Regulation of acetylcholinesterase expression by calcium signaling during calcium ionophore A23187- and thapsigargin-induced apoptosis

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Abstract

We have recently reported that acetylcholinesterase expression was induced during apoptosis in various cell types. In the current study we provide evidence to suggest that the induction of acetylcholinesterase expression during apoptosis is regulated by the mobilization of intracellular Ca2+. During apoptosis, treatment of HeLa and MDA-MB-435s cells with the calcium ionophore A23187 resulted in a significant increase in acetylcholinesterase mRNA and protein levels. Chelation of intracellular Ca2+ by BAPTA-AM (1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid–acetoxymethyl ester), an intracellular Ca2+ chelator, inhibited acetylcholinesterase expression. A23187 also enhanced the stability of acetylcholinesterase mRNA and increased the activity of acetylcholinesterase promoter, effects that were blocked by BAPTA-AM. Perturbations of cellular Ca2+ homeostasis by thapsigargin resulted in the increase of acetylcholinesterase expression as well as acetylcholinesterase promoter activity during thapsigargin induced apoptosis in HeLa and MDA-MB-435s cells, effects that were also inhibited by BAPTA-AM. We further demonstrated that the transactivation of the human acetylcholinesterase promoter by A23187 and thapsigargin was partially mediated by a CCAAT motif within the −1270 to −1248 fragment of the human acetylcholinesterase promoter. This motif was able to bind to CCAAT binding factor (CBF/NF-Y). These results strongly suggest that cytosolic Ca2+ plays a key role in acetylcholinesterase regulation during apoptosis induced by A23187 and thapsigargin.

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Keywords: Acetylcholinesterase; Apoptosis; Ca2+; mRNA stability; Transcriptional activity

Abbreviations: AChE, acetylcholinesterase, acetylcholine acetyl hydrolase; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid–acetoxymethyl ester; TG, thapsigargin; TUNEL, terminal deoxynucleotidyl transferase-mediated-dUTP nick end labeling

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1. Introduction

Acetylcholinesterase (AChE) is a type B carbamoylcholine esterase that rapidly hydrolyzes acetylcholine (ACh) at cholinergic synapses and neuromuscular junctions (Taylor & Radic, 1994). It is also involved in many non-cholinergic functions, such as cell adhesion, proliferation, and neurite outgrowth (Day & Greenfield, 2002; Johnson & Moore, 2004; Karpel et al., 1996; Koenigsberger, Chiappa, & Brimijoin, 1997; Whyte & Greenfield, 2003). Three different mature AChE mRNA transcripts, which encode AChE-T (tailed isoform), AChE-E (erythrocytic isoform) and AChE-R (readthrough isoform), have been produced (Massoulié et al., 1998).

The molecular mechanisms regulating ACHE gene expression have been studied mostly in muscle cells (Angus, Chan, & Jasmin, 2001; Luo, Fuentes, & Taylor, 1994; Tung et al., 2004), neurons and hematopoietic cells (Soreq & Seidman, 2001). It is noteworthy that in all three systems, post-transcriptional regulatory mechanisms seem to play an important role in the induction of AChE expression by stabilizing existing transcripts (Chan, Adatia, Krupa, & Jasmin, 1998; Coleman & Taylor, 1996; Deschenes-Furry, Belanger, Perrone-Bizzozero, & Jasmin, 2003; Luo et al., 1994). On the other hand, several regulatory elements within the AChE promoter have been reported to regulate the transcriptional activity of the ACHE gene in Torpedo, mouse, rat and human (Angus et al., 2001; Ben Aziz-Aloya et al., 1993; Chan, Boudreau-Lariviere, Angus, Mankal, & Jasmin, 1999; Ekstrom, Klump, Getman, Karin, & Taylor, 1993; Mutero, Camp, & Taylor, 1995; Siow et al., 2002).

Recent research in our laboratory revealed that AChE expression was also induced during apoptosis in various cell types, including those cells originating from non-muscle, non-nervous or non-hematopoietic systems, suggesting that AChE might be a novel regulator of apoptosis (Zhang et al., 2002). Our hypothesis was in agreement with a recent study by Park, Kim, and Yoo (2004), which elegantly demonstrated that AChE exerted a pivotal role in apoptosis by participating in the formation of apoptosisomes. However, the mechanisms that regulate the expression of AChE during apoptosis have not been reported.

Elevation of intracellular Ca$^{2+}$ increases AChE transcript levels during differentiation of myoblasts to myotubes (Luo et al., 1994). In cultured chick myotubes, ATP acts via the ATP receptor to stimulate AChE expression, which is mediated by protein kinase C and intracellular Ca$^{2+}$ release (Choi et al., 2003). Amyloid β-protein (Aβ), such as Aβ$_{40}$ and Aβ$_{25-35}$, was also demonstrated to increase AChE activity in P19 embryonic carcinoma and retinal cells, via a calcium influx mechanism (Melo, Agostinho, & Oliveira, 2003; Sberna, Saez-Valero, Beyreuther, Masters, & Small, 1997). These studies suggested that Ca$^{2+}$-dependent signaling pathways played a role in regulating AChE expression or activity in muscle and neuron.

Calcium is an important second messenger and intracellular Ca$^{2+}$ plays an important role in the modulation of apoptosis (Demlaurex & Distelhorst, 2003; Groenendyk, Lynch, & Michalak, 2004; Mattson & Chan, 2003). Overload of Ca$^{2+}$ has even been suggested to be the final common pathway of all types of cell death (Rizzuto et al., 2003). Our previous study demonstrated that AChE expression was induced upon induction of apoptosis by various stimuli in various cell types (Zhang et al., 2002), further suggesting that the induction of AChE expression is an universal property of apoptotic cells. We hypothesize that AChE expression during apoptosis is related to Ca$^{2+}$ mobilization.

