Multi-step DNA cleavage in rat liver nuclei is inhibited by thiol reactive agents

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Abstract DNA fragmentation in isolated rat liver nuclei is a Mg^{2+}-dependent, multi-step process which is potentiated by Ca^{2+} and cleaves the DNA into ≥ 700, 200–300 and 30–50 kilobase pair (kbp) fragments, prior to internucleosomal cleavage by Ca^{2+}/Mg^{2+}-dependent endonuclease(s). We now show that Cd^{2+}, Hg^{2+}, dichloroisocoumarin (DCI, a serine protease inhibitor) and *N*- ethylmaleimide (NEM) block both Mg^{2+} and Ca^{2+}/Mg^{2+}-dependent processes. Inhibition of DNA cleavage produced an increase in the size of the DNA fragments, from mono-/oligonucleosomes to 30–50, 200–300, ≥ 700 kbp and finally to intact DNA. NEM and DCI inhibition was blocked by dithiothreitol, and it is proposed that a critical thiol(s) is involved in the DNA cleavage reactions which are a feature of the apoptotic process.

Key words: Apoptosis; Multi-step DNA cleavage; Critical thiol

1. Introduction

Apoptosis is a morphologically distinct form of cell death which appears to be common to all multicellular organisms and is an essential mechanism for disposing of redundant, damaged and/or senescent cells [1]. The apoptotic process is extremely complex with a variety of cellular controls and checkpoints which remain to be elucidated. Indeed, the only common biochemical event is internucleosomal DNA cleavage (DNA laddering), which has been used as a biochemical 'hallmark' for apoptosis and has been correlated with the morphological features of chromatin condensation [2–6]. Internucleosomal cleavage is believed to be catalysed by a Ca^{2+}/Mg^{2+}-dependent endonuclease [3] and a number of studies have shown that increased cytosolic Ca^{2+} concentrations precede apoptotic cell death (for review see [5,6]). However, more recent studies [7] have reported that early rises in intracellullar Ca^{2+} are not required for glucocorticoid activated apoptosis in thymocytes.

It is now also increasingly clear that internucleosomal cleavage is a late or terminal step in the DNA degradation pathway and studies by Walker et al. [8] and Brown et al. [9] have shown that the early steps in the DNA fragmentation process in apoptotic thymocytes involve an initial cleavage of the DNA into high molecular weight fragments of approximately ≥ 700, 200–300 and 30–50 kilobase pair (kbp) sized fragments which are subsequently degraded by internucleosomal cleavage. Also, in a variety of cell lines, apoptotic cell death occurs with DNA cleavage into 300 and/or 50 kbp fragments prior to, or in the absence of internucleosomal fragmentation [10].

The cleavage of DNA into large fragments suggests that other endonucleases are involved and in this respect, isolated nuclei represent an ideal system to characterise the cleavage processes. Thus, in studies on isolated nuclei from liver and thymus, Ca^{2+} activates an endonuclease producing a DNA fragmentation pattern identical to that seen in apoptotic cells [4,11–13]. Furthermore, the large fragment formation can also be reproduced in isolated nuclei [12–15] and we have shown [12&13] that chromatin degradation in rat liver and thymocyte nuclei is a multi-step process which cleaves intact DNA into ≥ 700, 200–300, 30–50 kbp fragments and oligo-/mononucleosomes. This process has an absolute requirement for Mg^{2+}, which on its own can activate the initial steps i.e. the large kbp fragment formation. However, Ca^{2+} is then required for the subsequent and terminal internucleosomal cleavage which produces the oligo- and mono-nucleosomes.

In this study we provide further evidence for this stepwise degradation process by examining the effect of heavy metals, various protease inhibitors, and *N*-ethylmaleimide (NEM) on both the Mg^{2+} and Ca^{2+}/Mg^{2+} activated endonucleases. The results show that Hg^{2+}, Cd^{2+}, 3,4-dichloroisocoumarin (DCI, a serine protease inhibitor) and NEM were potent inhibitors of both processes and that inhibition of DNA cleavage was accompanied by a characteristic, step-wise increase in the size of the large fragments which progressed from the oligo-/mononucleosomes to 30–50, 200–300, ≥ 700 kbp sized fragments and subsequently to intact DNA. Furthermore, as all of these inhibitors react with thiol groups, we propose that they are inhibiting a sulphydryl group(s), which is essential for the cleavage of DNA into large fragments and oligonucleosomes.

2. Materials and methods

2.1. Materials

All chemicals unless stated otherwise were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Terminal deoxynucleotidyl transferase (TdT) was from Gibco, Paisley, Scotland, UK. Antidigoxigenin-fluorescein Fab fragments (anti-Dig), Digoxigenin-11-2′-deoxyuridine-5′-triphosphate (Dig-11-dUTP), pronase, DCI, *N*-α-tosyl-L-lysylchloromethylketone, and (TLCK), *N*-α-tosyl-L-phenylalanyl chloromethylketone (TPCK) were obtained from Boehringer Mannheim UK, Lewes, East Sussex, UK.

