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An Endogenous Calcium-Dependent, Caspase-Independent Intranuclear Degradation Pathway in Thymocyte Nuclei: Antagonism by Physiological Concentrations of K^+ Ions

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Abstract

Calcium ions have been implicated in apoptosis for many years, however the precise role of this ion in the cell death process remains incomplete. We have extensively examined the role of Ca^{2+} on nuclear degradation *in vitro* using highly purified nuclei isolated from non-apoptotic rat thymocytes. We show that these nuclei are devoid of CAD (caspase-activated DNase), and DNA degradation occurs independent of caspase activity. Serine proteases rather than caspase-3 appear necessary for this Ca^{2+}-dependent DNA degradation in nuclei. We analyzed nuclei treated with various concentrations of Ca^{2+} in the presence of both a physiological (140 mM) and apoptotic (40 mM) concentration of KCl. Our results show that a 5-fold increase in Ca^{2+} is required to induce DNA degradation at the physiological KCl concentration compared to the lower, apoptotic concentration of the cation. Ca^{2+}-induced internucleosomal DNA degradation was also accompanied by the release of histones, however the apoptotic-specific phosphorylation of histone H2B does not occur in these isolated nuclei. Interestingly, physiological concentrations of K^+ inhibit both Ca^{2+}-dependent DNA degradation and histone release suggesting a reduction of intracellular K^+ is necessary for this apoptosis-associated nuclear degradation in cells. Together, these data define an inherent caspase-independent catabolic pathway in thymocyte nuclei that is sensitive to physiological concentrations of interacellular cations.

Keywords

Apoptosis; Nuclease; $CaCl_2$; DNA fragmentation; Histone H2B

Introduction

Intra and extra-cellular ions play an important role in a variety of cellular functions and the concentration of intra-cellular ions is tightly regulated in cells by numerous energy-requiring cellular mechanisms [1]. For example, extra-cellular signaling pathways regulate cellular function by means of calcium ions acting through effector enzymes [2]. Alterations in cellular Ca^{2+} also occur during cell growth, differentiation, survival and apoptosis [3]. The regulation

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of cellular Ca^{2+} concentration is strongly associated with apoptotic states and the initiation of apoptosis [4,5]. During the early phase of apoptosis, the Ca^{2+} concentration increases in the cytoplasm in various apoptotic model systems, reaching a level much higher than in normal cells [6]. Additionally, Bcl-2 and related proteins are key components of the apoptotic process that modulate endoplasmic reticulum-associated Ca^{2+} influx and maintain Ca^{2+} homeostasis [7]. However, the mechanisms regulating the increase of intracellular Ca^{2+} along with a role for Ca^{2+} during apoptosis is not completely understood [8].

An increase of Ca^{2+} is observed when glucocorticoids induce apoptosis in lymphocytes [5,9] and primary thymocytes [10]. Treatment of cells *in vitro* with multiple apoptotic agents also induces an increase in intracellular Ca^{2+} (ionomycin, A23187 or thapsigargin) resulting in apoptosis morphologically and biochemically similar to glucocorticoid treatment of lymphatic cells [5,9,11]. This phenomenon is thought to result from the apoptotic stimulus inducing Ca^{2+} influx into the cytoplasm from the extra-cellular environment, as well as release of Ca^{2+} from the endoplasmic reticulum pools. The elevated Ca^{2+} concentration in the cytoplasm results in a further Ca^{2+} influx into both mitochondria and nuclei. In nuclei, Ca^{2+} has been reported to modulate gene transcription to activate apoptosis-associated enzymes that initiate DNA fragmentation and chromatin condensation [12]. Apoptotic DNA degradation is largely thought to occur by caspase-dependent endonucleases such as CAD [13]. Interestingly, Lechardeur et al. [14] has determined that ICAD/CAD exist in both the cytoplasmic and nuclear fraction of apoptotic cells, however the role of caspase-independent pathways in DNA degradation is less well defined [15].

Studies using *in vitro* systems have shown that DNA degradation can be induced in isolated nuclei from non-apoptotic thymocytes by the addition of Ca^{2+} [16], suggesting that Ca^{2+}-dependent endonucleases may pre-exist in the nuclei in a repressed state in non-apoptotic cells. Activation of these endonucleases during apoptosis to digest chromosomal DNA and produce DNA fragmentation has been proposed as a mechanism for the propagation of apoptosis [17]. Therefore, it has been proposed that the increase in Ca^{2+} concentration could be one of the triggers for the initiation of apoptosis [8], as well as being a late component of the process. Thus, an open issue is how the activation of these endogenous endonucleases is repressed in the nuclei of non-apoptotic cells.

Previous work from our laboratory has suggested that inhibition of apoptosis in non-apoptotic cells is caused by repressing the activation and activity of proapoptotic enzymes including endonucleases and proteases via maintenance of a homeostatic concentration of intracellular potassium [18]. Normal cells maintain high intracellular potassium in the range of 120 to 150 mM [19]. These studies have shown that apoptosis-associated enzymes are activated concomitantly with a decrease in K^+ concentration to approximately 30 to 50 mM [20,21]. Based on these data, we have hypothesized that the physiological concentration of K^+ prevents the activation of various apoptotic-associated enzymes. Consequently, these apoptotic enzymes are activated or disinhibited by ion fluxes that also result in a change in cell volume, or cell shrinkage during apoptosis [18].

We have now extensively examined the effect of Ca^{2+} on DNA degradation and chromatin structure in isolated nuclei obtained from non- apoptotic rat thymocytes, and evaluated the relationship between Ca^{2+}-dependent DNA degradation and intracellular K^+. We show here that thymocyte nuclei contain an endogenous calcium-activated, caspase-independent nuclease that results in apoptotic DNA fragmentation and histone release. Furthermore, both Ca^{2+}-dependent nuclear degradation and histone release are highly sensitive to inhibition by physiological levels of intracellular cations.

Materials and Methods

Isolation of nuclei

Adrenalectomized rats (male, Sprague Dawley) around one to two months old (body weight, 60 to 75 g) were decapitated. Thymus was surgically removed and was minced with scissors in PBS solution. The minced tissues were shaken and free thymocyte cells were released. The cell suspension was filtered through a nylon mesh (200 μm Nitex mesh, Tetko Inc., New York, NY) twice and free thymocytes were recovered by centrifugation at room temperature. Animal studies were done in accordance with institutional guidelines for the care and use of laboratory animals. For the isolation of nuclei from thymocytes, methods were modified from Widlak et al [22]. The thymocytes were washed once in isolation buffer (20 mM Hepes pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.25 M sucrose, 1 mM DTT and 1 mM PMSF). The cells were resuspended in isolation buffer, and treated with 0.25 % NP-40 in buffer for 10 min at 4°C. The buffer containing 1.2 M sucrose was underlayed as a cushion and centrifuged. The nuclear pellet was washed once in isolation buffer. Aliquoted nuclei were either used immediately, or stored at −80°C with 50% glycerol in the buffer and used within two weeks. We observed no comparable difference between fresh or frozen nuclei that were used interchangeable in our experiments within this two-week period.

Incubation of nuclei with $CaCl_2$

Nuclei (2×10^6) were suspended in 200 μl of incubation buffer (20 mM Hepes pH 7.5, 3 mM $MgCl_2$, 0.25 M sucrose, 1 mM DTT and 1 mM PMSF) and incubated with or without $CaCl_2$ for 60 or 90 min at 37°C. In this incubation system, effect of ions (monovalent and divalent cations), their chelaters, and various chemicals (inhibitors for caspase or non-caspases, and K^+ cations containing compounds) were examined. For the examination of the effect of osmolarity on the Ca^{2+}-dependent DNA degradation, nuclei were incubated with or without 1.0 mM $CaCl_2$ in the buffer containing additional sucrose (330 mM and 530 mM) that is equivalent to the addition of 40 mM and 140 mM KCl in the buffer, respectively.

DNA analysis with gel electrophoresis

For the analysis of DNA degradation, nuclei were harvested after incubation and were treated with 1 mg/ml of DNase-free RNase for 30 min at 37°C, and then 400 μg/ml proteinase K overnight. DNA (1 or 2 μg) was run on 1.8 % agarose gel for 90 min at 100 V. The DNA was stained with 5 μg/ml of ethidium bromide. A size marker of DNA (100 base pairs of DNA ladder, Invitrogen) was run together with the DNA samples. DNA patterns were photographed with a Spectroline UV-transilluminator. For the fluorometric TUNEL analysis, $CaCl_2$ treated nuclei were fixed with 4% paraformaldehyde and plated on cover slips. The nuclei were examined with a fluorometric TUNEL assay kit (Promega) and observed with an immunoflurescent microscope (Olympus IX70, AX10: Version 3.0) (Magnification, X 200).

Pulsed field gel electrophoresis

For the analysis of higher molecular weight DNA fragments, pulsed field gel electrophoresis (PFGE) was conducted by the method of Hughes and Cidlowski [23]. Nuclei (2×10^7) were incubated in isolation buffer containing 40 or 140 mM KCl with or without 1 mM $CaCl_2$. After 90 min at 37°C, the nuclei were then treated with RNase for 1 h. The nuclei were suspended in 0.5 % low melting temperature agarose, (NuSieve GTG Agarose, CAMBREX) in PBS 37° C, transferred to a plug mold, and cooled to 4°C before extruding into 100 mM EDTA and 1 % N-sarcosyl for lysis at 37°C overnight. The plugs were then treated with 1 mg/ml proteinase K in the same buffer overnight at 50°C. The plugs were embedded in a 1% agarose gel (SeaKem GTG, Cambrex) and subjected to PFGE using a Beckmann Geneline II system. The DNA was

stained with 0.5 μg/ml of ethidium bromide for 3 h. DNA size standards for PFGE were Lamda DNA ladder, MW, 48.5 to 1000 kb, and H. Wingei, MW, 1 to 3 mb (BioRad).