The calcium ionophore A23187 induces apoptosis by increasing cytosolic Ca$^{2+}$ in various cell types (Azmi, Dhawan, & Singh, 1996; Petersen, Castilho, Hansson, Wieloch, & Brundin, 2000). The sarcoplasmic reticulum Ca$^{2+}$-ATPase pump inhibitor thapsigargin (TG) depletes Ca$^{2+}$ within the endoplasmic reticulum (ER), resulting in a transient increase in cytoplasmic Ca$^{2+}$ levels (Sagara & Inesi, 1991), and perturbations in cellular Ca$^{2+}$ homeostasis have been also been reported to induce apoptosis in both normal and malignant cells (Krebs, 1998). In the current study, we found that, in cultured cells, the expression of AChE was markedly increased in both A23187-induced or TG-induced HeLa and MDA-MB-435s cell apoptosis, an effect which was inhibited by BAPTA-AM, a Ca$^{2+}$ chelator. In addition, A23187 and TG increased the activity of a 2.2 kb human AChE promoter. The promoter sequence of ACHE gene has been functionally characterized (Ben Aziz-Aloya et al., 1993; Siow et al., 2002; Wan, Choi, Siow, & Tsim, 2000). A CCAAT motif within the −1270 to −1248 fragment of the human AChE promoter was shown to be activated by A23187 and TG induction. Moreover, we suggested, for the first time, that the CCAAT binding factor (CBF/NF-Y) might be a potential transcriptional factor mediating AChE transcriptional activity during apoptosis induced by A23187 and TG.
2. Materials and methods

2.1. Cell culture and treatments

HeLa and MDA cells were maintained in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Gaithersburg, MD). Apoptosis was induced by A23187 (2 μM) (Calbiochem, La Jolla, CA) and TG (2 μM) (Calbiochem, La Jolla, CA) in HeLa cells and A23187 (2 μM) and TG (0.5 μM) in MDA cells. Given that MDA cells were much more sensitive to TG than HeLa cells, relatively lower concentrations of TG were used in our study. Apoptosis was assessed by morphology observation (rounding, shrinkage and detachment of cells), Hoechst staining (chromatin condensation), TUNEL staining (DNA fragmentation), and cleaved caspase-3 immunocytochemistry staining.

2.2. Double staining with immunocytochemistry and TUNEL reaction

Immunocytochemistry analysis protocol has been previously described (Zhang et al., 2002). AChE monoclonal antibody was from BD Biosciences (San Jose, CA) with the epitope mapping at the 411–609 peptide of the human AChE C-terminus. The AChE monoclonal antibody detected endogenous levels of a 68 kDa fragment of human AChE. Cleaved caspase-3 (Asp 175) antibody was from Cell Signaling Technology (Beverly, MA); Rhodamine coupled anti-mouse IgG and FITC coupled anti-rabbit IgG were from Santa Cruz Biotechnology. The anti-rabbit IgG and TUNEL reaction mixture (Roche Diagnostics Corporation (Santa Cruz, CA). Cell pellets were suspended in TUNEL reaction mixture (Roche Diagnostics Corporation Indianapolis, IN) for 1 h and stained with Hoechst 33258. Stained cells were analyzed with a Nikon fluorescence microscope (Nikon Inc.).

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

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and was subjected to reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, WI). Reverse transcription products were used for semiquantitative RT-PCR with oligonucleotide pairs specific for AChE and GAPDH. The primer pair (5′-CGG GTC TAC GCC TAC GTC TTT GAA CAC CGT GCT TC-3′; 5′-CAC AGG TGT GAG CAG CGA TCC TGC TGG CTG-3′) was designed to generate a 482 bp fragment of the tailed AChE-T mRNA, including exons E1–E2–E3–E4–E6, according to Homo sapiens acetylcholinesterase (YT blood group) (ACHE), transcript variant E4–E6, mRNA, NM_000665. The primer pair (5′-CGG GTC TAC GCC TAC GTC TTT GAA CAC CGT GCT TC-3′; 5′-ATG GGT GAA GCC TGG GCA GGT G-3′) was designed to generate two different fragments, 399 and 478 bp, to detect AChE mRNA encoding AChE-E (erythrocytic isoform, including exons E1–E2–E3–E4–E5) and AChE-R (readthrough isoform, including exons E1–E2–E3–E4–4′–E5), respectively. Primers for housekeeping gene GAPDH (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) were used as an internal control, with an expected product size of 588 bp.

Real-time PCR was performed with a PE Biosystems GeneAmp 5700 sequence detection system (PE Biosystems, Foster City, CA) using a SYBR green I PCR kit (PE Biosystems, Foster City, CA). Each reaction contained 2.5 μl of the 10× SYBR green buffer, 200 nM dATP, dGTP, and dCTP; 400 nM dUTP, 2 mM MgCl2, 0.25 U of uracil N-glycosylase (UNG), 0.625 U of AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, CA), 100 nM AChE primers (5′-AGC CGA GGC TGT GGT CCT GCA TTA CA-3′; 5′-CGC ATC AGT CGC TGG GCG AAG ATT TT-3′) or 25 nM GAPDH primers (as noted above); 1 μl 1:10 dilution of cDNA; and water to a final volume of 50 μl (n = 3/sample). AChE mRNA levels relative to GAPDH internal control was determined by the following formula: \(2^{(-\Delta \text{Ct AChE} - \Delta \text{Ct GAPDH}) \times \text{Time}}\), where the “Ct” was defined as the cycle number at which fluorescence crossed the threshold and “Time” was the indicated time point. β-Actin was used in MDA cells as the internal control, with primers (5′-GCA GGA AAT CGT GCG TGG TGC AAT TT-3′; 5′-GAT GGA GTT GAA GGT AGT TTC GTG-3′). AChE mRNA levels were determined utilizing the formula: \(2^{(-\Delta \text{Ct AChE} - \Delta \text{Ct β-actin}) \times \text{Time}}\).