2.2. Preparation of rat liver nuclei and autodigestion experiments

Rat liver nuclei were prepared as described previously [12] in ice-cold SSP-buffer (0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.1 mM EGTA, pH 7.5). The pellet was finally resuspended in 10 ml of incubation buffer

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Abbreviations: anti-DIG, antidigoxigenin-fluorescein FAB fragments; DCI, dichloroisocoumarin; DMSO, dimethyl sulphoxide; Dig-11-dUTP, digoxigenin-11-2′-deoxy-uridine-5′-triphosphate; DTT, dithiothreitol; kbp, kilobase pair; FIGE, field inversion gel electrophoresis; PBS, phosphate buffered saline; TdT, terminal deoxynucleotidyl transferase; TLCK, *N*-α-tosyl-L-lysyl chloromethylketone; TPCK, *N*-α-tosyl-L-phenylalanylchloromethylketone.

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containing 125 mM KCl, 2 mM potassium phosphate, 25 mM HEPES, 0.15 mM spermine, 0.5 mM spermidine, pH 7.0 and the nuclei counted using a Coulter-ZM cell counter and 256 Channelyzer (Coulter Electronics, Luton, Beds., UK).

Autodigestion experiments were carried out with 10×10^6 nuclei/ml which were incubated with and without additions at 37° C for 30 min. Protease inhibitors were dissolved in DMSO (TPCK and DCI), EtOH (NEM) or PBS (TLCK) and added immediately to the incubations at a final concentration of 0.1% v/v. Control samples received the same concentration of solvent. The digestion reaction was stopped with an equal volume of ice-cold incubation buffer containing 20 mM EGTA and aliquots taken for the analyses described below.

2.3. Electrophoresis of DNA

Conventional agarose gel electrophoresis was used to detect DNA ladders and was carried out as described previously [9] using 5×10^5 nuclei. Field inversion gel electrophoresis (FIGE), which was used to resolve large molecular weight DNA fragments was carried out on agarose plugs [16], containing 5×10^5 nuclei. The plugs were digested with 1mg/ml pronase for 48 h at 50°C and stored at 4°C, prior to electrophoresis which was carried out as described by Brown et al. [9]. Under these conditions, DNA fragments in the 4.4 to 460 kbp range were separated and in each experiment, appropriate standards of *Saccharomyces cerevisiae* chromosomes were used.

2.4. In situ end labelling (ISEL) and flow cytometry of autodigested nuclei

ISEL on isolated nuclei was carried out as described previously [12]. Briefly, nuclei (20×10^6 in 2 ml) after incubation were fixed in 1% formaldehyde (5°C) for 15 min before permeabilisation in 70% EtOH at −20°C for 1h. The nuclei were then resuspended in 1 ml of 50 mM Tris, 150 mM NaCl, pH 7.6 buffer (TBS) and counted. Aliquots containing 2×10^6 nuclei were resuspended in 100 μl of TdT labelling buffer (100 mM sodium cacodylate, 10 mM $CoCl_2$, 1 mM DTT, pH 7.2), 15 units of TdT enzyme, 2 μM Dig-11-dUTP and incubated for 60 min at 37°C. The reaction was stopped with 1 ml of ice-cold TBS + 5 mM EDTA and the labelled nuclei resuspended in 0.5 ml of antibody labelling buffer (5% non-fat dried milk, 0.1% v/v Triton X-100, 4x standard saline citrate, pH 7.2 and incubated for 15 min at 5°C to block non-specific antibody binding. The nuclei were then resuspended and incubated in the dark at 37°C for 60 min in 0.25 ml of antibody-labelling buffer containing 1.6 μg/ml of anti-Dig antibody. The antibody-labelled nuclei were then resuspended in 1 ml of PBS containing 10 μg/ml propidium iodide and analysed by flow cytometry [12].

3. Results

3.1. Inhibition of Mg^{2+} and Ca^{2+}/Mg^{2+} induced DNA fragmentation in isolated rat liver nuclei by Hg^{2+} and Cd^{2+}

Recently, we have shown that the extent and pattern of DNA cleavage in isolated rat liver nuclei is markedly dependent on the activating cation(s) [12]. Thus, if Mg^{2+} alone is used then the DNA is cleaved into large kbp sized fragments without internucleosomal cleavage. In contrast, Ca^{2+}/Mg^{2+} not only stimulates large fragment formation but also activates internucleosomal fragmentation thereby producing the typical 'DNA' ladder. These results can be interpretated as being evidence for either two or more separate enzymes with different cation requirements or alternatively as a single endonuclease which has both Mg^{2+} and Ca^{2+} binding sites that can activate and modify the catalytic activity of the enzyme. If there are separate and distinct enzymes, it should be possible to preferentially block one without affecting the other and in this study we have used a variety of inhibitors to test this hypothesis.