SDS gel electrophoresis and Western blotting

For the detection of histones, soluble chromatin in lysis buffer was directly mixed with SDS loading buffer and boiled for 5 min, then run on a 14% SDS gel (Invitrogen Inc.) at 150 V for 2 h. Proteins were stained with 0.2 % Commassie Brilliant blue. For the detection of caspase 3 and CAD, cytoplasmic or nuclear proteins (50 μg) were run on the SDS gel. Western blots were carried out using an anti-cleaved caspase 3 antibody (Cell Signaling) or anti-CAD antibody (Santa Crutz). During analysis of histone H2B phosphorylation, thymocytes were treated with 1 μM dexamethasone for 4 h and nuclei were incubated with $CaCl_2$ and harvested. Histones were extracted [24] and western blotted against anti-phospho S14H2B (Upstate) [25].

Analysis with Phase, Confocal and Electron Microscopy

Thymocyte cells and nuclei were observed by phase contrast microscopy (Nikon eclipse; TE200-E, SPOT version 4.0.6). For confocal microscopy, cells and nuclei were stained with Hoechst 33342 (5 μg/ml final) for 30 min, washed in their appropriate buffer and examined on a LSM 510 UV mounted on an Axiovert 200M microscope (Carl Zeiss, Inc.) with a Plan-Apo 63× oil N.A.=1.4 objective lens to obtain simultaneous DIC and UV images. Hoechst was excited at 361 nm from an Enterprise ion laser (Coherent Laser, Auburn, CA), and emission was collected with a 385LP filter. For the analysis by electron microscopy, pellets of the thymocytes or nuclei were fixed with a modified Karnovsky fixate. Each sample was subsequently processed and embedded in Spurr's resin. Thin sections (approximately 90 nm) from each epoxy block were cut, mounted on 200-mesh copper grid, stained with 10 % methanolic uranyl acetate and Reynold's lead citrate, and then examined on a JEOL 100S transmission electron microscope.

Measurement of caspase-3 activity

Caspase-3-like protease activity was measured using a fluorometric assay according to the recommendation of the manufacturer (Kamiya Biomedical Co., Thousand Oaks, CA). For the preparation of samples, thymocytes (5×10^7 cells) were lysed by sonication in lysis buffer or, 0.4 N NaCl soluble nuclear extracts were prepared from the nuclei with or without 1 mM $CaCl_2$. Debris was pelleted at 100,000 × g for 20 min. The supernatant was saved, and the amount of protein was measured. Reaction mixture (500 μl) consisted of 420 μl of caspase/CPP32 buffer (2 mM dithiothreitol, 0.1M HEPES pH 7.4, 1% sucrose, 0.1% CHAPS), 50 μl of 100 mM DTT, 10 μl of 2.5 mM substrate solution (Z-DEVD-AFC, Kamiya Biochemical Co.) and 20 μl of sample proteins (10, 25 and 50 μg). The mixture was incubated for 1 h at 30° C with or without 100 μM of noncompetitive inhibitor Z-DEVD-aldehyde (DEVD-al). Aliquoted samples were further incubated for 12 h at 30°C, and their fluorescence was determined at 400 nm (excitation) and 505 nm (emission). A standard curve of fluorescence versus free AFC (7-amino-4-trifluoromethyl coumarin, Sigma) was used for the positive control, and for the calculation of a specific activity of caspase-3-like enzymes in samples. FU value of each sample was subtracted with the FU value of no sample proteins.

Results

Morphology and the examination of CAD in isolated thymocyte nuclei

Following the isolation of rat thymocyte nuclei from non-apoptotic cells, we analyzed the nuclear structure by phase microscopy (Fig. 1a, left), confocal microscopy (Fig. 1a, middle) and electron microscopy (Fig. 1a, right), comparing primary thymocytes to isolated nuclei. The

isolated nuclei were judged intact, and free of contamination of cytoplasm by all three techniques. These data indicate that isolated nuclei from non-apoptotic thymocytes represent a pure population containing no contamination of intact thymocytes. Additionally, western blot analysis shows that only the cytoplasm of thymocytes contains CAD, while the nuclei isolated from non-apoptotic cells incubated in the absence or presence of 1mM $CaCl_2$ do not have detectable amounts of this nuclease (Figure 1b).

Ca^{2+} induces DNA degradation and histone release in isolated nuclei

Isolated nuclei were further examined by a fluorometric terminal deoxynucleotidyl transferase dUTP-biotin nick-end-labeling (TUNEL) assay to determine the extent of degraded DNA in the presence of 0.5 mM $CaCl_2$. All nuclei treated with $CaCl_2$ were TUNEL positive (Fig. 2a, top), whereas control nuclei showed only a few TUNEL positive nuclei (Fig. 2a, bottom). Apoptotic cells are known to shrink condensed chromatin during this cell death process, however we observed that nuclei treated with $CaCl_2$ were not significantly compacted as judged by DAPI staining (Fig. 2a). We next examined the effect of $CaCl_2$ treatment on DNA degradation in the concentration range of 0.1 μM to 1 mM final. Internucleosomal DNA ladders characteristic for apoptosis were observed in nuclei isolated from non-apoptotic thymocytes in buffer containing 50 μM to 1.0 mM $CaCl_2$ (Fig. 2b). Treatment of nuclei from non-apoptotic thymocytes with $CaCl_2$ concentration below 50 μM did not result in internucleosomal DNA degradation within the time frame of our experiments. During the incubation, internucleosomal DNA degradation was observed as early as 15 min after treatment, and reached a maximum level around 60 to 90 min post treatment (data not shown). These data strongly suggests that the Ca^{2+}-dependent DNA degradation machinery pre-exists in isolated nuclei obtained from non-apoptotic thymocytes. To examine the specificity of this Ca^{2+}-dependent DNA degradation, we examined the effects of EDTA, a chelater of divalent cations, or EGTA, which has a high specificity for Ca^{2+} than EDTA, on DNA degradation. Both EDTA and EGTA markedly inhibited Ca^{2+}-dependent DNA degradation (Fig. 2c). The inhibitory effect of EGTA (Fig. 2c, lane 8) appeared greater than EDTA (Fig. 2c, lane 7) as predicted based on their relative affinities for Ca^{2+}.

We also evaluated the effect of $CaCl_2$ treatment on histone release and observed the release of histones from $CaCl_2$ treated nuclei, specifically at the higher concentration of $CaCl_2$ (over 0.2 mM) that accompanies internucleosomal DNA fragmentation (Fig. 3a). Previous studies have shown that histone H2B is specifically phosphorylated during apoptosis in intact cells [24, 25], however it is unknown if Ca^{2+} treatment of isolated nuclei can also promote this apoptotic-specific phosphorylation of H2B. Figure 3b shows that H2B is phosphorylated in thymocytes induced to undergo apoptosis by dexamethasone, whereas H2B in isolated nuclei treated with 1 mM Ca^{2+} was not phosphorylated, despite the occurrence of degraded DNA. Thus our data suggest that while Ca^{2+} alone can induce the degradation of nucleosomes, as well as DNA, H2B phosphorylation only occurs in nuclei in intact cells and is not necessary for the occurrence of DNA degradation in isolated nuclei in the presence of elevated Ca^{2+}.

Examination of protease involvement during Ca^{2+}-dependent DNA degradation

It is well known that numerous proteases are activated in apoptotic cells. We therefore analyzed whether proteases exist or are activated in isolated nuclei during DNA degradation observed in response to $CaCl_2$. Figure 4a shows that none of the three classical caspase inhibitors (Z-VAD for pan caspases, DEVD for caspase-3, and IETD for casapase-8) altered the Ca^{2+}-dependent internucleosomal DNA degradation in isolated nuclei. Additionally, western blot analysis for caspase-3 showed that isolated non-apoptotic nuclei have a negligible amount of either intact or cleaved caspase-3 (Fig. 4a, ii).

Caspase-3-like protease activity was also directly analyzed in cytosolic and nuclear extracts by a fluorometric assay using a peptide of caspase-3 conjugated with a fluorescent dye, AFC (Figure 4b). In the normal cytosolic thymocyte lysate, caspase-3 activity is evident in a protein-dependent manner (filled circles in Fig 4b, left). This caspase-3 activity was completely inhibited by a caspase-3 inhibitor, DEVD-FMK (open circles in Fig 4b, left). In contrast, no significant caspase-3 activity was observed in the soluble nuclear proteins from isolated nuclei (Fig 4b, middle). Additionally, caspase-3 activity was also not detected in nuclei incubated with 1 mM $CaCl_2$ (Fig 4b, right), despite the extensive occurrence of nuclear DNA degradation (as shown in Fig. 2b). Together these data in conjunction with the data on the incubation of nuclei with caspase inhibitors and western blot analysis suggest that Ca^{2+}-dependent DNA degradation in nuclei is independent of caspase-3-like activity.

We further evaluated other protease inhibitors, such as the serine protease inhibitors TLCK, TPCK and PMSF, for their ability to inhibit Ca^{2+}-dependent DNA degradation. Among the three serine protease inhibitors examined, TLCK had not effect, whereas both TPCK and PMSF showed a partial inhibition of degraded DNA, with PMSF appearing to be more effective (Fig. 4c). Again, the caspase inhibitors DEVD and Z-VAD had no effect on preventing DNA degradation (Fig. 4c). Our experiments with protease inhibitors suggest that isolated nuclei do not have caspases, but rather caspase-independent protease activities, which may be involved in nuclear DNA degradation triggered by calcium.

Physiological concentrations of K^+ inhibit Ca^{2+}-dependent DNA degradation

Since the intracellular concentration of K^+ rapidly decreases in the early phase of apoptosis, the effect of K^+ ion concentration was extensively examined during Ca^{2+}-dependent DNA degradation *in vitro*. Forty mM of KCl was used to reflect the KCl concentration found in apoptotic cells [20,21], whereas 140 mM of KCl was used as a physiological concentration of KCl found in normal healthy cells [19]. In the presence of 0.5 mM $CaCl_2$, a fluorometric TUNEL assay was performed on isolated nuclei treated in the presence of either 40 or 140 mM KCl (Fig. 5a). TUNEL positive nuclei were observed for a majority of the nuclei incubated in the 40 mM KCl buffer in the presence of $CaCl_2$, whereas the occurrence of TUNEL-positive staining was significantly reduced at 140 mM KCl. These data suggest that Ca^{2+}-dependent DNA cleavage in nuclei can occur at low (apoptotic) concentrations of KCl, but is largely inhibited at the physiological concentrations of KCl. We also examined the effect of various KCl concentrations ranging from 5 to 150 mM in the presence of $CaCl_2$ to determine the extent of Ca^{2+}-dependent DNA degradation in isolated nulei. A significant inhibitory effect of KCl was observed at 90 mM KCl (Fig. 5b). Over 110 mM, KCl has a profound inhibitory effect on internucleosomal DNA degradation. Interestingly, at the lower concentration of K^+ previously reported to reflect the intracellular concentration of K^+ in apoptotic cells (30 to 50 mM K^+) [20,21], a distinct pattern of internucleasomal DNA degradation was observed (Fig. 5b).

