Calcium-activated DNA Fragmentation in Rat Liver Nuclei*

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Incubation of isolated rat liver nuclei with ATP, NAD*, and submicromolar Ca** concentrations resulted in extensive DNA hydrolysis. Half-maximal activity occurred with 200 nM Ca**, and saturation of the process was observed with 1 μM Ca**. ATP stimulated a calmodulin-dependent nuclear Ca** uptake system which apparently mediated endonuclease activation. Ca**-activated DNA fragmentation was inhibited by the inhibitor of poly(ADP-ribose) synthetase, 3-aminobenzamide, and was associated with poly(ADP-ribose)ylation of nuclear protein. The characteristics of this endonuclease activity indicate that it may be responsible for the Ca**-dependent fragmentation of DNA involved in programmed cell death (apoptosis) and in certain forms of chemically induced cell killing.

Accumulating evidence suggests that perturbations in intracellular Ca** homeostasis, characterized by sustained elevations of cytosolic Ca** concentration, play a critical role in toxic-induced cell killing (1). Among the cytotoxic effects induced by a sustained increase in cytosolic Ca** level are the cytoskeletal alterations associated with blebbing of the plasma membrane (2), activation of Ca**-dependent phospholipases with resultant membrane damage (3), and stimulation of Ca**-dependent neutral proteases (4).

Recently, we found (5) that a sustained rise in cytosolic Ca** (from normal values of 50–120 nM to 300–700 nM) precedes the DNA fragmentation that is characteristic of the glucocorticoid-induced killing of immature thymocytes known as "apoptosis" or programmed cell death (6). Pretreatment of the thymocytes with Ca** chelators, such as quin-2-AM, as well as incubation of the cells in a Ca**-depleted medium, prevented both the rise in cytosolic Ca** and DNA fragmentation and protected from cell death. Chromatin cleavage was also blocked by calmodulin inhibitors, suggesting that calmodulin mediates thymocyte apoptosis (5). Subsequent studies with several toxic agents, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (7), have indicated that an analogous process involving a sustained rise in cytosolic Ca** and associated activation of DNA fragmentation may also be of toxicological importance.

Earlier work had shown that a Ca**-dependent endonuclease exists in rat liver nuclei, although stimulation of the enzyme required the presence of millimolar concentrations of Ca** (8). Similarly, a recent study demonstrated that Ca** can arrest the topoisomerase 2 in a form that cleaves but does not religate DNA (9). Host defense mechanisms also elicit millimolar concentrations of Ca**. Because millimolar concentrations of Ca** do not occur in cells, the physiological significance of these observations has remained unclear. In the present study, we show that isolated rat liver nuclei contain a DNA endonuclease activity dependent upon Ca** in the submicromolar range when the nuclei are reconstituted with NAD* and ATP. An examination of the characteristics of this process revealed that it produced nucleosome-length DNA fragments, depended upon calmodulin and ATP for nuclear Ca** accumulation, and involved poly(ADP-ribose)sylation. Our results suggest that this endogenous endonuclease activity may be responsible for the DNA fragmentation occurring during programmed cell death and certain types of toxic cell killing.

EXPERIMENTAL PROCEDURES

Materials—ATP, ADP, NAD* (grade II), NADP*, and nicotinamide mononucleotide were purchased from Boehringer Mannheim. AMP, nicotinamide, hexokinase (type IV), [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 3-aminobenzamide, ethidium bromide, and diphenylamine (grade II) were from Sigma. Agarose for electrophoresis and d-sucrose for bacteriology were obtained from Fluka Chemie and CaCl2.*2H2O was from Merck. [carbonyl-14C]NAD* (44 mCi/mmol) and Ca** (10 mCi/mg) were purchased from Amer sham.

Calmidazolium (R24571) from Boehringer Mannheim was prepared as a 25 mM stock solution in dimethyl sulfoxide and stored at −20 °C. All other chemicals were reagent grade and obtained locally. Deionized water was used throughout except for high performance liquid chromatography, where distilled, deionized water was used.

