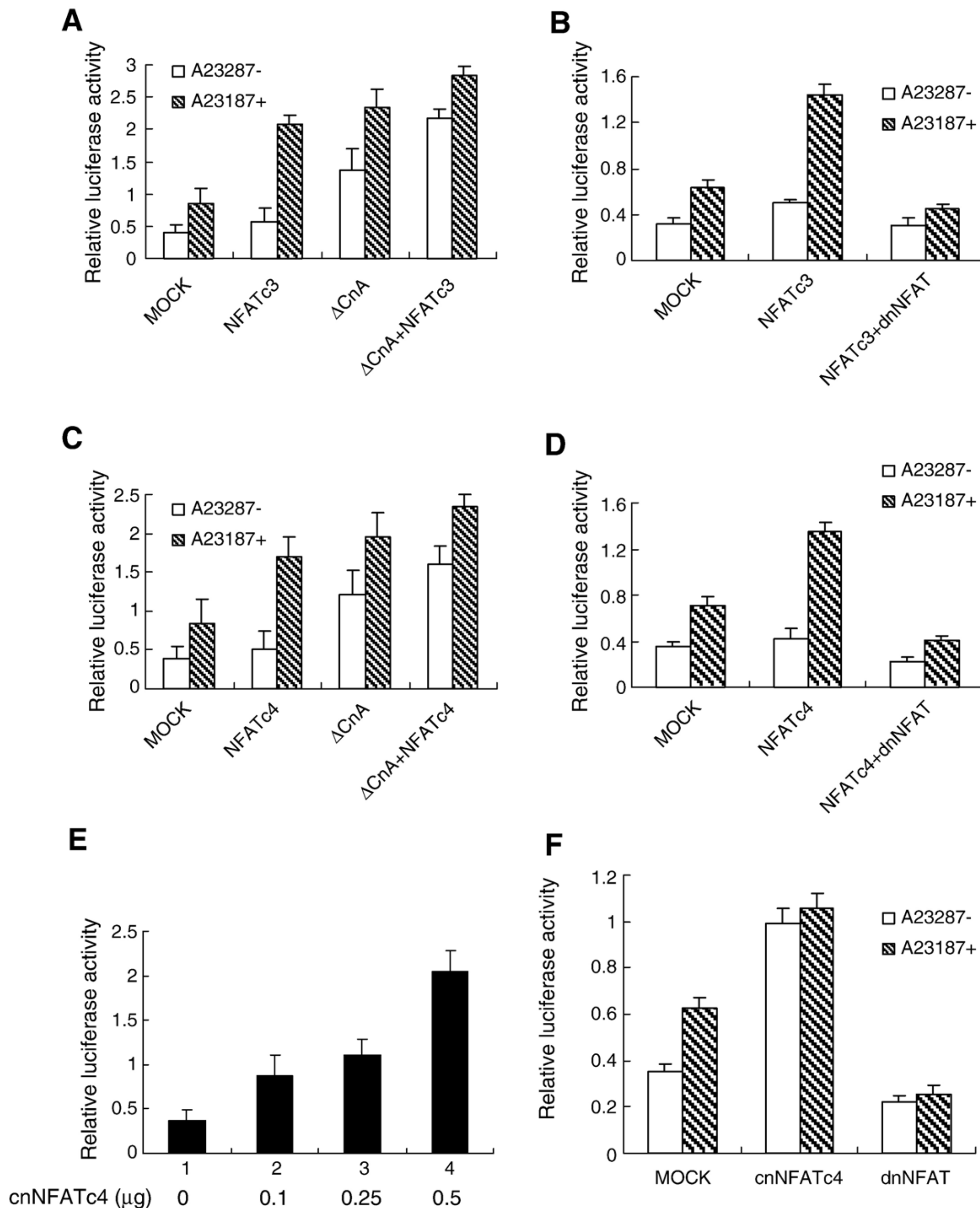

Fig. 8. NFAT proteins were involved in the A23187-induced activation of the human AChE promoter. (A, C) Each well of HeLa cells was co-transfected with pAChE-Luc, pRL-SV40, along with expression vectors for human NFATc3 (0.1 μg) (A)/ human NFATc4 (0.1 μg) (C), or constitutively active ΔCnA (0.1 μg), or NFATc3 (0.1 μg) (A)/ human NFATc4 (0.1 μg) (C) plus ΔCnA (0.1 μg). Each well was also co-transfected with CnB (0.1 μg) and relative empty vectors. Empty vectors appear as MOCK. (B, D) Each well of cells was co-transfected with pAChE-Luc, pRL-SV40, along with expression vectors for human NFATc3 (0.1 μg) (B)/ human NFATc4 (0.1 μg) (D), or NFATc3 (0.1 μg) (B)/ human NFATc4 (0.1 μg) (D) plus dnNFAT (0.1 μg). Each well was also co-transfected with relative empty vectors. Empty vectors appear as MOCK. AChE promoter activity was analyzed in each well after transfected cells were incubated with DMSO or A23187 (2 μM) for 24 h (A–D). (E) Constitutive nuclear NFATc4 (cnNFATc4) activated the human AChE promoter in HeLa cells. Each well of HeLa cells was co-transfected with pAChE-Luc, pRL-SV40, along with different amounts of constitutive nuclear NFATc4 (cnNFATc4) and empty vectors. After 24 h, AChE promoter activity was analyzed in each well. (F) Dominant-negative NFAT (dnNFAT) blocked A23187-dependent AChE promoter activity. Each well of HeLa cells was co-transfected with pAChE-Luc, pRL-SV40, along with expression vectors for cnNFATc4 (0.1 μg) or dnNFAT (0.1 μg). Empty vectors appear as MOCK. AChE promoter activity was analyzed in each well after transfected cells were incubated with DMSO or A23187 (2 μM) for 24 h.

A23187-induced AChE promoter activity. In addition, over-expression of constitutively active calcineurin activated the human AChE promoter independent of A23187. These data suggest that calcineurin mediates A23187-induced AChE expression at least through activation of specific transcriptional pathways. Importantly, it has been reported previously that

blocking AChE expression with antisense of AChE inhibited apoptosis [8]. Furthermore, siRNA-mediated knockdown of AChE abolished decrease of cell viability [41], suggesting that AChE is an important regulator of apoptosis. We show now that calcineurin plays a critical role in apoptosis by mediating the expression of the *ACHE* gene.