Previous studies examining the effect of high KCl on DNA degradation has focused mainly on increasing concentrations of KCl, thus we wished to determine how various concentrations of $CaCl_2$ at either an apoptotic or physiologic concentration of KCl would effect Ca^{2+}-dependent DNA degradation. At a physiological concentration of K^+ (140 mM), internucleosomal DNA ladders were observed at approximately 1 mM $CaCl_2$ (Fig. 6a, as indicated by an arrow), with DNA degradation being significantly reduced at the lower concentrations of Ca^{2+}. In contrast, in the presence of 40 mM K^+, a buffer reflecting an "apoptotic concentration" of K^+, the effective concentration of Ca^{2+} necessary for DNA degradation was significantly lower than observed in the 140 mM K^+ buffer (Fig. 6b). Under this apoptotic K^+ concentration, DNA ladders were observed at concentrations of $CaCl_2$ as low as 0.2 mM (Fig. 6b, indicated by an arrow). Together, these data suggest a 5-fold lower concentration of the stimulus is required in the presence of an apoptotic concentration of KCl,

compared to the physiological level of KCl. Interestingly, under both KCl conditions, $CaCl_2$ in the range of 8 to 10 mM had a slight inhibitory effect on the extent of DNA degradation. Since it is known that other cations can have a similar inhibitory effect on DNA degradation [18], our data suggests that high concentrations of $CaCl_2$ may prevent the occurrence of DNA fragmentation, even under conditions where $CaCl_2$ is used as the stimulatory agent.

To evaluate whether the change in osmolarity resulting from adding KCl to the buffer was responsible for this inhibitory effect, DNA degradation of nuclei in sucrose containing buffer with an equivalent osmolarity to KCl was examined. Nuclei in buffer prepared using either the normal concentration of sucrose (250 mM) in the buffer; 330 mM sucrose, which is equivalent to the 40 mM KCl buffer; or 530 mM sucrose, which is equivalent to the 140 mM KCl buffer were incubated with or without $CaCl_2$ and their DNA was analyzed by agarose gel (Fig. 6c). Increasing the osmolarity using sucrose did not prevent DNA degradation upon $CaCl_2$ treatment, suggesting that a change of osmolarity is not responsible for the inhibition of DNA fragmentation, thus demonstrating specificity based on either 40 or 140 mM KCl in the buffer.

We also examined degradation of large DNA molecules, in the mega-base pair range, in nuclei incubated in the absence and presence of $CaCl_2$ by pulse field gel electrophoresis (PFGE). First, DNA from nuclei maintained at 4°C was compared with DNA from nuclei incubated at 37°C (Fig. 6d, i). When nuclei were maintained at 4°C, DNA molecules between 600 kb to 2 mega base pairs (mb) were observed, whereas virtually no DNA of this size existed in nuclei at 37 °C. In contrast at 37 °C, the DNA was fragmented to an average size of approximately 300 kb, even in the absence of $CaCl_2$. However, addition of either 40 or 140 mM KCl consistently suppressed this degradation (Fig. 6d, ii, lanes 2 and 3), indicating that the physiological concentration of K^+ also inhibits fragmentation of high molecular weight DNA. The addition of 1 mM $CaCl_2$ resulted in the large DNA being degraded into fragments smaller than 100 kb even in the presence of 40 or 140 mM KCl (Fig. 6d, ii). Thus physiological K^+ can inhibit both mega base and internucleosomal DNA degradation in the absence of exogenous Ca^{2+}. In contrast, in the presence of Ca^{2+}, physiological K^+ can only inhibit DNA fragmentation at the level of internuclesomal ladder formation, and does not prevent the degradation of large DNAs of more than 100 kb in size.

Prevention of histone release at a physiological concentration of K^+

SDS gel electrophoresis was conducted to further evaluate the release of histones from nuclei treated with $CaCl_2$ as shown earlier (see Figure 3a), in the presence of either the apoptotic or physiologic concentration of KCl. In 40 mM KCl with 0.5 mM $CaCl_2$, core histones (H2A, H2B, H3 and H4) were clearly observed around 13 Kd, and H1 was observed around 32 K (Fig. 7, right), along with a robust amount of degraded DNA (Fig. 7, left). In contrast, the amount of histone released in the presence of 140 mM KCl was negligible (Fig. 7, right), coordinate with the inhibition of internucleosomal DNA (Fig. 7, left). Together these data indicate that a physiological concentration of K^+ prevents both histone release along with internucleosomal DNA degradation during Ca^{2+}-treatment of isolated nuclei.

Discussion

Mechanism of DNA degradation in isolated nuclei by Ca^{2+}

We have examined apoptotic DNA degradation that occurs in the presence of Ca^{2+} during incubation with highly purified nuclei from non-apoptotic cells. Our studies showed that Ca^{2+} induces internucleosomal DNA degradation in isolated nuclei from non-apoptotic thymocytes in the absence of CAD. We also analyzed in detail the effects of Ca^{2+} on DNA degradation in isolated purified nuclei in regards to the effects of various concentrations of KCl known to prevent apoptotic nuclease activity. Our data indicate that greater than 1 mM

Ca^{2+} is required for DNA fragmentation in the presence of a physiological concentration of KCl. In contrast, at an apoptotic concentration of KCl, a 5-fold lower Ca^{2+} concentration results in a similar extent of DNA fragmentation. We show that the inhibition of DNA cleavage is not due to an overall increase in osmolality of the buffer, as comparable concentrations of sucrose did not prevent nuclease activity. The presence of physiological concentrations of KCl also prevents histone release, while the release of histones is observed at an apoptotic concentration of KCl. Finally, the phosphorylation of histone H2B, a characteristic often implicated with apoptotic cells, does not occur in isolated nuclei upon Ca^{2+} treatment, suggesting that this phosphorylation is not required for Ca^{2+}-dependent DNA degradation. Thus, our studies indicate that the DNA degradation machinery that exists in nuclei from primary thymocytes and is activated upon $CaCl_2$ treatment is similar to that reported in typical apoptotic cells.

Interestingly, Ca^{2+} can induce apoptotic DNA degradation without influence of cytoplasmic molecules, suggesting an additional mechanism to induce apoptosis. Calcium ions can easily penetrate from cytoplasm to nuclei through the nuclear envelope. Therefore, small changes of cytosolic Ca^{2+} cause equally rapid changes in nuclear Ca^{2+}, consistent with the free diffusion of Ca^{2+} through nuclear pores [26]. It is also known that the Ca^{2+} concentration is approximately 100 nM in the cytoplasm and nuclei, but 100 to 1000 μM in the endoplasmic reticulum [19] and the intracellular concentration of Ca^{2+} can increase to approximately 300 to 500 nM upon treatment with Vitamin D compounds [27,28]. The concentration of $CaCl_2$ necessary for extensive degradation such as internucleosomal DNA degradation in our study was 0.2 to 1.0 mM, in contrast to approximately 5 mM in a previous report [29], which is much higher than a physiological concentration of Ca^{2+}. Our data suggest that in apoptotic cells, other factors or unique mechanisms likely exists to facilitate DNA degradation. One possible regulatory mechanism we identified is the lowering the intracellular K^+ as observed during the loss of cell volume during apoptosis to facilitate Ca^{2+}-dependent DNA degradation.

A caspase-associated endonuclease (CAD) has been identified which is Mg^{2+}-dependent, activated by caspases, and has often been implicated in the generation of internucleosomal DNA degradation [13,30]. We did not detect the presence of CAD in isolated nuclei from non-apoptotic thymocytes. Additionally, we found isolated nuclei to be devoid of caspase-3 protein and caspase-3-like activity in the presence and absence of Ca^{2+}. Therefore, this model system does not appear to have either caspase activity or CAD, suggesting other endonucleases are involved in the internucleosomal DNA degradation response to $CaCl_2$. While Ca^{2+}-induced DNA degradation mimics the internucleosomal, ladder-like pattern observed in apoptotic cells, we can not rule out if single or multiple nucleases exists in cells that may be responsible for DNA fragmentation *in vivo*. In whole cells, cytoplasmic factors may influence the resulting activation of apoptotic nucleases. Additionally, this activation may depend on the specific cell type or stimulus employed to induce cell death. However, our data shows that enzymes exist in isolated nuclei that are exquisitely sensitive to the level of Ca^{2+} present under various cationic conditions.

Our laboratory initially described a calcium-dependent endonuclease (NUC18) from apoptotic rat thymocytes [17]. NUC18 activity was detected in nuclear extracts of thymocytes of both control and glucocorticoid-treated thymocytes, and was associated with apoptotic DNA degradation. Furthermore, we have shown that partial protein sequencing of pure NUC18 generated two peptide sequences that have a remarkable homolog to rat cyclophilin A, and other cyclophilin family members such as cyclophilin B and C [31]. These cyclophilins have a calcium/magnesium-dependent nuclease activity with biochemical and pharmacological properties similar to those of NUC18, and result in high molecular weight DNA cleavage [31]. These data in conjunction with recent genetic analysis in C. elegans raise the intriguing possibility that cyclophilin-related proteins may play a role in DNA degradation during apoptosis [32]. Another possible endonuclease among the potential apoptosis-associated

endonucleases [33] is DNase-gamma as this enzyme is also Ca^{2+}- and Mg^{2+}-dependent, and was originally isolated from the nuclei of rat thymocytes [34].