2.4. Western blotting

Immunoblotting protocol has been previously described (Zhang et al., 2002). Anti-β-actin mAb was from Sigma (St. Louis, MO); HRP-conjugated secondary antibody was Santa Cruz Biotechnology. The immunoreactive protein was visualized using a chemiluminescence detection kit (ECL, Santa Cruz Biotechnology).

2.5. Transient transfections and luciferase assays

The 2.2 kb DNA fragment of the human AChE promoter (Ben Aziz-Alloya et al., 1993) was subcloned
into \textit{BgIII} and \textit{HindIII} sites of the pGL3 basic vector (Promega, Madison, WI) with a downstream tagged firefly luciferase gene, pAChE-Luc (Wan et al., 2000). A distal CCAAT binding mutation within the –1270 to –1248 fragment of the human AChE promoter was created using a PCR-based mutagenesis procedure with the forward (F) and reverse (R) end primers (F, 5′-ACG GTA CCG AGC AGC TCT TAC GC-3′; R, 5′-CCG GTA CCG CCG ATA TCA GGG GGT GTG TGC-3′). The PCR product, including the CCAAT binding mutation, was replaced in the wild type pAChE-Luc with two Kpn I sites to construct Distal CCAAT binding mutant. A proximal CCAAT binding mutation within the –594 to –571 fragment of the human AChE promoter was created with the forward (F) and reverse (R) end primers (F, 5′-TAG CCC GGG CTC GAG ATC T-3′; R, 5′-TAC CG GAA TGC CAA GCT T-3′). The following primers (F, 5′-CAG AGA TTG CAT GAG AAG GGG CA-3′; R, 5′-TCA GGG TAC CTC CAG CGA GCG-3′). The proximal CCAAT binding mutation was incorporated in the wild type pAChE-Luc to construct proximal CCAAT binding mutant. All polymerase chain reaction-derived constructs were confirmed by sequencing.

HeLa and MDA cells were cotransfected with firefly luciferase reporter construct pAChE-Luc, Distal CCAAT binding mutant or Proximal CCAAT binding mutant, with Renilla luciferase reporter plasmid pRL-SV40 (Promega, Madison, WI) used as the internal control utilizing LipofectAMINE regent (Invitrogen, Carlsbad, CA). Apoptosis was induced, 24 h after transfection, utilizing LipofectAMINE regent (Invitrogen, Carlsbad, CA), and activity was measured with a luminometer BGP Luciferase Reporter Assay System (Promega, Madison, WI) used as the internal control with Renilla luciferase reporter plasmid pRL-SV40 (Promega, Madison, WI) used as the internal control.

2.6. Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts of HeLa cells were prepared according to Dignam et al. (Dignam, Martin, Shastry, & Roeder, 1983) with some modifications. Electrophoretic mobility shift assays were performed with nuclear extract protein (4 μg) in binding buffer (10 mM Tris–HCl, 1 mM MgCl2, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM DTT, 4% glycerol, pH 7.5) and poly (dl–dc) (1 μg). Binding reactions were incubated (20 min, room temperature) with 20,000 cpm (0.1–0.5 ng) double-stranded oligonucleotides end-labeled with \([\gamma-32P]\) ATP using T4 polynucleotide kinase (Invitrogen). An excess of unlabeled double-stranded oligonucleotides was used as competitors, where indicated. Double-stranded oligonucleotides corresponding to the distal CCAAT binding site and the proximal binding site were 5′-ACC CCC TAA TGG GGG TCG TAC CC-3′ and 5′-TGG ACA TTA TGG GAT GAG AAG GG-3′, named Wt CCAAT Dist and Wt CCAAT Prox, respectively. Mutant double-stranded oligonucleotides corresponding to the distal CCAAT binding site were 5′-ACC CCC TGA TAT CGG CCG TAC CC-3′, named Mut CCAAT Dist. The consensus sequences of the CCAAT motif oligonucleotides were as follows: C/EBP\(^a\) (5′-TGG AGA TTG CCC AAT CTG CA-3′) (sc-2525; Santa Cruz), CCAAT protein 1 (CP1) (5′-AGA TTA TTG AAA AAG ACT AAA CAA TTT CAA CTC ACG AAA C-3′) (Elizondo, Corchero, Sterneck, & Gonzalez, 2000), CCAAT protein 2 (CP2) (5′-GGT TTA CTC GGG ATC ACA AGA CCG TAC GGA AGA TCT CTT-3′) (Elizondo et al., 2000), CCAAT transcription factor (CTF-2) (5′-TAC CTT ATT TTG GAT GAA CCG AAC TAT GAT AAT-3′) (Elizondo et al., 2000), C/EBP (5′-CTAGGGCTTGCGCAATCTATATTCG-3′) (Elizondo et al., 2000), CCAAT transcription factor (CTF-1) (5′-CCT TTG GCA TGC CCA CCA ATG-3′) (Roy & Lee, 1995), and CBF transcription factor (CBF wt) (5′-AGA CCC TCG TAT GTG ATT GTT TGA TCT CTT-3′) (sc-2591; Santa Cruz). Mutant oligonucleotides of the consensus binding site for the CBF transcription factor were (CBF mut) (5′-AGA CCC TCG TAT GTG ATT GTT TGA TCT CTT-3′) (sc-2592; Santa Cruz). Antibody supershift assays were carried out in the same buffer, with further addition of 2 μl of anti-CBF-A/NF-YB (sc-13045X), anti-CBF-B/NF-YA (sc-10779 X), anti-CBF-C/NF-YC (sc-13044X) antibodies (Santa Cruz, CA), and anti-Oct 4 antibody (Santa Cruz, CA). The reaction was electrophoresed on a 4%, 0.5× TBE native polyacrylamide gel then autoradiographed to detect specific DNA–protein complex formation.