Isolation of Nuclei—Male, Sprague-Dawley rats (200–300 g, fed ad libitum) were anesthetized with sodium pentobarbital. Livers were cannulated and perfused in situ with approximately 50 ml of ice cold TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2), which contained 2 mM calcium chloride, and 2.3 mM sucrose. The homogenate was filtered through three layers of cheesecloth. The nuclei were pelleted by centrifugation at 700 × g for 10 min. The pellets were homogenized (five strokes) in 40 ml of the same solution containing 0.25 M sucrose. The homogenate was washed through three layers of cheesecloth. The nuclei were pelleted by centrifugation at 700 × g for 10 min. The pellet was resuspended in 24 ml of the same solution by homogenization (three to five strokes), and 6 ml were added to each of four tubes containing 12 ml of TKM containing 2.3 mM sucrose. The tubes were gently mixed, and a 6-ml cushion (TKM containing 2.3 M sucrose) was carefully layered on the bottom of each tube. The tubes were centrifuged at 37,000 × g for 30 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet of highly purified nuclei was resuspended in the incubation medium (125 mM KCl), 2 mM potassium phosphate, 25 mM Hepes, 4 mM MgCl2, pH 7.0 by hand homogenization. Assay of marker enzymes (glucose-6-phosphatase, 5'-nucleotidase, succinate-cytochrome c reductase) showed that there was less

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1 The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
DNA Fragmentation Assay—Nuclei from one liver were suspended in 40 ml of incubation medium and divided into the appropriate number of flasks with additions as needed. At the indicated times, 2-
mi aliquots were removed and added to 3 ml of ice-cold lysis medium (5 mM Tris-HCl, 20 mM EDTA, 0.5% Na2EDTA, pH 8.0). After 10 min, samples were centrifuged for 20 min at 27,000 × g to separate the intact chromatin (pellet) from the fragmented DNA (supernatant) (6). The supernatants were decanted and saved; the pellets were resuspended in 5 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Pellet and supernatant fractions were assayed for DNA content with the diphenylamine reaction (10).

Gel Electrophoresis—Nuclei were lysed and centrifuged as described above to separate DNA fragments from the intact chromatin. The samples of DNA fragments were extracted sequentially with equal volumes of phenol, phenolchloroform (1:1), and chloroform, precipitated in 67% ethanol, 0.17 M NaCl at -20 °C for 18 h, and redissolved in 10 mM Tris-HCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, pH 8.0, prior to electrophoresis for 2 h at 60 V in 1.4% agarose gels. DNA was visualized by UV fluorescence after staining with ethidium bromide (6 μg/ml).

Buffering of Calcium Concentration—The concentrations of total EGTA and Ca2+ needed to establish free Ca2+ concentrations in the range of 0.05 to 10 μM were determined as described by Bartfai (11) with 2 mM EGTA. Ca2+ and appropriate additions of EGTA and CaCl2 to give the desired values. Calculated values were consistent with amounts measured by fluorescence with fura-2 (12).

High Performance Liquid Chromatography of Adenine and Pyridine Nucleotides—ATP, ADP, AMP, NAD+, NADP+, nicotinamide, and nicotinamide mononucleotide were separated on a reverse phase C18 column (Waters Radial Pak, 5 μm, 8 mm diameter) essentially as described (13). Solvent A was 100 mM potassium phosphate buffer, pH 5.9, and solvent B was A plus 10% (v/v) methanol. Flow rate was 4 ml/min. Metabolites were assayed by absorbance at 266 nm. For some experiments, [carboxyl-14C]NAD+ was added (17), and fractions from HPLC runs were collected and counted in the presence of a scintillation fluid to derive the rate of the nicotinamide from NAD+.

Ca2+ Uptake—Isolated nuclei (150–200 μg of DNA/ml) were resuspended in Ca2+-supplemented (0.1 mM CaCl2) Ca2+-buffered medium described above so that the final concentration of free Ca2+ was 400 nM. Samples were incubated in the presence or absence of 1 mM ATP for 5 min at 37 °C. Aliquots of 0.1 ml were then placed on 0.22-
μm nitrocellulose filters (Sartorius) and washed four times with 0.25 ml of an ice-cold buffer containing 50 mM Tris, 150 mM KCl, pH 7.0. Filters were placed in 5 ml of scintillation mixture (Beckman Ready Safe) prior to the determination of Ca2+ radioactivity in a Beckman LS 1801 liquid scintillation counter.

RESULTS

DNA Fragmentation in Freshly Isolated Nuclei—Previous work has shown that rapid autodigestion of DNA can occur when nuclei isolated from a variety of different tissues, including rat liver, are incubated in a buffer containing Ca2+ and Mg2+ (8, 14). To examine the Ca2+ dependence of this endogenous DNA fragmentation process in more detail, isolated rat liver nuclei were incubated with EGTA or various amounts of Ca2+ prior to quantitation of DNA cleavage. As was observed previously (8), time-dependent endonuclease activation occurred with 1 mM Ca2+, although an increase in activity was also observed at much lower Ca2+ levels (1–10 μM) compared to controls with a high EGTA concentration (Fig. 1). Thus, we sought to optimize conditions for the activation of DNA fragmentation in micromolar Ca2+ concentrations.