Caspase independent DNA degradation

During the incubation of nuclei with $CaCl_2$, we observed that isolated nuclei from non-apoptotic cells do not have caspase activity. Thus, our data also suggest that non-caspase proteases are activated in nuclei, and DNA degradation can be activated with Ca^{2+} in the absence of caspase-3. Previous studies indicate that endonucleases other than CAD or serine proteases are involved in DNA degradation [33]. TLCK and TPCK are known irreversible serine protease inhibitors of trypsin or trypsin-like proteases and chymotrypsin or chymotrypsin-like proteases, respectively. PMSF is known to be a more general serine protease inhibitor, along with inhibiting various cysteine proteases. However, these inhibitors have also been shown exhibit other biochemical effects on cells. Specifically, TLCK and TPCK have been shown to block activation of NFκB [35], prevent the activation of pp70(s6k), a mitogen-related kinase [36], and to suppress the processing of caspases in some model systems [37, 38]. In contrast, these two proteases have been shown to both induce and enhance cell death upon treatment with various cytotoxic agents [39–41].

McConkey [29] reported that TPCK, a serine protease inhibitor, prevented Ca^{2+}-dependent DNA degradation in isolated thymocyte nuclei, and suggested the existence of an alternative pathway of DNA degradation mediated by a Ca^{2+} stimulated nuclear protease. Hughes et al. [42] has also reported that non-caspase proteases are required for chromatin degradation during apoptosis. In a study of apoptosis in human melanoma cells induced by etoposide, Bruin et al. [43] recently found that a serine protease is involved in the initiation of DNA damage-induced apoptosis. Our data showed that the broad-spectrum protease inhibitor PMSF was most effective in preventing DNA degradation upon Ca^{2+} treatment. Thus, it is possible that there are at least two different signaling pathways in isolated nuclei to activate endonucleases: one where by Ca^{2+} activates endonucleases directly; and another where by endonucleases are activated indirectly by a proteolysis resulting from Ca^{2+}-dependent proteases.

Regulatory mechanism of apoptosis by K^+ ions during DNA degradation

It has been previously reported that K^+ prevents Ca^{2+}-dependent DNA degradation [18,44]. In the present study, we also found a suppressive effect of KCl on Ca^{2+} dependent DNA degradation occurring at a KCl concentration of 90 to 100 mM, with a maximum effect around the physiological range of 140 to 150 mM KCl. However, we further examined the effect of both a physiological and apoptotic concentration of KCl on Ca^{2+}-dependent DNA degradation in response to increasing concentrations of $CaCl_2$. In the presence of a physiological concentration of KCl (140 mM), a 5-fold high concentration of Ca^{2+} was required to induce internucleosomal DNA cleavage, compared to an apoptotic concentration of KCl (40 mM). Interestingly, at higher concentrations of $CaCl_2$, a repressive effect was also observed, suggesting that while Ca2+ can induced DNA degradation, this cation may also inhibit nuclease activity in a similar manner of K^+. Similarly, KCl at 140 mM in media also prevented apoptotic DNA degradation in thymocytes, as well as cultured HL-60 cells upon apoptotic stimulation (data not shown). The mechanism of preventing apoptosis by altering ionic strength is unknown, however possibilities include K^+ or cationic inactivation of the endonuclease activity, or by blocking the interaction between Ca^{2+} and endonuclease, thus preventing the activation of the enzyme itself. Overall, these findings support a hypothesis that K^+ homeostasis or maintaining a high concentration of intracellular K^+ in the normal physiological range protects cells from apoptosis. Additionally, there appears to be direct relationship between the concentration of KCl and the amount of stimulus required to induce DNA degradation. Consequently, a rapid reduction of K^+ in the early phase of apoptosis triggers the activation of apoptotic machinery.

Chromatin structure in isolated nuclei treated with Ca^{2+}

In this study, we found little evidence for chromatin condensation in nuclei treated with $CaCl_2$ at all KCl concentrations examined (0, 40 and 140 mM), supporting the notion that chromatin condensation may not be necessarily linked to or is independent of DNA degradation [45]. Previous studies have shown that histones are released in various apoptotic cells [46, 47]. Our findings indicate that 1) histones are also released in isolated nuclei treated with Ca^{2+} and 2) the amount of histones released corresponds approximately to the amount of internucleosomal DNA. The release of histones is prevented under physiological concentrations of KCl. Together, these findings aid our understanding of the basic mechanism of chromatin degradation in apoptotic cells.

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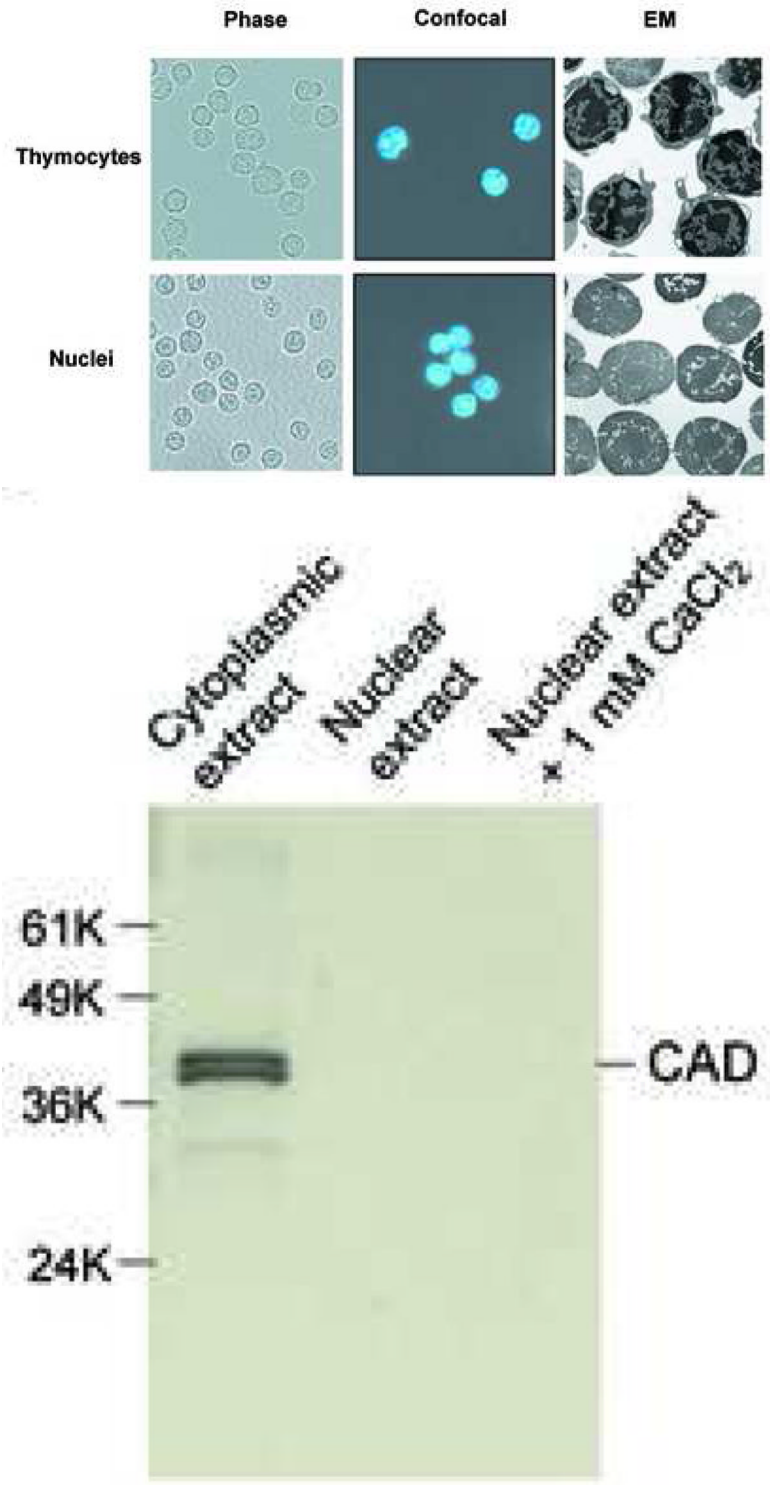
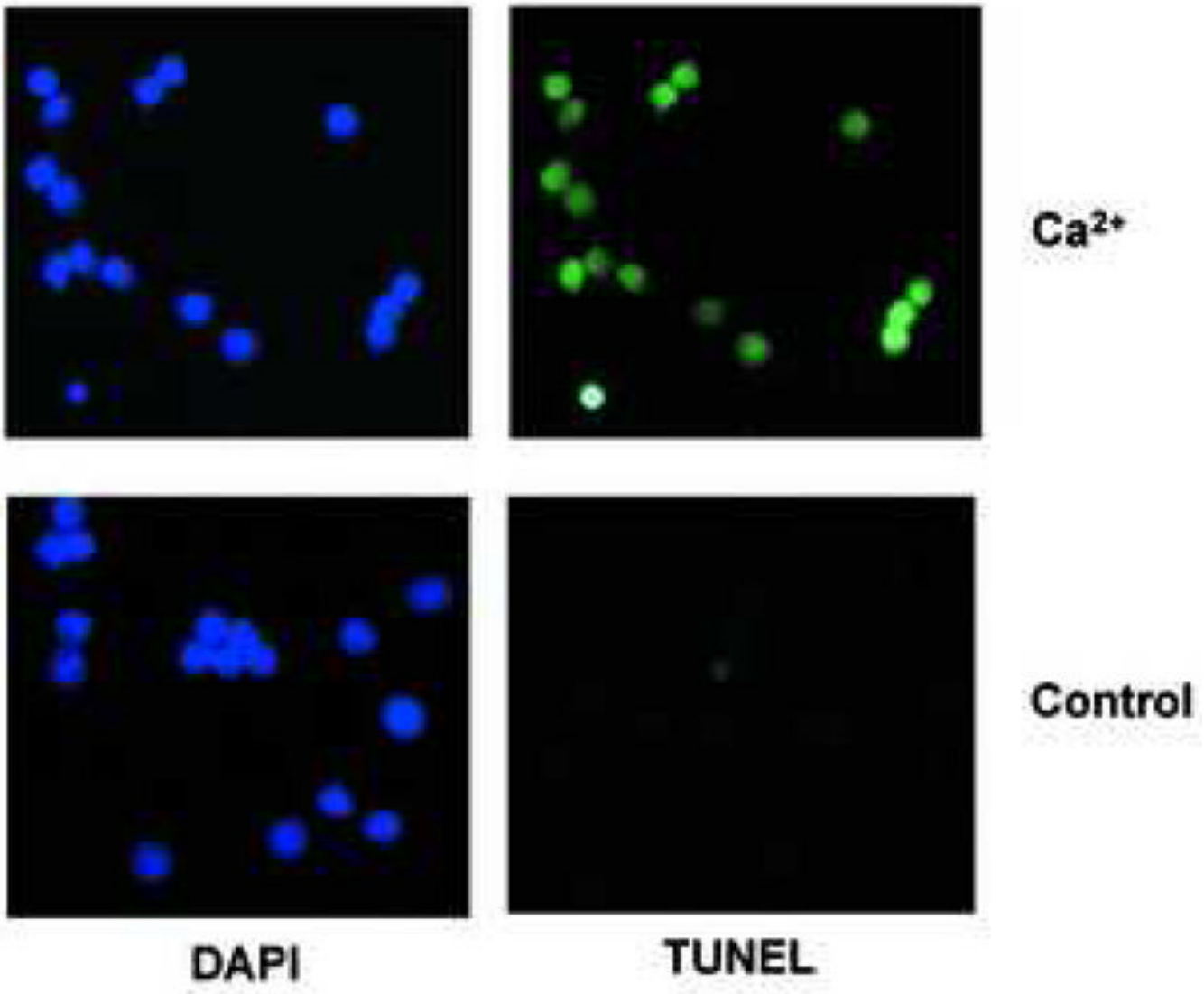
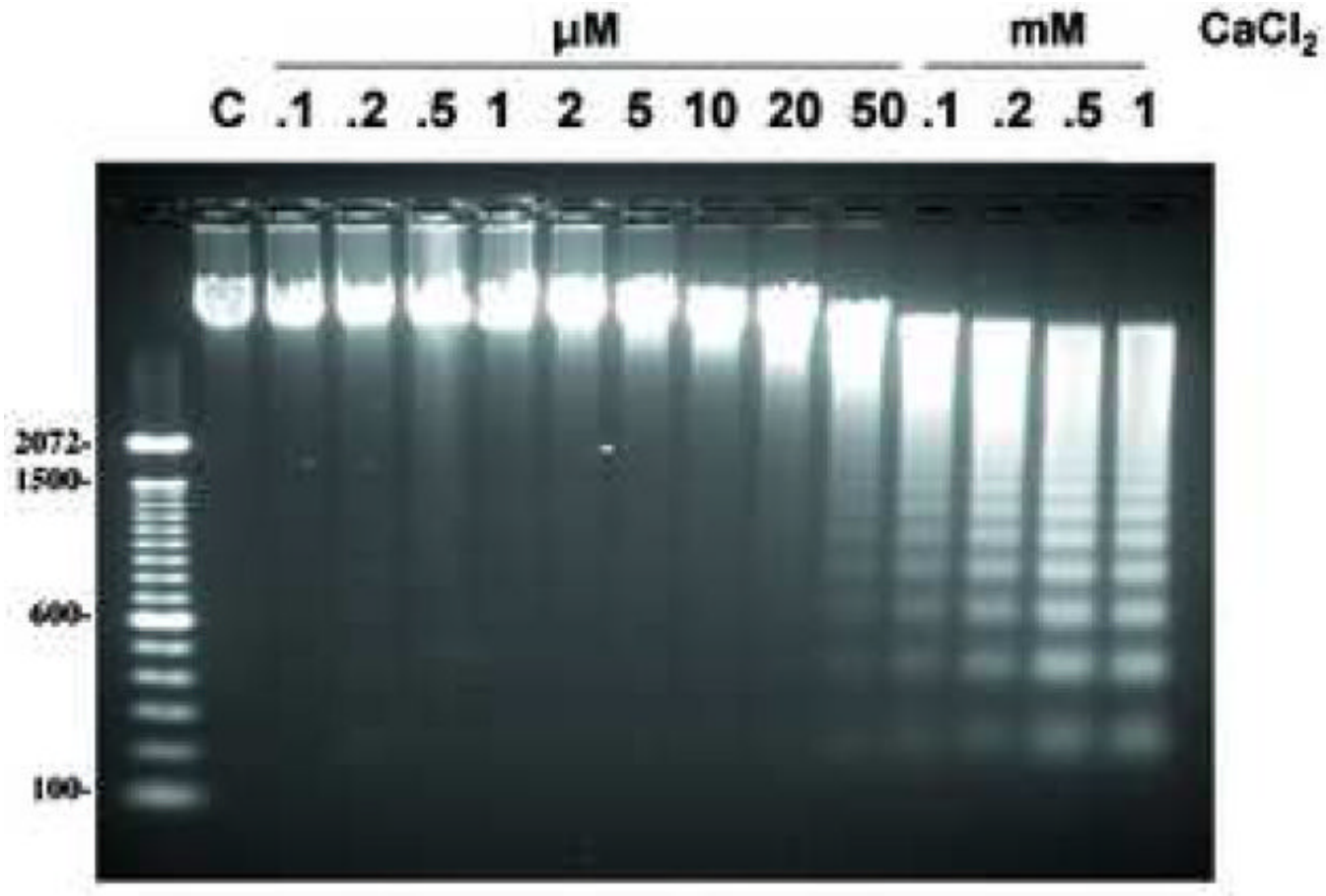