2.7. Chromatin immunoprecipitation assay

Assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotech, Lake Placid, NY) according to manufacturer’s protocol. Cells were fixed with 1% formaldehyde (15 min, 37 °C). Cell pellets were resuspended in SDS lysis buffer and sonicated using an ultrasonic homogenizer VP-5S (TITiC, Tokyo, Japan). After centrifugation, the supernatant was diluted in chromatin immunoprecipitation dilution buffer then incubated (overnight, 4 °C) with anti-CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC antibodies (Santa Cruz Biotechnology). Immune complexes were recovered by the addition of 60 μl of salmon sperm DNA/protein A-agarose slurry, followed by incubation.
(2 h, 4 °C). Beads were washed with both low and high salt buffers, a LiCl buffer and a Tris/EDTA buffer and immune complexes were eluted with an elution buffer (1% SDS, 100 mM NaHCO₃, and 1 mM dithiothreitol). To reverse DNA cross-linking, elutes were added to 5 M NaCl (8 μl) and incubated (4 h, 65 °C), followed by treatment with proteinase K. DNA was recovered by phenol-chloroform extraction and ethanol precipitation and pellets were resuspended in 50 μl of Tris/EDTA buffer. PCR was carried out for 35 cycles using 5 μl of sample DNA solution, and PCR products were separated on 2% agarose gels in 1× Tris–acetate/EDTA.

2.8. Statistical analyses

Each experiment was repeated a minimum of 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. A23187 and TG triggered induction of AChE expression during apoptosis in HeLa and MDA cells

The HeLa and MDA-MB-435s cell lines are derived from cervical epithelia and human breast ductal carcinomas, respectively. Living HeLa and MDA cells do not express AChE obviously. In order to establish our system, we treated HeLa and MDA cells with A23187 and TG. A23187, a Ca²⁺ ionophore, equilibrates Ca²⁺ gradients across membranes and can cause a rapid rise in intracellular Ca²⁺ levels (Bartlett, Luethy, Carlson, Sollott, & Holbrook, 1992; Waser, Mesaeli, Spencer, & Michalak, 1997). Thapsigargin (TG), by inhibiting of the sarcoplasmic reticulum Ca²⁺-ATPase pump, causes a transient increase in cytoplasmic Ca²⁺ from ER Ca²⁺ stores, and a later influx of Ca²⁺ from the extracellular milieu, leading to the induction of apoptotic cell death in various cell types (Jackisch et al., 2000; Kaneko & Tsukamoto, 1994; McColl et al., 1998). In our system, A23187 and TG are both capable of inducing apoptosis in HeLa cells and MDA cells assessed by morphology observation, Hoechst staining, TUNEL staining, and activated caspase-3 staining. Fig. 1 shows that at 48 h following A23187 and TG treatments, apoptosis of HeLa cells was detected, as characterized by rounding, shrinking, chromatin condensation, DNA fragmentation and caspase-3 activation.

AChE expression during apoptosis induced by A23187 and TG was also investigated. Fig. 1B and C demonstrate the correlation between changes in cell morphology, chromatin condensation, DNA fragmentation and AChE protein expression in HeLa cells. Apoptotic cells with condensed nuclei were positive for both AChE and TUNEL staining while living cells were negative for both. Additionally, A23187 and TG induced AChE expression in cleaved caspase-3 staining positive cells (Fig. 1D and E). Similar results were noted in MDA cells (data not shown). Our data demonstrated that AChE protein expression was significantly increased in HeLa and MDA cells when they were induced to undergo apoptosis by A23187 and TG.

Semiquantitative RT-PCR confirmed our previous report that the 482 bp tailed AChE-T fragment, but not the AChE-E and AChE-R fragments, was induced during apoptosis (Yang, He, & Zhang, 2002; Zhang et al., 2002) (Fig. 2). Although transcriptional activation of the ACHΕ gene is often associated with a shift in its splicing pattern, leading to accumulation of the rare AChE-R variant under psychological, chemical, and physical stresses (Kaufer, Friedman, Seidman, & Soreq, 1998; Shohami et al., 2000), AChE-R variant accumulation has been noted mostly in mammalian brain neurons, with the shift dependant on neuronal activity (Soreq & Seidman, 2001). AChE-R mRNA transcript has been suggested to represent a general nervous system stress-response element (Shohami et al., 2000). In various cell apoptosis, we found that AChE-T variant, which is the major transcript, increased obviously (Yang et al., 2002; Zhang et al., 2002).