Addition of 1 mM ATP and 1 mM NAD+ stimulated the rate of DNA fragmentation in nuclei incubated with 10 μM Ca2+ (Table I). In the presence of ATP and NAD+, Ca2+-dependent DNA fragmentation was nearly as extensive with 10 μM Ca2+ as with 1 mM Ca2+ in the absence of these compounds (Fig. 1). Analysis of the digested chromatin by agarose gel electrophoresis revealed a ladder-like pattern of cleavage characteristic of the production of nucleosome-length fragments (a sample of DNA fragments from glucocorticoid-treated thymocytes is shown for comparison) (Fig. 2).

To examine the requirements for ATP and NAD+ in more detail, a variety of experiments were performed with related biomolecules and different concentrations of ATP and NAD+ (Table I). Addition of AMP or ADP to nuclei with 1 mM NAD+ gave little or no stimulation above that seen with NAD+ alone. Since liver nuclei contain some ATP that is not use of the endogenous ATP, although addition of hexokinase plus glucose in an attempt to remove this ATP had no effect on the Ca2+-dependent DNA fragmentation in the presence of NAD+. Substitution of nicotinamide for NAD+ in nuclear fractions incubated with 1 mM ATP stimulated DNA fragmentation to about half the maximal rate, whereas in the

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**Table I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>DNA Fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM ATP, 1 mM NAD+</td>
<td>100</td>
</tr>
<tr>
<td>1 mM ATP, 0.5 mM NAD+</td>
<td>78</td>
</tr>
<tr>
<td>1 mM ATP, 0.2 mM NAD+</td>
<td>60</td>
</tr>
<tr>
<td>1 mM ATP, 0.1 mM NAD+</td>
<td>58</td>
</tr>
<tr>
<td>1 mM ATP, 0.05 mM NAD+</td>
<td>58</td>
</tr>
<tr>
<td>1 mM ATP, 0.01 mM NAD+</td>
<td>44</td>
</tr>
<tr>
<td>0.5 mM ATP, 1 mM NAD+</td>
<td>26</td>
</tr>
<tr>
<td>0.2 mM ATP, 1 mM NAD+</td>
<td>21</td>
</tr>
<tr>
<td>0.1 mM ATP, 1 mM NAD+</td>
<td>15</td>
</tr>
<tr>
<td>1 mM AMP, 1 mM NAD+</td>
<td>37</td>
</tr>
<tr>
<td>1 mM AMP, 1 mM NAD+</td>
<td>26</td>
</tr>
<tr>
<td>1 mM AMP, 1 mM nicotinamide</td>
<td>26</td>
</tr>
<tr>
<td>1 mM AMP, 1 mM nicotinamide</td>
<td>19</td>
</tr>
<tr>
<td>1 mM adenosine, 1 mM nicotinamide</td>
<td>0</td>
</tr>
<tr>
<td>1 mM NAD+</td>
<td>6</td>
</tr>
<tr>
<td>10 mM Ca2+</td>
<td>13</td>
</tr>
<tr>
<td>5 mM EGTA</td>
<td>0</td>
</tr>
<tr>
<td>1 mM ATP, 1 mM NAD+</td>
<td>0</td>
</tr>
</tbody>
</table>
absence of ATP or in the presence of AMP, no stimulation was observed with nicotinamide. NADP⁺ did not substitute for NAD⁺ either with (not shown) or without ATP. Thus, the results show that both ATP and NAD⁺ are required for maximal endonuclease activity, but endogenous sources of these as well as the precursor nicotinamide are sufficient to allow for some Ca²⁺-dependent DNA fragmentation.

An analysis of the concentration dependence showed that maximal rates of Ca²⁺-dependent DNA fragmentation were obtained with 0.5 mM ATP and 1 mM NAD⁺ (Table I). Higher concentrations of NAD⁺ gave results identical with that observed with 1 mM. From the concentration dependences, half-maximal activity was obtained with 0.1–0.2 mM ATP and 0.1–0.2 mM NAD⁺.