In this respect, the first compounds we investigated were Cd^{2+} and Hg^{2+} which have been shown by Lohmann and Beyersmann [17] to be potent inhibitors of the Ca^{2+}/Mg^{2+} stimulated internucleosomal cleavage. However, the effect of these metals on large fragment formation has not been investigated and the experiments described in Fig. 1 not only confirm that Hg^{2+} is a very potent inhibitor of internucleosomal cleavage but also that this metal and Cd^{2+} are equally effective at blocking large fragment formation. The figure shows that the inhibitory effects of Hg^{2+} on Ca^{2+}/Mg^{2+} activated DNA cleavage were produced over a very narrow concentration range (i.e. between 10–15 μM, Fig. 1A,B). The inhibition of DNA fragmentation by Hg^{2+} blocked the internucleosomal cleavage (Fig. 1B) and resulted in the appearance of large DNA fragments of increasing size, comprising 200–300, $\geq$700 kbp and intact DNA (Fig. 1A). Only trace amounts of the smaller 30–50 kbp fragments were detected. In other experiments (results not shown) Cd^{2+} was also shown to be a good inhibitor, although, higher (25–800 μM) concentrations were needed.

Both Cd^{2+} and Hg^{2+} (Fig. 1C,D) also inhibited large fragment formation in nuclei incubated with 8 mM Mg^{2+} alone for 60 min at 37°C. Under these conditions, FIGE revealed that in the absence of the heavy metals the DNA was cleaved into 30–50 and 200–250kbp sized fragments (zero lanes, Fig. 1C,D). The inhibition of the formation of these large fragments was accompanied by marked effects on the size of the fragments. Thus, between 25–100 μM Cd^{2+} there was a change in the DNA size distribution with the appearance of intact DNA in the sample well and an increased amount of the 200–300 kbp fragments. This was accompanied by a corresponding decrease in the amount of the smaller 30–50 kbp fragments. At higher Cd^{2+} concentrations (200 and 400 μM), the inhibition was greater and resulted in the DNA being partially cleaved into 200–250 and $\geq$700 kbp fragments with more of the intact DNA remaining in the sample well. The highest concentration of 800 μM Cd^{2+} produced an almost total inhibition of cleavage leaving virtually all of the DNA retained at the origin of the gel. Both Mg^{2+} (Fig. 1C) and Ca^{2+}/Mg^{2+} (results not shown) dependent DNA cleavage were inhibited at similar Cd^{2+} concentrations, indicating that both fragmentation processes had a similar sensitivity to the heavy metal. The inhibitory effects of Hg^{2+} on Mg^{2+} activated cleavage were produced over a very narrow concentration range (10–15 μM) and were accompanied by an abrupt shift in the DNA size from 30–50 and 200–300 kbp fragments to intact DNA (Fig. 1D). As with Cd^{2+}, both the Mg^{2+} and Ca^{2+}/Mg^{2+}-dependent DNA cleavage reactions were inhibited by similar concentrations of Hg^{2+}.

3.2. Inhibition of Ca^{2+}/Mg^{2+} induced DNA fragmentation in isolated rat liver nuclei by protease inhibitors and N-ethylmaleimide

There is increasing evidence for the role of proteases in apoptosis and recently, Weaver et al. [18] have reported that the serine protease inhibitors TPCK and DCI inhibited DNA laddering but not large fragment formation in apoptotic thymocytes. They also reported that these inhibitors did not block internucleosomal cleavage or the formation of large fragments in isolated nuclei obtained from rat thymus and rat liver. However, in contrast Zhivotovsky et al. [15] have shown that TPCK and another serine protease, TLCK inhibited DNA fragmentation in isolated rat liver nuclei.

We have also investigated the effects of DCI, TPCK and TLCK on cation stimulated DNA cleavage and as well as the more usual gel techniques, we have also used an ISEL labelling technique [12] to quantify the inhibitory effects of these agents.