Calcineurin controls gene expression via de-phosphorylation of transcription factors, such as NFAT, MEF2, NF-κB, CREB, and Elk-1, affecting their nuclear localization and influences the regulation of proteins involved in the survival and apoptotic pathways [45]. For example, the $InsP_3$ receptor is a downstream target for NFAT mediated by Ca^{2+}-dependent calcineurin/NFAT pathway [47]. Our results indicate that calcineurin signaling is both necessary and sufficient to regulate human AChE promoter activity during A23187-induced cell apoptosis. This activity may be mediated directly by the NFAT family of transcription factors. Overexpression of NFATc3 and NFATc4 greatly increased the human AChE promoter activity in A23187-treated cells whereas dominant-negative NFAT (dnNFAT) blocked the A23187-induced AChE promoter activity. Overexpression of constitutive nuclear NFATc4 directly activated human AChE promoter. These results suggest that NFAT proteins were also necessary and sufficient for AChE promoter activity during ionophore-induced apoptosis. NFATs are expressed in many cell types and contribute to diverse cellular functions [28]. The calcineurin/NFAT signaling pathway has recently been implicated in development and function of the nervous system, the cardiovascular system, the musculoskeletal development, and in cardiac growth and function [31,48]. It is also reported that there is an induction of a NFAT-dependent luciferase reporter by ionomycin and PMA in HeLa cells, indicating NFAT is activated by calcium in a calcineurin-dependent fashion in HeLa cells [49]. The calcineurin/NFAT signaling pathway is also activated by the calcium ionophore A23187 [11,28,50]. Our studies are the first to suggest that calcineurin/NFAT pathway regulates human *ACHE* gene expression during Ca^{2+}-induced cell apoptosis.

Regulation of calcium, apoptosis, and calcineurin signaling has important consequences for disease, particularly Alzheimer's disease (AD). AD is a neurodegenerative disorder characterized by extensive neuronal loss which may be due to apoptosis [51,52]. Dysregulation of intracellular calcium signaling has been implicated in the pathogenesis of AD [53], and increased levels of calcineurin A mRNA have been detected in Alzheimer's diseased brain by microarray [54]. Elevated cleavage and activation of calpain has also been associated with early stage AD [55–57], which in turn leads to calcineurin activation. Interestingly, AChE activity has also been reported to be increased in plaques and tangles early in the onset of AD [58,59]. Our results suggest that the activation of calcineurin in AD may lead to AChE expression and neuronal death, suggesting a new mechanism for the pathogenesis of AD. Further investigation into the mechanisms of AChE regulation and function during apoptosis could contribute to the development of fruitful therapeutic approaches to treatment of AD.

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