Ca²⁺ Concentration Dependence in the Presence of 1 mM ATP and 1 mM NAD⁺—To measure the Ca²⁺ dependence of the endonuclease at submicromolar Ca²⁺ concentrations, free Ca²⁺ was buffered at desired values by using high (millimolar) concentrations of EGTA (11). Under these conditions, DNA fragmentation was more rapid than with 10 μM Ca²⁺ which was unbuffered. As shown in Fig. 3, the process was fully activated with 1 μM extranuclear Ca²⁺ and a half-maximal rate was observed with 100–200 nM extranuclear Ca²⁺.

The apparently high affinity of the endonuclease for Ca²⁺, the calmodulin dependence of glucocorticoid-stimulated thymocyte DNA fragmentation (5), and the recent report that rat liver nuclei contain calmodulin (15) led us to determine the role of calmodulin in Ca²⁺-dependent rat liver nuclear chromatin cleavage. Incubation of nuclei with calmidazolium, a potent inhibitor of calmodulin (16), almost completely inhibited Ca²⁺-dependent DNA fragmentation (Fig. 4). Thus, Ca²⁺-dependent endonuclease activity in rat liver nuclei appears to be dependent on calmodulin.

Requirement for ATP—ATP can support NAD⁺ synthesis from its precursors in isolated nuclei, and some stimulation of DNA fragmentation by addition of nicotinamide in the presence of ATP (Table I) indicated that ATP could be used for synthesis of NAD⁺ from nicotinamide to promote this process. However, under conditions where excess NAD⁺ was added, ATP was still required for maximal DNA fragmentation. Thus, the major requirement for ATP appeared to be for a reaction distinct from NAD⁺ synthesis.
that NAD⁺ turnover in the nucleus can be extremely rapid. If nuclei were suspended in buffered media with 400 nM Ca²⁺ containing Mg²⁺, Procedure. Mean S.E. from three separate experiments. B, effect of Ca²⁺ ionophore A23187 on the ATP requirement of Ca²⁺- and NAD⁺-dependent DNA fragmentation in rat liver nuclei. Nuclei were incubated in 5 mM EGTA (O) or in the buffered media with 400 nM Ca²⁺ and NAD⁺ in the absence (O) or presence of 1 mM ATP (A) or 10 μM A23187 (A). Mean ± S.E. from three separate experiments.

Table II

Effect of Ca²⁺ on NAD⁺ loss and poly(ADP-ribosylation) of protein in isolated rat liver nuclei

<table>
<thead>
<tr>
<th>Condition</th>
<th>NAD⁺ loss*</th>
<th>5’-AMP present</th>
<th>pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without added NAD⁺</td>
<td>0.15</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>+5 mM EGTA</td>
<td>0.86</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>+10 μM Ca²⁺</td>
<td>8.0</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>With 200 μM NAD⁺ added</td>
<td>162</td>
<td>17.7</td>
<td></td>
</tr>
</tbody>
</table>

* NAD⁺ loss after 20 min at 37 °C. Without added NAD⁺, content was 1 to 1.5 nmol/ml of incubation. Nuclei content was equivalent to 80 μg of DNA/ml.

Poly(ADP-ribosylation) was measured as the amount of 5’-AMP released from washed protein pellets from 20-min incubations hydrolyzed in 1.3 M KOH at 60 °C for 30 min. Initial 5’-AMP was 0.03 to 0.06 nmol.

FIG. 6. Effect of 3-aminobenzamide on DNA fragmentation in isolated rat liver nuclei. Nuclei were incubated as described in Fig. 2 either with (open symbols) or without (closed symbols) 3 mM 3-aminobenzamide. Circles represent incubations with 10 μM Ca²⁺, and triangles are incubations with 5 mM EGTA. Mean ± S.E. from four separate experiments.

from the Ca²⁺-dependent endonuclease activity.

Requirement for NAD⁺—Previous studies have established that NAD⁺ turnover in the nucleus can be extremely rapid. Nuclear NAD⁺ turnover involves cleavage of the nicotinamide group from NAD⁺, in contrast to the reduction and reoxidation reactions that occur in the cytosol and mitochondria. To examine whether NAD⁺ cleavage was dependent on Ca²⁺, we measured NAD⁺ loss and nicotinamide production in nuclei incubated in the presence of NAD⁺ with or without micromolar concentrations of extranuclear Ca²⁺. The results showed that with concentrations of NAD⁺ at or below 200 μM, NAD⁺ loss was substantially stimulated by Ca²⁺ concentrations of 1 or 10 μM (Table II). With [carboxyl-¹⁴C]NAD⁺ added as a tracer, the rate of nicotinamide formation was found to equal the rate of NAD⁺ loss (91–104% recovery). Further experiments revealed that Ca²⁺-stimulated concomitant poly(ADP-ribosylation) of nuclear protein (Table II), as estimated by the 5’-AMP released from the protein pellet under alkaline conditions (17). Thus, the data show that the Ca²⁺-stimulated endonuclease activity is associated with the breakdown of NAD⁺ to nicotinamide and with the formation of poly(ADP-ribose).