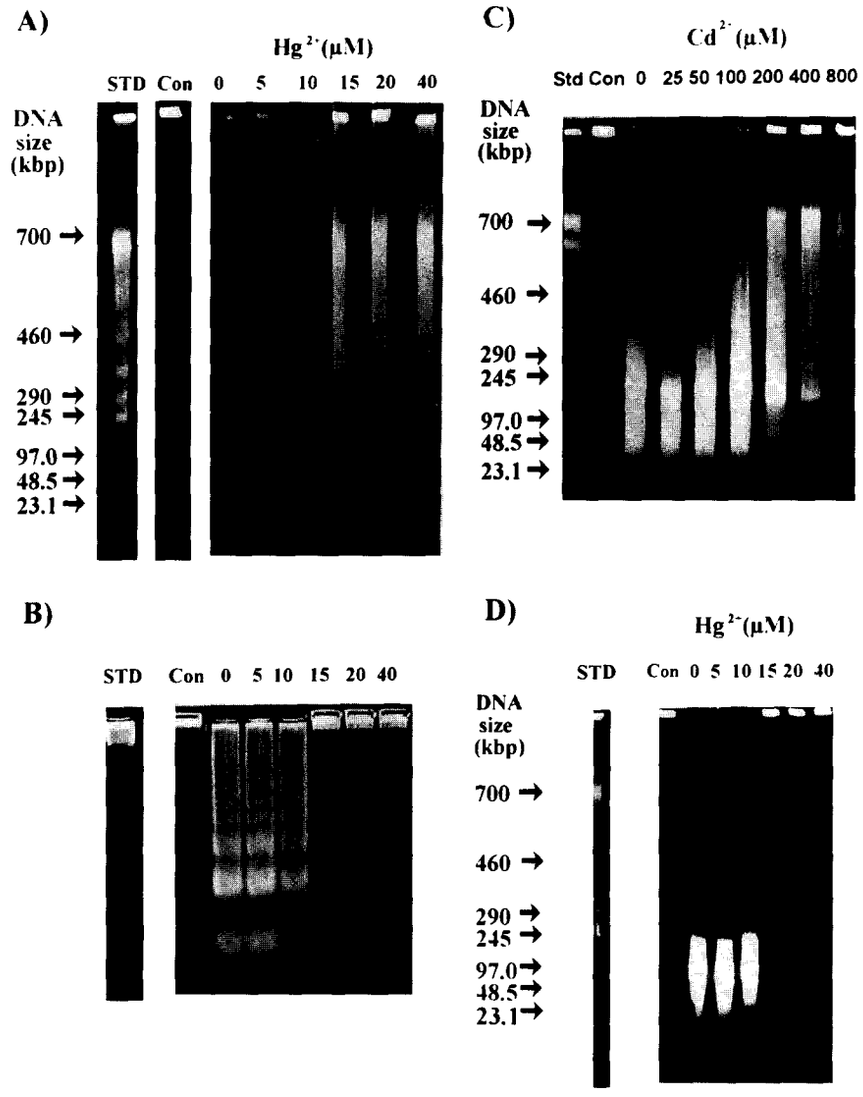

Fig. 1. Cd^{2+} and Hg^{2+} inhibition of DNA cleavage in rat liver nuclei. Rat liver nuclei were isolated and incubated with and without inhibitors with either 100 μM Ca^{2+}/8 mM Mg^{2+} for 30 min or 8 mM Mg^{2+} for 60 min as described in section 2. The reactions were terminated and the nuclei from the incubations analysed by FIGE and/or conventional agarose gel electrophoresis. The effect of Hg^{2+} on Ca^{2+}/Mg^{2+} activated cleavage is shown in panel A (FIGE) with the corresponding internucleosomal cleavage shown in the panel (B) below. The effect of Cd^{2+} and Hg^{2+} on large fragment formation as stimulated by 8mM Mg^{2+} alone is shown in panels (C) and (D). In these cases there was no internucleosomal cleavage and the conventional agarose gel electrophoresis results are not shown. The 'con' lanes refer to nuclei incubated without cations for the appropriate time and thus demonstrate that in the absence of added cations there was no endogenous DNA cleavage. In the FIGE gels (A,C and D), *S. cerevisiae* chromosomal DNA markers are shown as 'STD' with the sizes indicated by arrows. In the conventional agarose gel (B) the 'STD' lane refers to laddering standards of 123 bp or multiples thereof. The concentrations of Hg^{2+} and Cd^{2+} are as indicated in the legends above the gels.

This is shown in Fig. 2. which demonstrates that DCI was a potent inhibitor of Ca^{2+}/Mg^{2+} stimulated DNA cleavage. The end-labelling experiments (Fig. 2C) showed that the I_{50} value for this protease inhibitor was 10–12 μM and that cleavage of the DNA was almost completely blocked at approximately 50 μM DCI. The gel electrophoresis studies demonstrated that inhibition of ISEL was accompanied by the abolition of internucleosomal cleavage (Fig. 2B, lanes 2–5) and the concomitant appearance (Fig. 2A, lanes 2–5) of larger kbp sized fragments of DNA. The size of these fragments was determined by the extent of the inhibition which was accompanied by a stepwise increase in the size of the large fragments which progressed from >30 kbp (lane 1, no inhibitor), to 30–50 and 200–250 kbp (lane 2; 11.5 μM DCI), to 30–50, 200–300, and ≥700 kbp and intact DNA (lanes 3–5; 29–115 μM DCI). TPCK was a poor inhibitor of both ISEL (Fig. 2C), DNA laddering and large fragment formation (lanes 7–11, Fig. 2A,B). In other studies (data not shown), TLCK was found to be essentially ineffective as an inhibitor of DNA fragmentation as measured by ISEL, conventional agarose gel electrophoresis and FIGE.