3.2. Effect of calcium on AChE expression during apoptosis induced by A23187 and TG in HeLa and MDA cells

We next examined whether the induction of AChE expression and the increase in intracellular Ca²⁺ were causally related. BAPTA-AM, an intracellular Ca²⁺ chelator, significantly reduced Ca²⁺ concentrations, maintaining it at low levels in the cytoplasm (Muallem, Khademazad, & Sachs, 1990). BAPTA-AM also prevented the dramatic increase in cytosolic Ca²⁺ mediated by A23187 and TG (Bartlett et al., 1992; He et al., 2002). Pre-treatment of HeLa and MDA cells with BAPTA-AM (10 μM, 45 min) inhibited the rapid rise in cytosolic Ca²⁺ induced by A23187 and inhibited the transient rise in cytosolic Ca²⁺ caused by TG (data not shown), in agreement with previous studies. Treatment with A23187 resulted in an increase in AChE mRNA levels at 6 h, an early stage of apoptosis while HeLa and MDA cells did not possess obvious apoptotic morphologic features. However, pre-treatment of both cell lines with BAPTA-AM (10 μM, 45 min) followed...
Fig. 1. Induction of AChE expression during apoptosis of HeLa cells treated with A23187 and TG. At 48 h following DMSO (A), A23187 (2 μM) (B) and TG (2 μM) (C) treatments, apoptosis of HeLa cells was confirmed by: (a) morphology; (b) Hoechst 33258 staining; (c) TUNEL staining; (d) AChE immunocytochemistry staining. At 48 h following A23187 (2 μM) (D) and TG (2 μM) (E) treatments, apoptosis of HeLa cells was confirmed by: (a) Hoechst 33258 staining; (b) AChE immunocytochemistry staining; (c) cleaved caspase-3 immunocytochemistry staining. Thin arrows indicate living cells; bold arrows indicate apoptotic cells.

by A23187 exposure resulted in a significant inhibition of AChE mRNA expression (Fig. 3A and C), with AChE mRNA levels reduced by approximately 50%. AChE protein expression was greatly increased in both cell lines 12 h after apoptosis induction by A23187, which was also inhibited by pre-treatment with BAPTA-AM (10 μM, 45 min) (Fig. 3B and D). Similarly, in TG-induced apoptosis, AChE mRNA and protein levels were
significantly increased at 6 h, and pre-treatment with BAPTA-AM (10 μM, 45 min) also inhibited TG-induced AChE expression (Fig. 4). These data strongly suggested that cytosolic Ca²⁺ plays an important role in regulating AChE expression during apoptosis.

3.3. Effect of calcium on AChE mRNA stability during A23187-induced apoptosis of HeLa and MDA cells

AChE mRNA levels increased dramatically in A23187 treated HeLa and MDA cells compared with untreated controls at indicated time points (Fig. 5A and C). The small increase in AChE mRNA levels in untreated cells may have resulted from apoptosis, which occurred in a few cells, with time. We further investigated the effects of A23187 on AChE mRNA stability so as to clarify whether elevated AChE mRNA levels during apoptosis resulted from an increase in the rate of AChE transcription or an increase in the AChE mRNA stability, or both. To do so, we employed a classical technique involving the measurement of AChE mRNA relative decay rates in transcriptionally arrested cells by using actinomycin D (Act D) in the absence or presence of A23187. The effect of BAPTA-AM on AChE mRNA decay rates was also examined. GAPDH and β-actin mRNA levels were used as internal controls in HeLa and MDA cells, respectively. Since there is a low level of AChE mRNA in untreated cells, it is difficult to analyze AChE mRNA decay rates in the presence of Act D without A23187 pre-treatment. HeLa and MDA cells were first treated with A23187 (2 μM) for 6 h. As shown in Fig. 5B and D, the removal of A23187 as well as the addition of BAPTA-AM with A23187 resulted in an increase in the decay rate of AChE mRNA levels, suggesting that AChE mRNA stability was enhanced by A23187 and that the post-transcriptional mRNA stability is an important mechanism accounting for elevation

![Fig. 2. Semiquantitative RT-PCR analysis of the induction of tailed AChE-T mRNA. HeLa cells were treated with DMSO (control, lane 1) or A23187 (2 μM) for 6 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 48 h (lane 5).](image)

![Fig. 3. Effects of intracellular Ca²⁺ on AChE expression during A23187-induced apoptosis. Real-time quantitative RT-PCR of AChE mRNA levels were normalized to GAPDH and β-actin mRNA levels in HeLa (A) and MDA cells (C), respectively. (Open bars) DMSO treatment (6 h), (filled bars) A23187 (2 μM, 6 h) and (cross-hatched bars) pre-incubated with BAPTA-AM (10 μM, 45 min) then incubated with A23187 (2 μM, 6 h). ** p < 0.005 significant difference relative to cells incubated with A23187. (B) and (D) AChE protein levels were analyzed by Western-blot after 12-h treatment with DMSO, A23187 (2 μM) or pre-incubated with BAPTA-AM (10 μM, 45 min) prior to A23187 (2 μM).](image)
of AChE expression during A23187-induced apoptosis in HeLa and MDA cells. Our data also suggested that calcium also played a role in this event.