A well characterized nuclear enzyme that cleaves NAD⁺ to produce nicotinamide is poly(ADP-ribose) synthetase. This enzyme is involved in control of DNA excision repair following DNA damage (18), and it is thought to have a more general role in regulation of enzyme activity and chromatin structure through reversible covalent modification of proteins by ADP-ribose polymers (17). To determine whether the stimulation of DNA fragmentation involved the function of the poly(ADP-ribosylase) synthetase activity, the effect of the synthetase inhibitor 3-aminobenzamide on Ca²⁺-dependent DNA fragmentation was assessed. The results (Fig. 6) showed that 3-aminobenzamide inhibited the Ca²⁺-dependent DNA fragmentation by about 50%. These results suggest that Ca²⁺-dependent endonuclease activity in liver nuclei involves the activation of poly(ADP-ribosylation).

DISCUSSION

The fundamental role of the calcium ion in the regulation of biological processes has been studied in detail and frequently reviewed (19, 20). Several systems function to maintain intracellular Ca²⁺ homeostasis, and perturbations of these systems are involved in a variety of pathological and toxicological processes (1). Several recent studies have indicated that Ca²⁺ plays an important role in regulation of nuclear functions (5, 8, 21–24), and we recently found that a sustained increase in cytosolic Ca²⁺ level precedes the activation of DNA fragmentation that is characteristic of programmed cell death (5, 7).

Programmed cell death is important in development and characterized by a morphological pattern known as apoptosis, involving early cell membrane blebbing, generalized chromatin condensation, and the compacting of cytoplasmic organelles (25). The process has been studied most extensively in glucocorticoid-treated thymocytes, and Wyllie and Morris (26) showed that the condensed chromatin of apoptotic cells consists of endogenously digested chromatin fragments that are produced by the activity of a constitutive endonuclease. The Ca²⁺ ionophore A23187 has been shown to cause endonuclease activation in immature thymocytes (14, 27), indicating that Ca²⁺ may mediate the process. More recently, we found that cytosolic Ca²⁺ concentrations increase severalfold in thymocytes following treatment with glucocorticoids and that suppression of this increase by incubation in Ca²⁺-free media or inclusion of a high concentration of quin-2 to buffer cytosolic Ca²⁺ inhibited the initiation of apoptosis (5). Moreover, we have found that a similar process involving an increase in cytosolic Ca²⁺ level mediates the 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced killing of thymocytes (7) and the killing of hepatocytes with moderately toxic concentrations of menadione (28). In addition, we found that Ca²⁺-dependent DNA fragmentation was the lesion that killed thymocytes...
exposed to A23187 or glucocorticoid hormone (29). Thus, it appears likely that a Ca\textsuperscript{2+}-activated endonuclease is of central importance in certain types of cell killing.