As Cd^{2+} and Hg^{2+} are known to have great affinity for thiols [19] we also investigated the effect of NEM, the classical -SH blocking reagent on Ca^{2+}/Mg^{2+} dependent DNA cleavage. The results of these studies are also shown in Fig. 2. which demon-

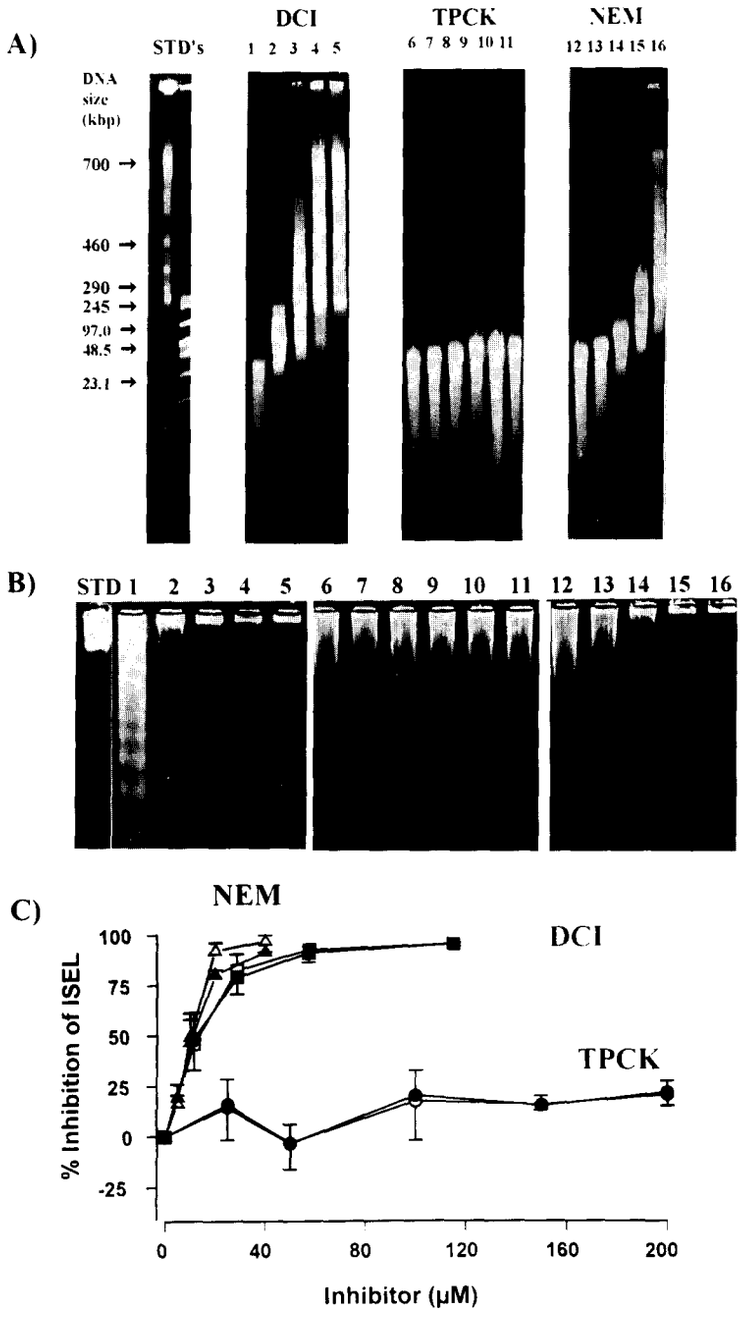

Fig. 2. The effect of NEM and protease inhibitors on DNA cleavage activated by 100 μM Ca^{2+}/8 mM Mg^{2+} TPCK, DCI and NEM were incubated with rat liver nuclei in the presence of 100 μM Ca^{2+}/8 mM Mg^{2+} for 30 min. The reactions were terminated and aliquots taken for FIGE (A), conventional agarose gel electrophoresis (B) and ISEL (C) which were carried out as described in section 2. The gel patterns are typical examples taken from separate experiments and the inhibitor concentrations were; DCI (lanes 1–5) = 0, 11.5, 28.8, 57.5 and 115 μM; TPCK (lanes 6–11) = 0, 25, 50, 100, 150 and 200 μM; NEM (lanes 12–16) = 0, 5, 10, 20, and 40 μM. Analysis of ISEL was carried out as described previously, from bivariate cytograms of propidium iodide (DNA content) versus green (anti-DIG) fluorescence. The peak mean fluorescence values, which were a measure of the end (anti-dig) labelling of the 2n (open symbols) and 4N (closed symbols) nuclei, were corrected for control fluorescence values (i.e. nuclei incubated without cations). The corrected values were expressed as a% of the uninhibited cleavage and are shown as the mean ± S.E.M of 3 separate nuclei preparations.

strates that NEM was also a very potent inhibitor of DNA cleavage. The inhibition of end-labelling by NEM (I_{50} = 10–12 μM) was accompanied by the abolition of internucleosomal cleavage (Fig. 2B, lanes 13–16) and a stepwise (inhibition-dependent) increase in the size of the large fragments (Fig. 2A, lanes 13–16) from >30 kbp, to 30–50 and 200–300 kbp, ≥ 700 kbp and intact DNA. NEM produced an almost total (90–95%) inhibition of ISEL. It should be emphasised that all the inhibitors had no effect on DNA when incubated in the absence of cations.