### 3.4. Effect of calcium on AChE promoter activity during A23187 and TG-induced apoptosis in HeLa and MDA cells

We subsequently investigated the effects of A23187 and TG on the transcription of the AChE genomic promoter-reporter gene. The 2.2 kb human AChE promoter fused to the firefly luciferase reporter gene, pAChE-Luc and internal control Renilla luciferase reporter plasmid pRL-SV40 were transiently cotransfected to HeLa cells and treated with or without A23187 (2 μM) and TG (2 μM) for indicated times. Cells transfected with promoterless control plasmids showed no detectable luciferase activity (data not shown). There was a significant time-dependent increase in the induction of luciferase activity in treated cells compared to control (Fig. 6A), indicating that A23187 and TG activated AChE promoter activity. Although an approximate three-fold induction of AChE promoter activity was seen at 48 h after treatment, at 6 h after A23187-induced apoptosis, the promoter activity increase was modest in HeLa cells, whereas, the AChE mRNA level increased significantly. This may have been due to the limitation that the truncated portion of the gene promoter, in conjunction with additional regulatory elements, did not reveal all behaviors of the endogenous gene transcription. The more obvious increase in AChE promoter activity induced by TG may also have been due to additional regulatory mechanisms. To test whether AChE promoter activation by A23187 and TG was affected by changes in cytosolic Ca\(^{2+}\) concentrations, HeLa cells were pre-loaded with BAPTA-AM (10 μM, 45 min) followed by incubation with A23187 or TG for 24 h. BAPTA-AM alone had minimal effects on AChE promoter activity (data not shown) while BAPTA-AM pre-treatment resulted in an approximate 30% reduction in A23187- and TG-dependent activation of AChE promoter (Fig. 6B).

A one- to five-fold induction of AChE promoter activity in MDA cells, 6–48 h post-treatment, was induced by A23187 (2 μM) and TG (0.5 μM), relative to untreated controls, with an even greater increase noted in HeLa cells (Fig. 6C). Similar to what was noted in other assays, A23187- and TG-dependent activation of the AChE promoter was also reduced in BAPTA-AM pre-treated cells (Fig. 6D), suggesting that the cytosolic Ca\(^{2+}\) signaling pathway may also have been involved in the transcriptional activity of AChE during apoptosis.

![Fig. 4. Effects of intracellular Ca\(^{2+}\) on AChE expression during TG-induced apoptosis. HeLa cells were incubated with TG (2 μM), and MDA cells were incubated with TG (0.5 μM) for 6 h. AChE mRNA levels were analyzed by real-time quantitative RT-PCR. *p<0.05 significant difference relative to cells incubated with TG (also referred to in Fig. 3A and C). Western blot of AChE protein levels in HeLa cells incubated with TG (2 μM) and MDA cells incubated with TG (0.5 μM) for 6 h (also referred to in Fig. 3B and D).](image-url)
Fig. 5. Effects of calcium on AChE mRNA relative decay rates during A23187-induced apoptosis. HeLa (A) and MDA cells (C) were incubated with A23187 (2 µM), or DMSO. AChE mRNA levels were analyzed by real-time quantitative RT-PCR, normalized to GAPDH and β-actin mRNA levels; mRNA levels at 6 h in untreated cells were considered as 1. Measurement of AChE mRNA levels in transcriptionally arrested cells using Act D (15 µg/ml). HeLa (B) and MDA cells (D) were initially treated with A23187 (2 µM) for 6 h, at this time point (0 h), they were divided into three sets: A23187 was washed out; allowed to remain in the cultures; and BAPTA-AM (10 µM) was added for 45 min in the culture containing A23187. Then the three sets of cells were treated with Act D (15 µg/ml). Total RNA was extracted from cells at indicated times and AChE mRNA levels were analyzed by RT-PCR and normalized to GAPDH and β-actin mRNA levels in HeLa cells and MDA cells, respectively. Relative mRNA levels at 0 h were considered as 1.

3.5. A CCAAT motif within the −1270 to −1248 fragment of AChE promoter functions as an A23187- and TG-responsive element

Investigation of AChE promoter regions was utilized to identify the cis-regulatory elements that might be responsible for the A23187- and TG-dependent activation of the ACHE gene. Transcription of human AChE mRNA is driven by a 2.2-kb sequence upstream from initiator AUG in the ACHE gene, with multiple potential sites for binding universal and tissue-specific transcription factors. These include clustered MyoD elements, E-box, SP1, EGR1, AP-2, and the development-related GAGA motif (Ben Aziz-Aloya et al., 1993). It has been reported that the most proximal CCAAT element to the TATA motif within the Grp78 (BiP) promoter is important for A23187 and TG induction, and that this Ca^{2+} response element is sufficient to confer Ca^{2+} stress inducibility to a heterologous promoter (Li, Alexandre, Cao, & Lee, 1993; Roy & Lee, 1995). Examination of the AChE promoter sequence also revealed two CCAAT motifs, with the distal CCAAT motif upstream of the TATA box, located within the −1270 to −1248 fragment of the AChE promoter and a second motif, located within the −592 to −571 fragment of the AChE promoter (Fig. 7A). Mutations of the two CCAAT sites were constructed so as to further elucidate the role of these motifs in A23187- and TG-induced increases in AChE promoter activity. As shown in Fig. 7B, in comparison to the wild-type construct, the distal mutation of the CCAAT site reduced A23187 and TG inducibility by 35% and 30%, respectively, suggesting that the regulatory element identified for the A23187 induction also mediated the TG response. However, mutation of the proximal CCAAT
Fig. 6. Effects of calcium on AChE promoter activity during A23187- and TG-induced apoptosis. HeLa and MDA cells were cotransfected with pAChE-Luc and pRL-SV40. HeLa cell transfectants (A) were incubated with DMSO (un-induced control) or induced by A23187 (2 μM) or TG (2 μM). MDA cells transfectants (C) were incubated with DMSO (un-induced control) or induced by A23187 (2 μM) or TG (0.5 μM). HeLa cells transfectants (B) were incubated with DMSO (un-induced control), A23187 (2 μM) and TG (2 μM), or A23187 (2 μM) and TG (2 μM) plus BAPTA-AM (10 μM) for 24 h; MDA cells transfectants (D) were incubated with DMSO (un-induced control), A23187 (2 μM) and TG (0.5 μM), or A23187 (2 μM) and TG (0.5 μM) plus BAPTA-AM (10 μM) for 24 h. AChE promoter activity was analyzed by luciferase activity and represented as the fold induction relative to un-induced controls. * p < 0.05 significant difference relative to cells incubated with A23187 or TG alone.