Fig. 1.

Morphological observation and the detection of CAD in nuclei isolated from viable rat thymocytes. (**a**) Thymocytes and isolated nuclei were obtained as described in Materials and Methods. They were observed by phase contrast microscopy (left panel; (Magnification, 200X), confocal microscopy (middle panel) or electron microscope (right panel,

Magnification, 5,400X). The pictures of the nuclei indicate that cell membrane and cytoplasm are completely removed. Figures of phase contrast and electron microscopical analyses other than conforcal microscopy represent one of three independent experiments. (**b**) Isolated nuclei do not contain CAD. Fifty ug of cytosolic and nuclear proteins were analyzed by western blot with anti-CAD antibody. The data shown represents one of four independent experiments.





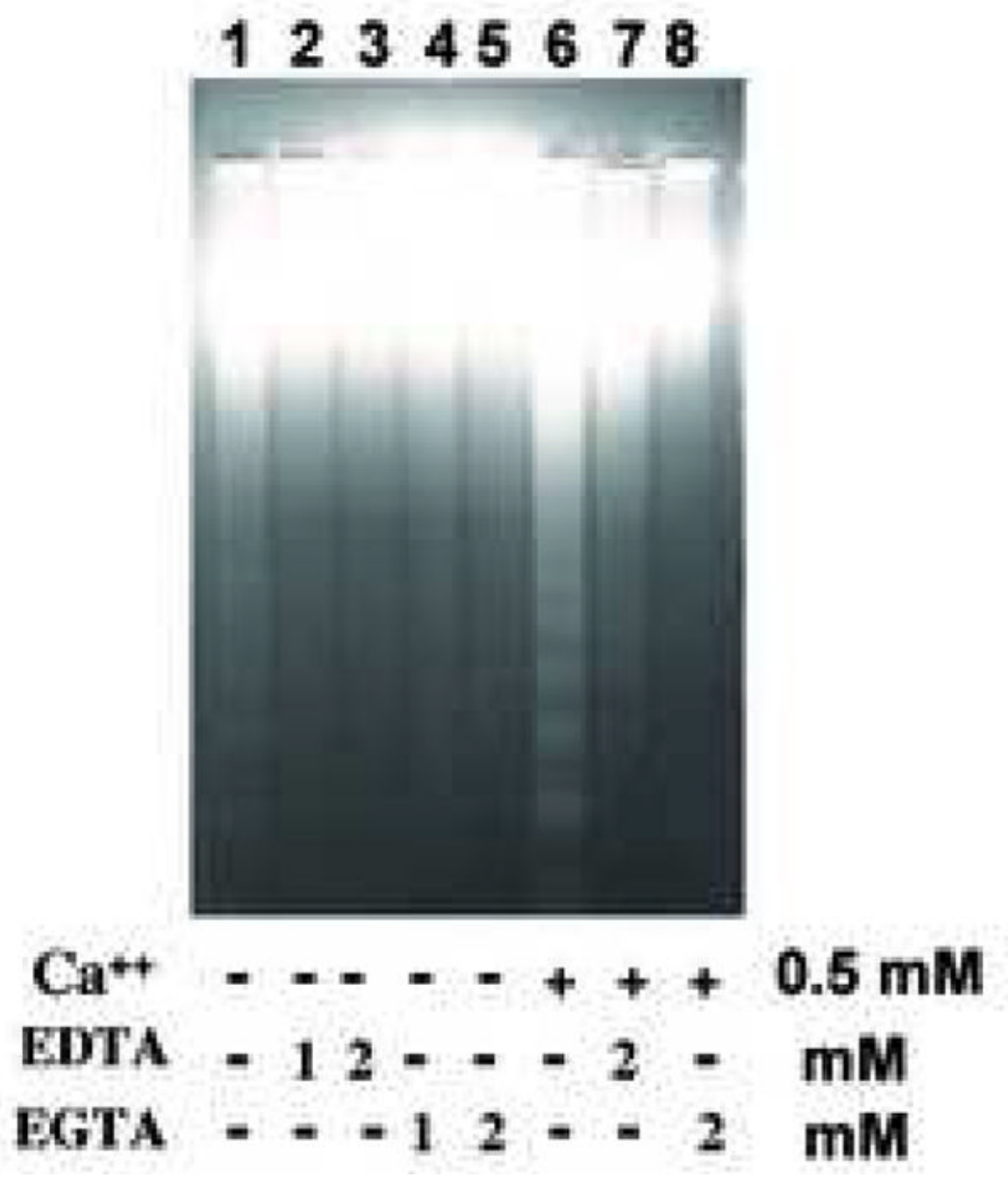


Fig. 2.