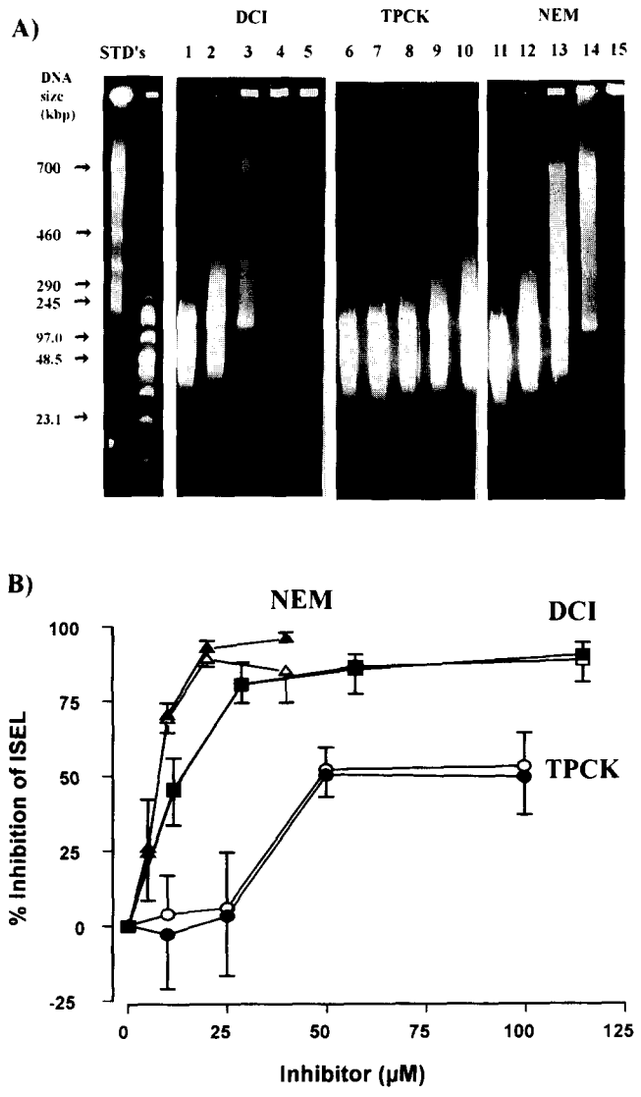

Fig. 3. The effect of NEM and protease inhibitors on DNA cleavage activated by 8 mM Mg^{2+} alone. TPCK, DCI and NEM were incubated with rat liver nuclei in the presence of 8 mM Mg^{2+} for 60 min. The reactions were terminated and aliquots taken for FIGE (A) and ISEL (B) which were carried out as described in Fig. 2. The gel patterns are typical examples from separate experiments and the concentrations for the inhibitors were ; DCI (lanes 1–5) = 0, 11.5, 28.8, 57.5 and 115 μM; TPCK (lanes 6–10) = 0, 25, 50, 100, 150 and 200 μM; NEM (lanes 11–15) = 0, 5, 10, 20, and 40 μM. Inhibition of end-labelling was carried out as described in Fig. 2. and the results are expressed as the mean ± S.E.M of 4–6 separate nuclei preparations.

3.3. *Inhibition of Mg^{2+}-dependent degradation of DNA into 'large fragments' by protease inhibitors and N-ethylmaleimide*

As shown in Fig. 3, large fragment formation activated by Mg^{2+} alone was also inhibited by DCI and as the end-labelling data demonstrates (Fig. 3B), the sensitivity of this process (I_{50} = 12–14 μM) to the inhibitor was similar to that shown by the Ca^{2+}/Mg^{2+} dependent (Fig. 2C) cleavage reaction. In the presence of Mg^{2+}, only the large 30–50 and 200–300 kbp sized fragments were produced which were detected by FIGE (lane 1, Fig. 3A) and increasing inhibition of the cleavage process was accompanied by a step-wise increase in the size of the DNA fragments (lanes 2–5). TPCK partially inhibited the Mg^{2+} stimulated DNA cleavage (compare with the Ca^{2+}/Mg^{2+} activated process, Fig. 2C), producing a maximum of 50% inhibition and only a small increase in the size of the DNA fragments (lanes 7–10, Fig. 3A) . TLCK (data not shown) was ineffective at inhibiting the Mg^{2+}-dependent reaction. However, NEM was a potent inhibitor of the end-labelling stimulated by Mg^{2+} (I_{50} = 6–8 μM, Fig. 3B.) and as shown in Fig. 3A (lanes 12–15) this inhibition was accompanied by a step-wise increase in the size of the cleaved DNA fragments.