Binding site did not reduced A23187 or TG inducibility as dramatically. Similar responses were seen in MDA cells (Fig. 7C) suggesting that the distal CCAAT element upstream of the TATA box was important for A23187- and TG-induced AChE promoter activation. Given that A23187 and TG are known to induce perturbations in cellular Ca2+ homeostasis, our data further suggested that the distal CCAAT binding motif was involved in Ca2+ signal transduction. It is relevant to note that residual levels of A23187 and TG induction were observed with mutations in the distal CCAAT, suggesting other elements in the AChE promoter compensated for the function of the mutated elements in A23187 and TG responses.

3.6. Identification of the CCAAT binding factor (CBF/NF-Y) binding to the distal CCAAT element

In order to investigate which binding proteins were able to bind to the two CCAAT motifs, distal and the proximal CCAAT site probes (Wt CCAAT Dist and Wt CCAAT Prox) were used in EMSAs together with nuclear extracts from HeLa cells. The Wt CCAAT Dist probe was found to form DNA–protein complex (Fig. 8A, lane 2), whereas, Wt CCAAT Prox probe did not bind to any protein (data not shown). As shown in Fig. 8A, an excess of unlabeled probe that incorporated mutation in the distal CCAAT binding site did not inhibit the complex formation (Fig. 8A, lane 4). However, an excess of unlabeled probe, specific for the distal CCAAT site, competed for complex formation with the labeled Wt CCAAT Dist probe (Fig. 8A, lane 3). Given that the distal CCAAT motif found in the human ACHE gene promoter region could potentially represent a binding site for a number of nuclear factors, including C/EBP, C/EBP, CTF1, CTF2, CP1, CP2, and CBF/NF-Y, the binding of these nuclear factors was tested in a competitive EMSA. Our data showed that an excess of the consensus oligonucleotides of CBF/NF-Y (CBF wt) inhibited the binding of the labeled Wt CCAAT Dist probe (Fig. 8A, lane 6), whereas, C/EBP, C/EBP, CTF1, CTF2, CP1, and CP2 consensus oligonu-
Fig. 7. A CCAAT motif within the $-1270$ to $-1248$ fragment of the AChE promoter is an A23187- and TG-responsive element. (A) The two CCAAT mutants from the human AChE promoter were constructed as described in Section 2. Wild type and mutated human AChE promoters were tagged with reporter luciferase, pAChE-Luc, Distal CCAAT binding mutant, and Proximal CCAAT binding mutant, respectively. HeLa and MDA cells were cotransfected with pAChE-Luc or Distal CCAAT binding mutant or Proximal CCAAT binding mutant, and pRL-SV40. Transfectants of HeLa cells (B) were incubated with DMSO (un-induced control) or induced by A23187 ($2 \mu M$) or TG ($2 \mu M$) for 24 h. Transfectants of MDA cells (C) were incubated with DMSO (un-induced control) or induced by A23187 ($2 \mu M$) or TG ($0.5 \mu M$) for 24 h. AChE promoter activity was analyzed by the luciferase activity and represented as the fold induction relative to un-induced controls. * $p < 0.05$ significant difference from cells cotransfected with pAChE-Luc and pRL-SV40.

4. Discussion

In our previous study, we reported the novel finding that apart from its catalytic function in hydrolyzing acetylcholine, AChE expression was induced in various cell types upon treatment with different apoptosis complexes were immunoprecipitated with antibodies and the recovered DNA fragments, including the distal CCAAT motif, were monitored by PCR using primers for the $-1312$ to $-1111$ region of the AChE promoter. DNA fragments immunoprecipitated with polyclonal antibodies against CBF/NF-Y were amplified by PCR (Fig. 8D, lane 3) as well as the positive control (Fig. 8D, lane 2), whereas, those immunoprecipitated with anti-Oct 4 antibody (Fig. 8D, lane 4) or without antibody (Fig. 8D, lane 5) were not. These results indicated that CBF/NF-Y was able to bind directly to the distal CCAAT motif in the AChE promoter.

4. Discussion

In our previous study, we reported the novel finding that apart from its catalytic function in hydrolyzing acetylcholine, AChE expression was induced in various cell types upon treatment with different apoptosis
inducers (Zhang et al., 2002). Blocking the expression of AChE with antisense inhibited apoptosis (Zhang et al., 2002), and silencing of the ACH gene by siRNA abolished the decrease in cell viability (Park et al., 2004), suggesting that AChE could be a novel regulator of apoptosis. Elucidating the regulatory mechanisms of AChE expression during apoptosis may improve therapeutic methods to regulate apoptosis, an important factor in prominent diseases, including cancers (with apoptosis-resistant tumor cells) and neurodegenerative disorders, such as Alzheimer’s and Parkinson’s disease (Fadeel, Orrenius, & Zhivotovsky, 1999). In the present study, we choose HeLa and MDA cells, two cell lines that do not express AChE strongly in normal states, as our model systems. We found that intracellular Ca2+ played a critical role in the expression of AChE during A23187- and TG-induced apoptosis.