Ca^{2+} induces DNA cleavage and histone release in isolated nuclei from non-apoptotic thymocytes. (**a**) Induction of DNA cleavage in nuclei with 0.5 mM $CaCl_2$ treatment. 1×10^4 nuclei were treated with a fluorometric TUNEL assay (right) and counter stained with DAPI (left). The data shown represents one of three independent experiments. (**b**) Induction of DNA degradation with various concentrations of $CaCl_2$ ranging from 0.1 μM to 1 mM. C is a control. Nuclei (2×10^6) were incubated *in vitro* at 37°C for 90 min with or without $CaCl_2$ as described in Materials and Methods. Nuclei were harvested and treated with protease K and RNase. DNA (2 μg) from the nuclei was run on an agarose gel and stained with ethidium bromide. Typical internucleosomal DNA ladders were observed starting around 50 μM $CaCl_2$. The data shown

represents one of at lease three independent experiments. (c) Divalent cations and Ca^{2+} specific DNA degradation in isolated nuclei. EDTA and EGTA block Ca^{2+} dependent DNA degradation in nuclei obtained from thymocytes. The data shown represents one of two independent experiments.

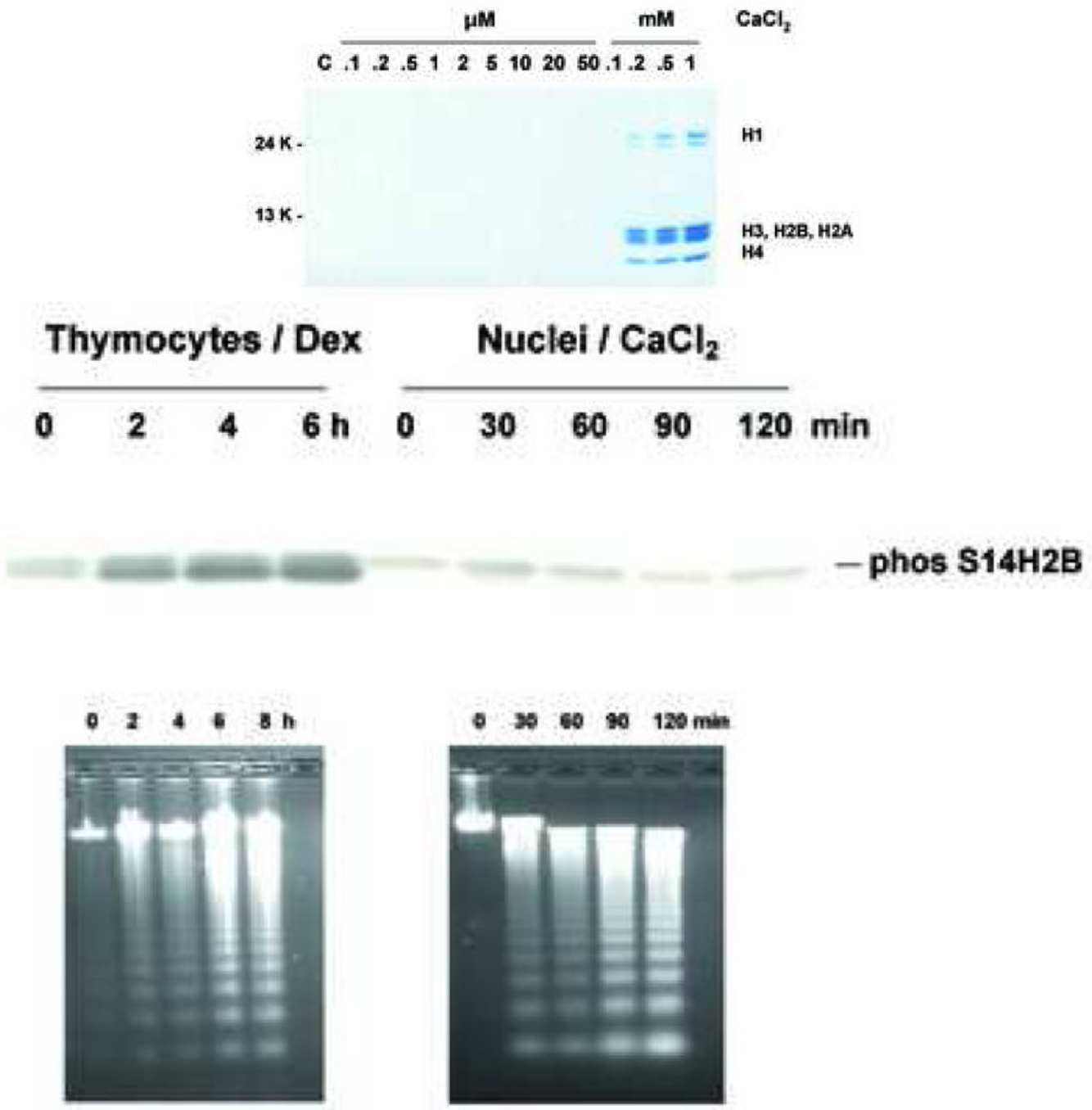


Fig. 3.

Histone release without H2B phosphorylation. (**a**) Histone release accompanying DNA fragmentation during incubation with $CaCl_2$. After incubation of nuclei at each $CaCl_2$ concentration as described for figure 2b, aliquots of lysis buffer soluble fractions were analyzed by SDS PAGE. Proteins were stained with Commasie blue. The data shown represents one of three independent experiments. (**b**) Ca^{2+} does not induce apoptosis-specific H2B phosphorylation in isolated nuclei. Western blot analysis with anti-phospho Ser14H2B for thymocytes cultured with dexamethasone or nuclei incubated with 1 mM of $CaCl_2$ at 37°C for the indicated times. Histones were extracted were extracted from these samples and 10 μg were

blotted against anti-phospho S14H2B. DNA was extracted and analyzed by agarose gel electrophoris. The data shown represents one of three independent experiments.

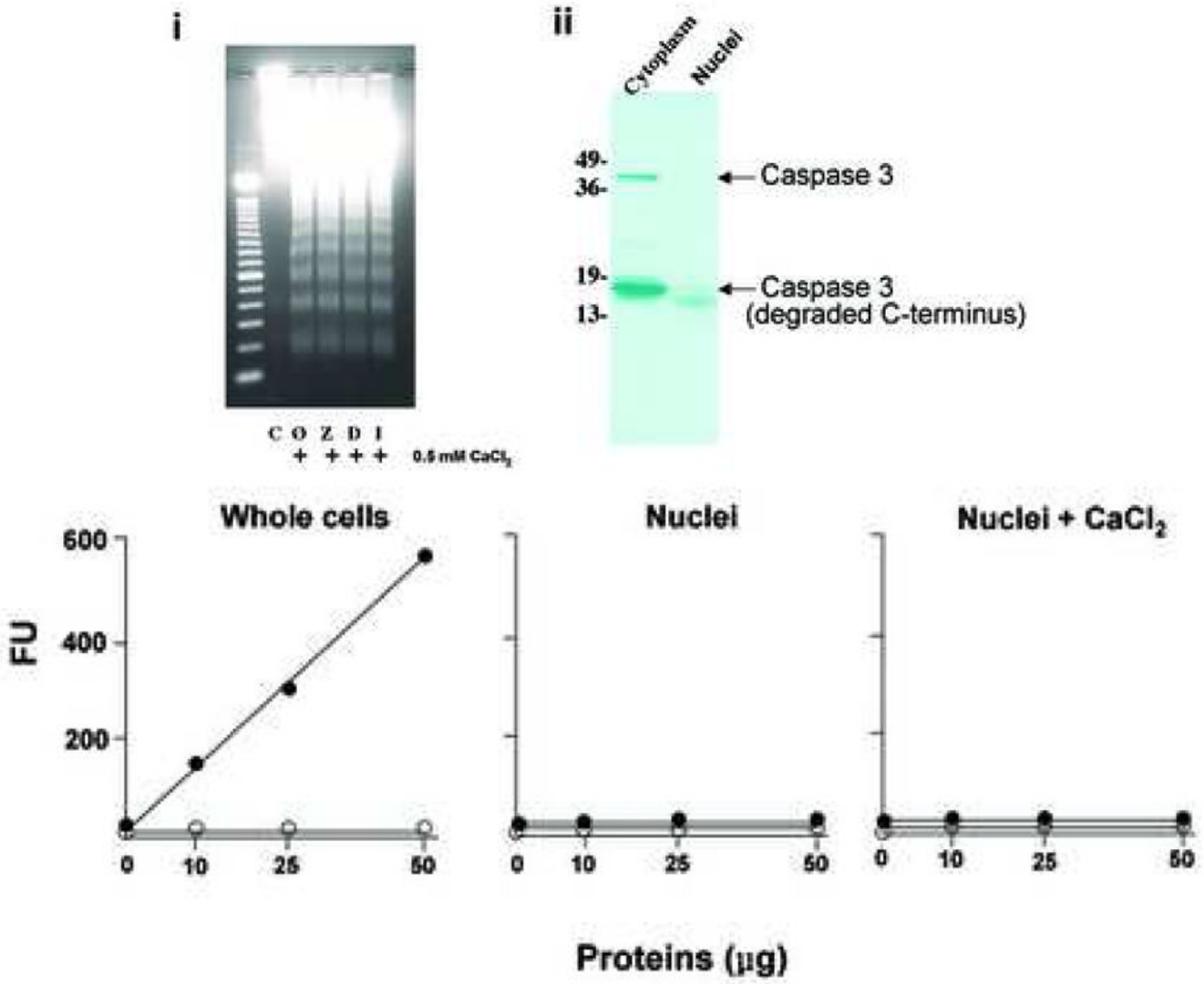


Fig. 4.

Effect of protease inhibitors on Ca^{2+}-dependent DNA degradation. (**a**) i; Analysis of caspase inhibitors on Ca^{2+}-dependent DNA degradation. Nuclei were incubated with 0.5 mM $CaCl_2$ and 100 μM of caspase inhibitors in buffer containing 40 mM KCl prior to DNA was analyzed. C and Ø are control and calcium treatment without inhibitor, respectively. Z, D and I are: Z-VAD, DEVD and IETD with calcium, respectively. ii; Western blotting of caspase 3. Anti-caspase 3 antibody was used against protein extracts (100 μg of protein) from cytoplasm and nuclear extracts from non-apoptotic thymocytes. The data shown represents one of three independent experiments. (**b**) Measurement of caspase-3 activities in the nuclear proteins. Cell lysate were prepared from thymocytes and 0.4 N NaCl soluble nuclear extracts were obtained

from nuclei or nuclei incubated with 1 mM $CaCl_2$ for 90 min at 37°C. Capase-3 activity was measured based on the ability to cleave specific fluorogenic substrates (Z-DEVD-AFC-cleavable activity) in the extracts. FU: Fluorescent units. The data shown represents two experiments. (**c**) Effect of protease inhibitors on the Ca^{2+}-dependent DNA degradation. Nuclei were incubated with 1 mM Ca^{2+} and a protease inhibitor (100 μM each) in buffer containing 40 mM KCl. The data shown represents one of two independent experiments.