3.4. *Effect of dithiothreitol on DCI and NEM inhibition of DNA cleavage*

The results of the above studies clearly showed that DCI was a potent inhibitor of DNA cleavage and contrasted with the results of Weaver et al. [18] who maintained that the Ca^{2+}/Mg^{2+} endonuclease activity of nuclei isolated from thymocytes and rat liver (data not shown) was not inhibited by DCI. However, it should be noted that in the latter experiments the incubation buffer contained 0.2 mM dithiothreitol (DTT) which has been shown to inactivate DCI and thereby prevent its inhibition of the proteolytic activity of the multi-proteinase complex [20]. It was therefore possible that DTT could also block the effects of DCI on DNA cleavage and the experiments shown in Fig. 4. verify this hypothesis. Thus, incubation of nuclei with 100 μM Ca^{2+}/8 mM Mg^{2+} (con lane, Fig, 4A,B) for 30 min/37°C produced a typical DNA ladder with a small amount of ≥ 30 kbp large fragments. The same fragmentation pattern was obtained in the presence of 500 μM dithiothreitol (DTT lane) demonstrating that this dithiol did not effect the fragmentation process. DCI (115 μM) blocked the internucleosomal cleavage (Fig. 4B) and resulted in most of the DNA being retained in the sample well of the FIGE gel with only a small amount of the chromatin degraded to 200–300 and ≥ 700 kbp fragments. Co-incubation of DTT with DCI essentially blocked the inhibitory effect of DCI on internucleosomal cleavage and large fragment formation. DTT (200 μM) also blocked the inhibitory effects of NEM (Fig. 4A,B) which is a potent thiol reagent. The blocking affect of DTT on the inhibitory effects of DCI and NEM on large fragment formation were also observed in nuclei activated with 8 mM Mg^{2+} (Fig. 4C).

4. Discussion

The biochemical and morphological changes in nuclei are an important part of the apoptotic process and isolated nuclei provide an appropriate model system for investigating the mechanisms/enzymes catalysing the cleavage of DNA into large fragments and subsequently to oligo-mononucleosomes. In this study we have shown that a variety of compounds with widely different structures will inhibit both Mg^{2+} and Ca^{2+}/Mg^{2+}-dependent DNA cleavage reactions. Although, the compounds used in this study have little or no structural relationship, it is clear that the inhibitory effects produced by all these compounds were remarkably similar. Thus, increased inhibition was always correlated with a sequential, step-wise increase in the size of the DNA fragments and in many respects the size distribution pattern was the exact opposite of that produced by the normal step-wise degradation process which we and others have described for thymocyte and rat liver nuclei [8,12–14]. The

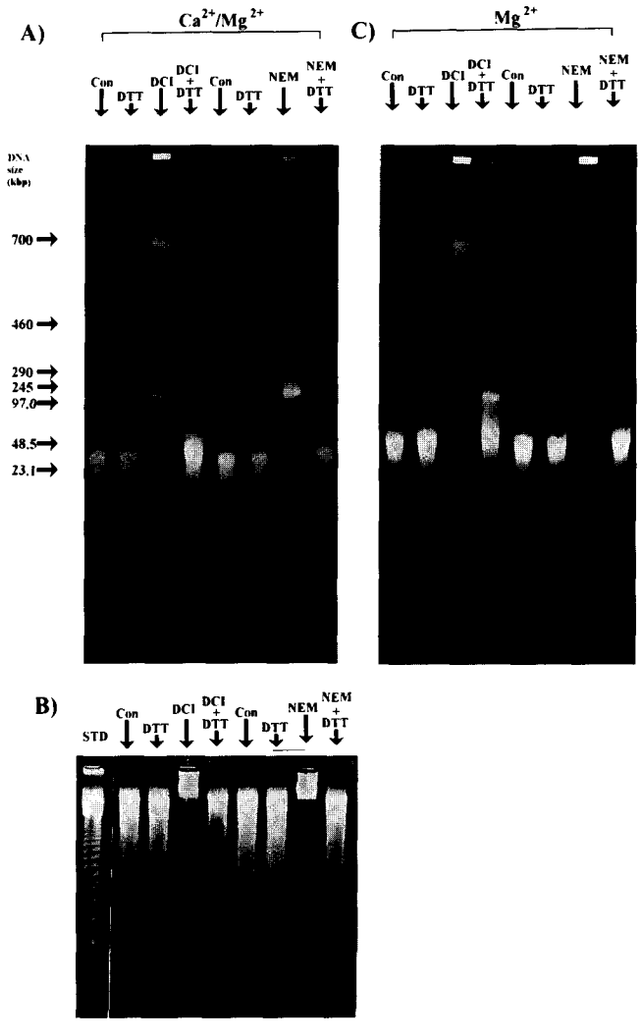

Fig. 4. The effect of DTT on the inhibition of DNA cleavage by NEM and DCI. Nuclei activated with either 100 μM Ca^{2+}/8 mM Mg^{2+} for 30min (A,B) or 8 mM Mg^{2+} for 60 min (C) and were inhibited with either 40 μM NEM or 115 μM DCI in the presence or absence of DTT (i.e. 500 μM for DCI and 200 μM for NEM). The reactions were terminated and DNA cleavage analysed by FIGE (A and C) and conventional agarose gel electrophoresis (B). The control lanes are from nuclei incubated with the appropriate cations, without any other additions. The remaining lanes are as the con lanes except that the nuclei have incubated with the indicated additions. The standards are not shown for the FIGE gels, although key sizes are labelled with arrows.