We further examined the mechanisms that upregulated AChE mRNA levels during apoptosis induced by A23187. A23187 can cause a sustained high intracellular free Ca2+ level. Our results indicated that a post-transcriptional mechanism, operating at the level of transcript stability, appeared to be an important regulatory step involved in the regulation of AChE expression induced by A23187, and that this regulatory processes was mediated by cytosolic Ca2+. It has previously been demonstrated that the regulation of mRNA stability plays a crucial role in the basic mechanisms underlying gene expression (Guhaniyogi & Brewer, 2001; Mitchell & Tollervey, 2000). This regulatory mechanism involves not only distinct cytoplasmic factors, but also the contribution of sequence determinants located both within and outside the coding regions of mature transcripts, such as AUUUA-rich elements (AREs), identified by their ability to target host mRNAs towards rapid degradation (Barreau, Paillard, & Osborne, 2005). Various exogenous factors, including calcium ionophores, modulate ARE-directed mRNA degradation (Ruth, Esnault, Jarzembowski, & Malter, 1999). Interestingly an AUUUA-element has been located in the 3′ untranslated region (UTR) of AChE mRNA. It has been recently reported that the RNA-binding protein HuD regulated AChE expression, at a post-transcriptional level, by binding directly to the AChE 3′ UTR, containing an ARE element, in nerve growth factor-treated PC12 cells (Deschenes-Furry et al., 2003). Taylor and colleagues proposed that the ACH gene was ubiquitously transcribed, and that the transcript was readily degraded in most tissues, whereas, it was specifically stabilized in the tissues where transcripts could be detected (Mutero et al., 1995). The rise in cytosolic Ca2+ during A23187-induced apoptosis in HeLa and MDA cells may directly or indirectly block destabilizing factors or activate stabilizing factors critical for AChE mRNA stabilization. Further research is needed to fully elucidate this question.

Our results also demonstrated there was a Ca2+-dependent increase in AChE promoter activity during A23187 and TG-induced apoptosis, indicating that ACH gene expression was modulated at the transcriptional level, with the regulation of AChE promoter activity facilitated by Ca2+-dependent signaling pathways. In order to examine the transcriptional regulation of the ACH gene, we focused on a CCAAT motif within the −1270 to −1248 fragment of the human ACH promoter. A23187 and TG are known to disturb Ca2+ homeostasis. The mutation of distal CCAAT binding site significantly decreased A23187 and TG-induced AChE promoter activation, suggesting that the Cu2+ signal transduction was mediated, at least in part, through the CCAAT motif. The CCAAT motif is a common promoter element present in the proximal promoter of numerous mammalian genes transcribed by RNA polymerase II (Maity & de Crombrugghe, 1998). Mutations in the CCAAT motifs of several gene promoters alter transcriptional regulation by various inducing agents (Maity & de Crombrugghe, 1998). Li et al. have reported that the stimulation of grp78 transcription by A23187...
and TG, following ER Ca\(^{2+}\) discharge, acted through a novel pathway in which a Ca\(^{2+}\) signal was transduced through the most proximal CCAAT element to the TATA motif, achieving specific gene regulatory events (Li et al., 1993). Our result may give another example that Ca\(^{2+}\) signal is transduced through elements containing CCAAT motifs within the promoter regions.

This is, to our knowledge, the first demonstration that the CCAAT binding factor (CBF/NF-Y) may be a potential transcriptional factor mediating AChE transcriptional activity during apoptosis through a CCAAT motif. CBF/NF-Y was originally identified as a protein complex able to bind to the MHC class II conserved Y box in Ea promoters, the yeast CYC1 U AS CCAAT box, and the \(\alpha 2(I)\) collagen gene in the proximal promoter region (Matsuo et al., 2003). CBF/NF-Y was not only involved in high-basal-level transcriptional activation (Matsuo et al., 2003), but also contributed to induced transcription of the grp78 gene under calcium stress conditions (Roy & Lee, 1995). Having established that CBF/NF-Y was recruited to the CCAAT motif upstream of the TATA sequence, within the AChE promoter, we are currently investigating the mechanism by which it regulates the A23187- and TG-induced transcription of the ACHe gene. It is possible that the interactions of CBF/NF-Y with other transcription factors are effected by A23187 and TG, or that the binding CBF/NF-Y to the CCAAT motif is influenced by intracellular calcium concentrations, similar to what has been reported in the grp78 gene (Roy & Lee, 1995).

It has been suggested that neuronal death in Alzheimer’s disease (AD) is due to apoptosis (Li, Chan, Lai, & Yew, 1997; Su, Deng, & Cotman, 1997). Dysregulation of intracellular calcium signaling also has been implicated in the pathogenesis of AD (LaFerla, 2002; Mattson & Chan, 2003). In vivo and in vitro studies have demonstrated that A\(\beta\) destabilizes neuronal Ca\(^{2+}\) homeostasis, leading to a significant increase in cytosolic Ca\(^{2+}\) and triggering neuronal apoptosis (Goodman & Mattson, 1994; Mattson, Tomaselli, & Rydel, 1993). Increased AChE activity has been reported in plaques and tangles early in the process of amyloid deposition of AD (Moran, Mufson, & Gomez-Ramos, 1993; Ulrich, Meier-Ruge, Probst, Meier, & Ipsen, 1990), promoting the assembly of A\(\beta\) into fibrils (Inestrosa, Alvarez, & Calderon, 1996) and increasing the cytotoxicity of these peptides (Alvarez et al., 1998). We believe that further investigation of AChE up-regulation, its targets and functions during apoptosis, will further elucidate apoptosis mechanism of action as well as facilitate the development of therapeutic approaches that selectively target AChE expression pathways in the treatment of AD.

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