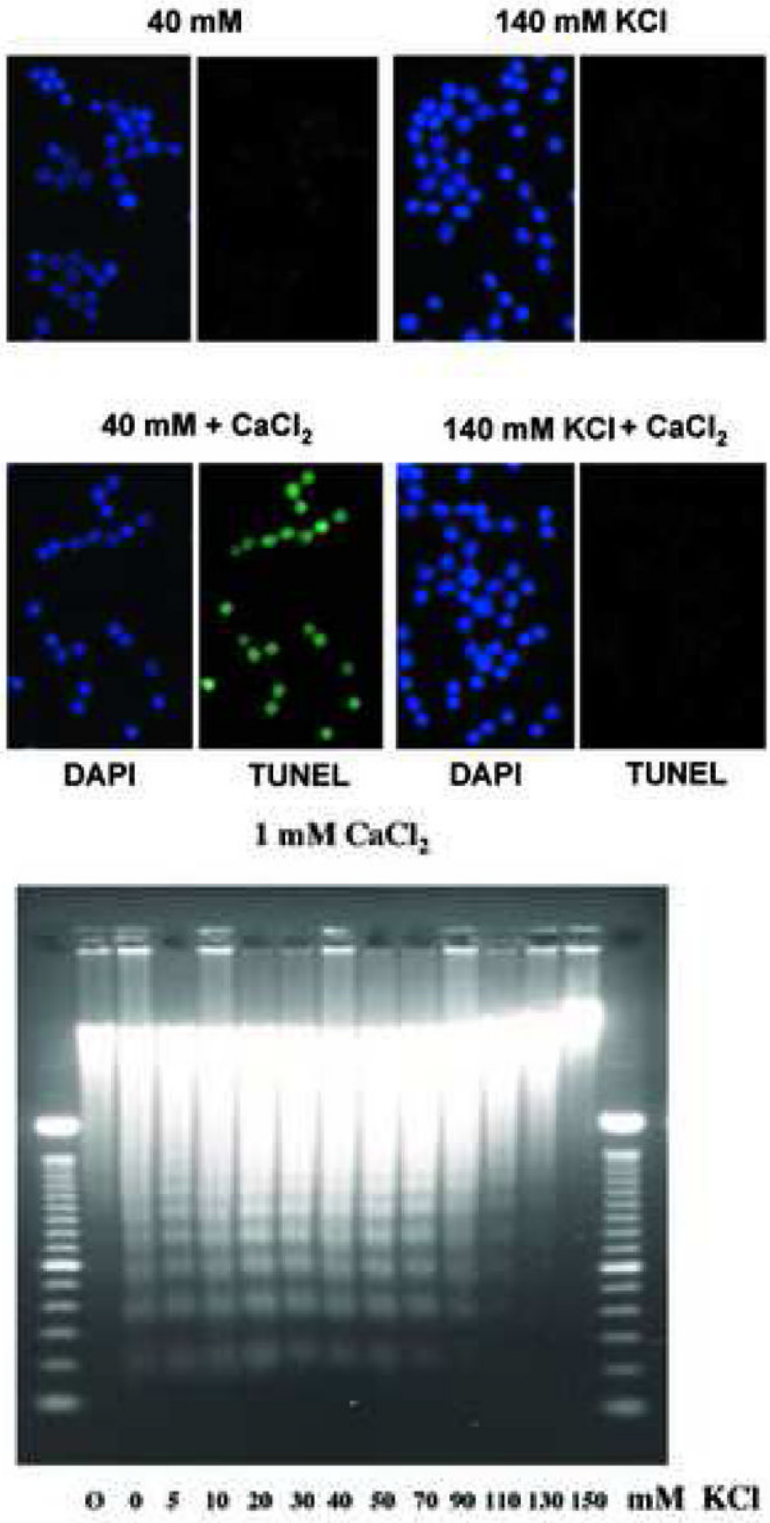
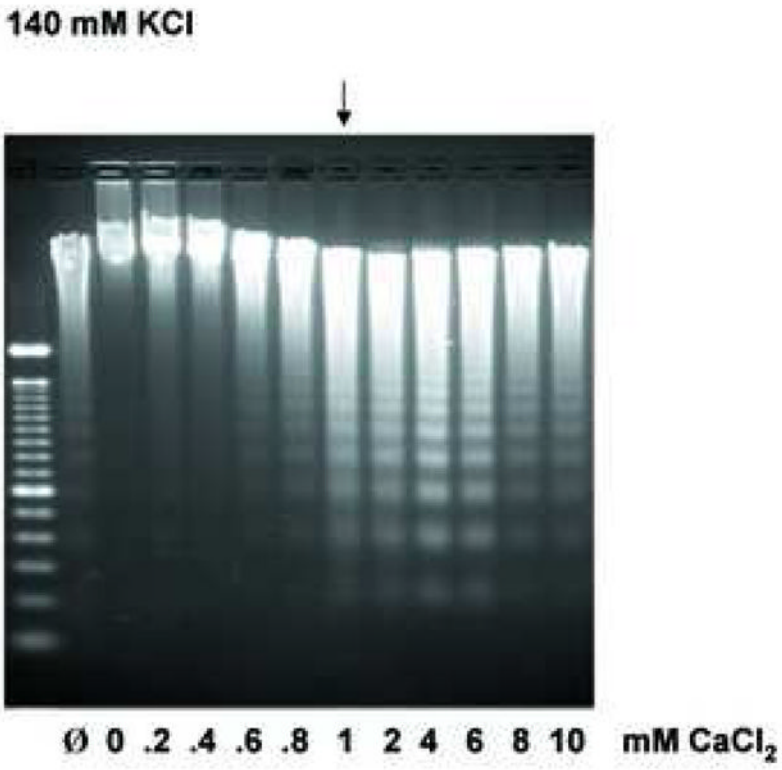
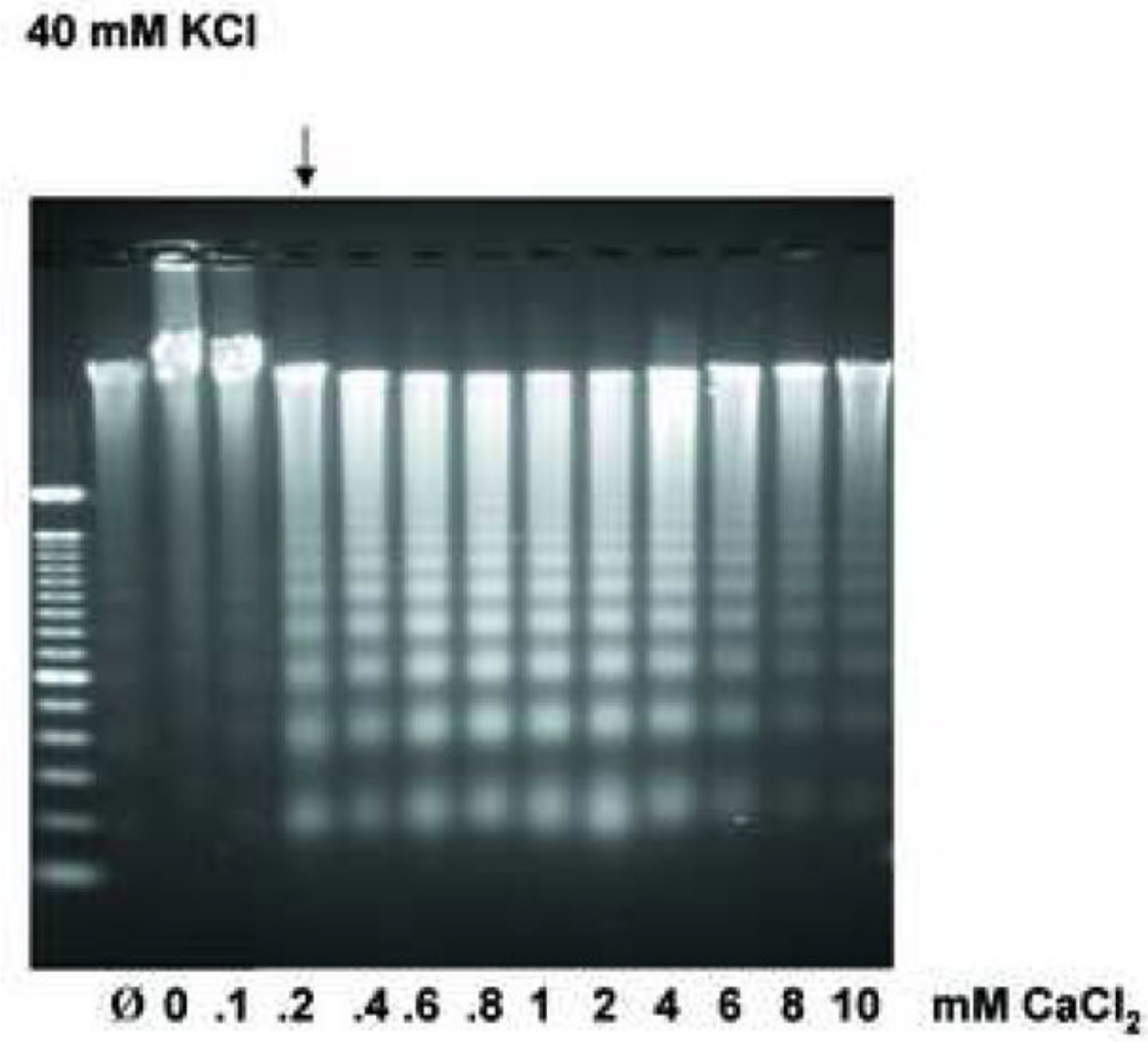