latter studies suggested that DNA cleavage is an ordered and stepwise process which progressively reduces the size of the DNA into its constituent higher order structural components. The inhibitor studies support this conclusion and lend support to the hypothesis that the sizes of the large fragments are similar to the 'loops' (30–50 kbp) and rosettes (200–300 kbp), which have been suggested by Filipski et al. [21] to be essential components of higher order chromatin structure. It is therefore possible that the DNA cleavage patterns which are seen in these inhibitor studies are the result of these compounds blocking the degradation process at the various stages in the unravelling of the DNA. This would suggest that the various stages of degradation are catalysed by different enzymes and Weaver et al. [18] have reported that in thymocytes treated with dexamethasone, DCI will block internucleosomal fragmentation but not large fragment formation or the morphological signs of apoptosis. Furthermore, Walker et al. [14] have reported that in liver nuclei, DCI will block DNA laddering but not large fragment formation, These results have been interpreted as evidence that proteases are involved in internucleosomal cleavage but not in large fragment formation. The results presented here, however do not support this concept and the data in Figs. 2–4 clearly show that DCI will inhibit large fragment formation whether stimulated by Ca^{2+} /Mg^{2+} or Mg^{2+} alone. Furthermore, the ISEL data show that the sensitivity of both these cation stimulated processes is the same.

The identity of the enzyme(s) which cleave DNA during apoptosis is as yet unknown and as reviewed by Peitsch et al. [6], various candidates have been put forward. The data presented in this and our previous studies [12,13] provide evidence for either a Mg^{2+}-dependent enzyme whose activity is markedly altered and stimulated in the presence of Ca^{2+}, or for two (or more) enzymes, one (or group) catalysing the large fragment formation and the other, the internucleosomal cleavage. If there were separate enzymes it is likely that they would show differential sensitivity to inhibitors. However, the findings described in this paper demonstrate that Cd^{2+}, Hg^{2+}, NEM and DCI were equally effective at inhibiting the Mg^{2+} and Ca^{2+}/Mg^{2+} activated DNA cleavage. Only, TPCK appeared to have differential effects in that it was slightly more potent at inhibiting the Mg^{2+} process. But even in this case the maximum inhibition of approximately 50% was much less than that produced by DCI or NEM which gave 80–100% inhibition. Thus, the inhibition data is in the main, consistent with the idea that both the Mg^{2+} and Ca^{2+}/Mg^{2+} activities are catalysed by the same endonuclease enzyme.

Both the Mg^{2+} and Ca^{2+}/Mg^{2+} activated DNA cleavage were inhibited by Cd^{2+} and Hg^{2+} which are noted for their affinity for -SH groups [19]. It is therefore likely that they are targeting a critical thiol group(s) (see later) on the endonuclease. DNA cleavage was also blocked by concentrations of DCI which are similar to the levels used for inhibiting serine proteases [22] and would suggest that the most likely target was a protease. However, TLCK a trypsin like serine protease inhibitor did not inhibit DNA degradation and TPCK a chymotrypsin like serine protease inhibitor was a weak inhibitor of the Mg^{2+}-dependent DNA fragmentation. DCI is believed to be a general serine protease inhibitor in that it inhibits both chymotrypsin and trypsin like proteases [23]. However, recent studies have shown that DCI will also inhibit calpain I, a thiol protease [24] and the finding that DTT inactivates the inhibitor [20] suggests that DCI reacts with sulphydryl groups. It is therefore significant that our results, showed that NEM which is well known for its reactivity towards thiols [25,26] was also a potent inhibitor of DNA fragmentation. Furthermore, co-incubation with DTT blocked the inhibitory effects of NEM and also those of DCI. The protective effect of DTT suggests that DCI and NEM were reacting with a thiol and we have found (K. Cain and S.H. Inayat-Hussain, unpublished results) that diamide which oxidises protein -SH groups to disulphides [27] also protects against the inhibitory effects of these compounds. These results, coupled with the findings with Cd^{2+} and Hg^{2+} which are classical sulphydryl inhibitors, strongly suggest that a critical thiol is involved in the DNA fragmentation process. The thiol group may be located on the endonuclease itself as Nikonova et al.

[28] have reported that three nucleases extracted from thymocyte nuclei were inhibited by NEM and idoacetamide. Also, Ribeiro and Carson [29] have described the purification of a Ca^{2+}/Mg^{2+} endonuclease from human spleen, which was partially inhibited by NEM. Alternatively, the sensitive thiol group may be located on a protease and in this respect there are a number of studies which have indicated that proteases are involved in apoptosis and DNA degradation [18,30,31]. There is some confusion and contradiction as to the precise effects of protease inhibitors and it is likely that more than one protease is involved. In this respect it is of interest that Motizuki et al. [32] have reported the purification of a thiol protease from rat liver nuclei which was activated by dithiothreitol and inhibited by iodacetamide.

The results of our study provide evidence that DCI and NEM may be targeting a critical thiol group which is essential for endonuclease activity and suggests that these compounds will be useful probes for further investigating this important element of the apoptotic process.

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