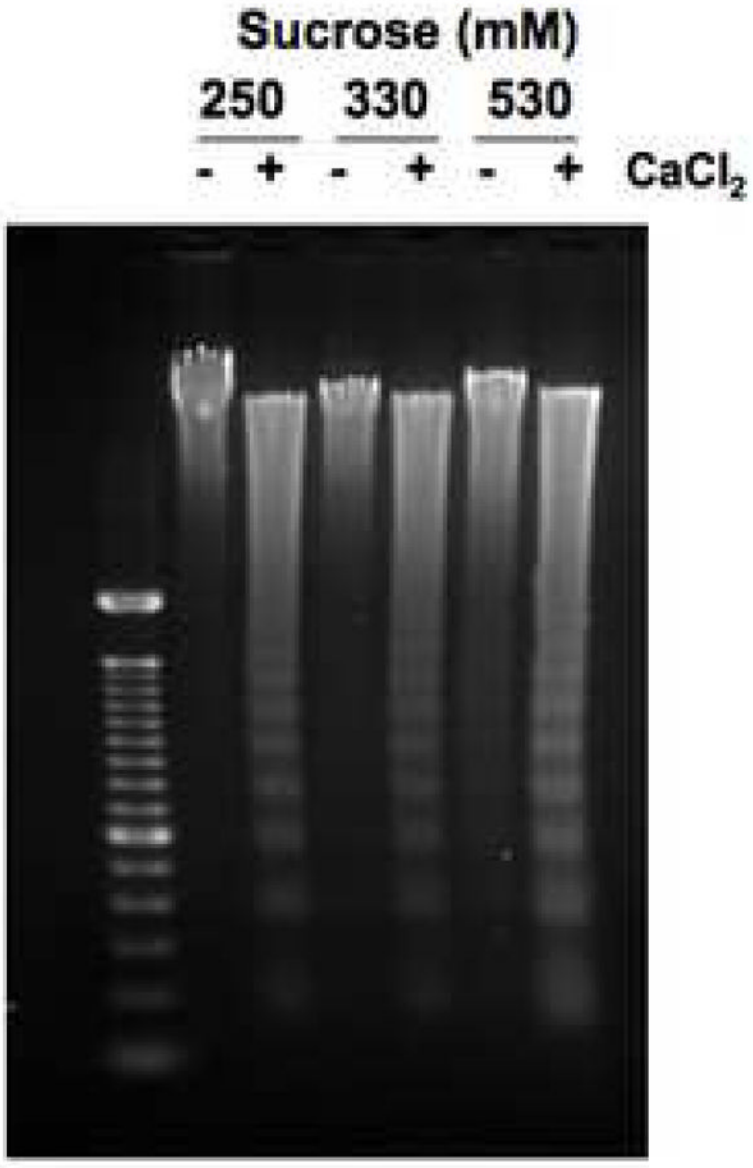
Fig. 5.

Physiological concentration of K^+ blocks Ca^{2+}-dependent DNA degradation in nuclei. (**a**) TUNEL assay, Nuclei (1×10^4) were incubated under "apoptotic" (40 mM) and "physiological" concentration (140 mM) of K^+ with 1 mM $CaCl_2$. *Right,* fluorescent TUNEL

assay. *Left*, counter staining with DAP I. The data shown represents at least three experiments. (**b**) Analysis of Ca^{2+}-dependent DNA degradation under various concentration of K^{+}. DNA was extracted and run on agarose gel electrophoresis. Nuclei (2×10^6) were incubated with 1 mM $CaCl_2$ together with an increasing concentration of KCl. For each sample, 1 μg of DNA was analyzed. "Ø" denotes control nuclei maintained on ice. The data shown represents one of three independent experiments.







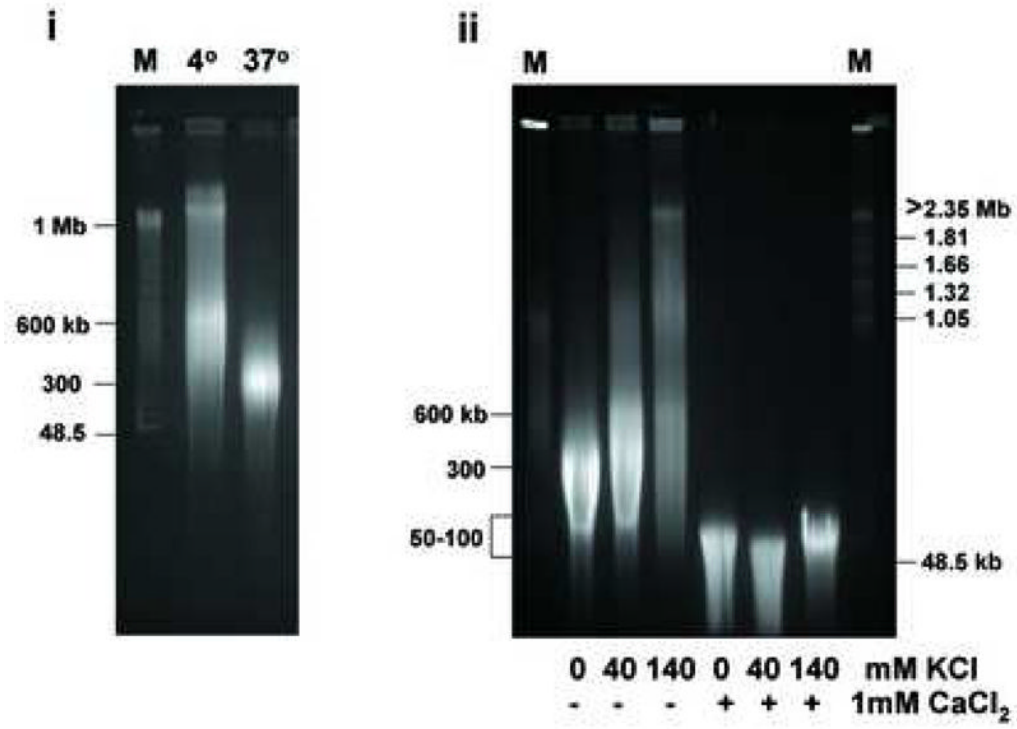


Fig. 6.

Extensive analysis of KCl effect on DNA degradation. (**a**) The effect of Ca^{2+} under the physiological concentration of K^+ at 140 mM on Ca^{2+}-dependent DNA degradation. Nuclei were incubated in isolation buffer containing 140 mM KCl with an increasing concentration of $CaCl_2$. The arrow indicates the initial concentration of $CaCl_2$ with considerable internucleosomal DNA degradation. "Ø" denotes control nuclei maintained on ice that was not exposed to either 40 mM or 140 mM KCl buffer. "0" denotes nuclei resuspended in the respective KCl buffer however in the absence of $CaCl_2$. The data shown represents one of three independent experiments. (**b**) The effect of Ca^{2+} in the "apoptotic concentration" of K^+ at 40 mM on Ca^{2+}-dependent DNA degradation. Nuclei were incubated in the same condition as above except with the buffer containing 40 mM KCl. In a buffer containing 40 mM KCl, a 5 times smaller Ca^{2+} concentration is sufficient to trigger the degradation of internucleosomal DNA. The data shown represents one of three independent experiments. (**c**) Effect of osmolarity of sucrose in buffer on the Ca^{2+}-dependent DNA degradation. Nuclei (2×10^6) were incubated in buffer containing sucrose (330 and 530 mM equivalent concentration to the addition of 40 and 140 mM KCl, respectively), with or without 1.0 mM $CaCl_2$. The data shown represents one of two independent experiments. (**d**) Pulsed field gel electrophoresis (PFGE) of DNA degradation products in the presence of K^+. Nuclei (2×10^6) were incubated in buffer containing 40 or 140 mM KCl with or without 1 mM $CaCl_2$. i, DNA degradation during the incubation of nuclei. ii, Inhibition of the Ca^{2+}-dependent and independent DNA degradation by K^+. M; size marker of DNA, C; Control. The data shown represents two experiments.

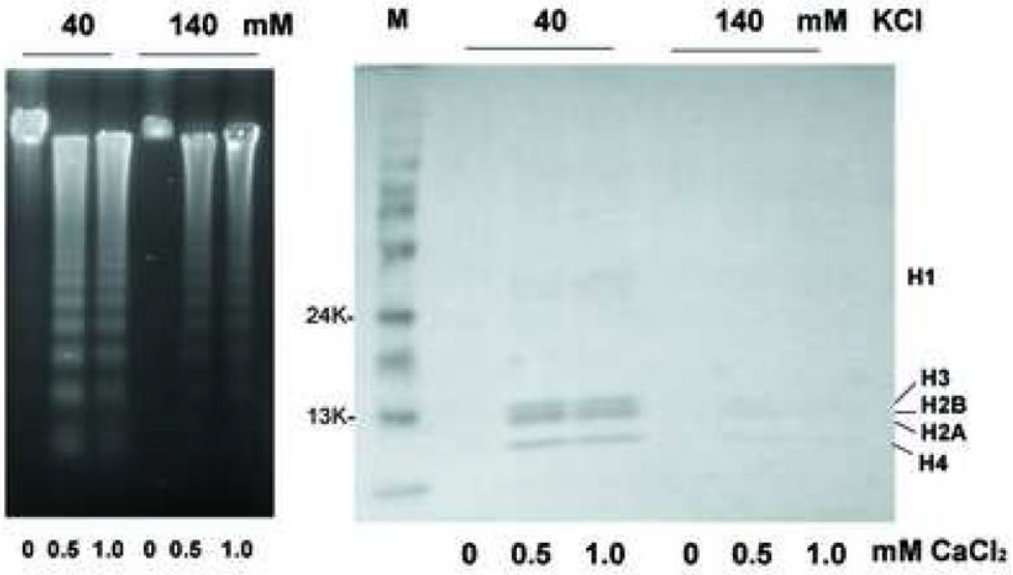


Fig. 7.

The prevention of histone release by physiological concentration of KCl. Nuclei were treated with 0.5 or 1 mM $CaCl_2$ under the KCl at either 40 or 140 mM KCl. Soluble chromatin was run on SDS gel electrophoresis and stained with Coommacie Briliant Blue (*right*). The DNA was analyzed from the identical chromatin fractions (*left*). The data shown represents one or three independent experiments.