Several previous studies have examined Ca\textsuperscript{2+}-activated endonuclease activities in nuclei. Burgoyne et al. (30) and Hewish and Burgoyne (31) showed that incubation of nuclei with millimolar concentrations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} caused a remarkable fragmentation of DNA. Nakamura et al. (32) purified a Ca\textsuperscript{2+}-dependent DNA endonuclease from calf thymus and showed that this enzyme is distinct from DNase I, which is also Ca\textsuperscript{2+}-activated. Cohen and Duke (14) studied a constitutive endonuclease in the nuclei of thymocytes which could be activated by 5 mM Ca\textsuperscript{2+} in the presence of Mg\textsuperscript{2+} and produced oligonucleosome-length fragments of DNA. It was concluded that this activity was involved in glucocorticoid-induced DNA fragmentation and cell death because the process was inhibited by Zn\textsuperscript{2+}, both in glucocorticoid-treated thymocytes and in isolated thymocyte nuclei. Vanderbilt et al. (8) studied the properties of an endonuclease activity in nuclei from a variety of tissues and found that autodigestion was dependent on Ca\textsuperscript{2+} in the millimolar range. They showed that cationic precipitation of chromatin inhibited DNA fragmentation and concluded that activation of DNA autodigestion resulted from variations in the cationic composition of the nucleoplasm. Taken together, these studies provide secure evidence for the widespread occurrence of constitutive endonuclease activity that is dependent upon Ca\textsuperscript{2+} and produces oligonucleosome-length fragments of DNA. However, in spite of this knowledge, the significance of the Ca\textsuperscript{2+} dependence of this activity has remained uncertain because all of these previous studies showed Ca\textsuperscript{2+}-dependent endonuclease activity only with millimolar Ca\textsuperscript{2+} concentrations, while the cytosolic Ca\textsuperscript{2+} concentration is maintained in the submicromolar range in mammalian cells (33). This discrepancy is further illustrated by the finding that cytosolic Ca\textsuperscript{2+} concentrations in thymocytes undergoing apoptosis and DNA fragmentation only reach 400–700 nM (see above). Thus, it was essential to determine whether Ca\textsuperscript{2+}-dependent activation of DNA fragmentation could occur with the submicromolar Ca\textsuperscript{2+} concentrations present in mammalian cells and to define the factors that contribute to this sensitivity.

The present findings show that isolated rat liver nuclei contain an endonuclease that generates oligonucleosome-length DNA fragments and can be activated by submicromolar Ca\textsuperscript{2+} concentrations when physiological levels of ATP and NAD\textsuperscript{+} are present. Since this activity is Ca\textsuperscript{2+}-dependent over the range of cytosolic Ca\textsuperscript{2+} measured in cells during initiation of apoptosis and during the onset of certain types of chemically induced cell death, it is probably responsible for the DNA fragmentation that occurs in cells under these conditions.

The requirements for ATP and NAD\textsuperscript{+} were isolated to two distinct processes: ATP was required for Ca\textsuperscript{2+} accumulation by nuclei, while NAD\textsuperscript{+} was required for subsequent endonuclease activation. Recent detailed studies of ATP-dependent nuclear Ca\textsuperscript{2+} accumulation (34) revealed that the Ca\textsuperscript{2+} uptake reported here results in an increase in the nuclear free Ca\textsuperscript{2+} concentration as measured by fura-2, which can be blocked by calmidazolium and thiol reagents. Nuclear Ca\textsuperscript{2+} uptake does not depend on NAD\textsuperscript{+}, and the ability to eliminate the ATP requirement for activation of the endonuclease by inclusion of the Ca\textsuperscript{2+} ionophore A23187 in the nuclear incubation medium clearly distinguishes this ATP-dependent step from subsequent endonuclease activation.

Several lines of evidence indicate that the NAD\textsuperscript{+} requirement for the Ca\textsuperscript{2+}-dependent endonuclease activity involves the function of poly(ADP-ribose) synthetase. The synthetase is present in nuclei, and its activation has previously been shown to result in rapid NAD\textsuperscript{+} cleavage with release of nicotinamide (17). The activation of the Ca\textsuperscript{2+}-dependent endonuclease of liver nuclei was associated with release of nicotinamide from NAD\textsuperscript{+} and a concomitant increase in the amount of 5'-AMP that was releasable from the protein pellet upon mild alkaline hydrolysis. Although this latter assay is only an indirect measure of poly(ADP-ribose)lation, 5'-AMP formation was inhibited by 3-amino benzamide and qualitatively corresponded to the rate of NAD\textsuperscript{+} loss (not shown). Furthermore, 3-amino benzamide inhibited the Ca\textsuperscript{2+}-dependent DNA fragmentation. Thus, it appears likely that the Ca\textsuperscript{2+}-dependent endonuclease activity is regulated either directly or indirectly by poly(ADP-ribose)ylation.

Previous studies have shown that oxidants such as H\textsubscript{2}O\textsubscript{2} can stimulate poly(ADP-ribose) synthetase activity in cells and result in NAD\textsuperscript{+} and ATP depletion which may be responsible for cell death (35, 36). This activation appears to be due to the generation of oxidant-damaged DNA which activates poly(ADP-ribose) synthetase. However, oxidants also inhibit Ca\textsuperscript{2+}-ATPases (37) and result in sustained increases in cytosolic Ca\textsuperscript{2+} (38). The present results show that DNA fragmentation can be initiated by submicromolar increases in Ca\textsuperscript{2+} in the absence of an oxidant. Thus, it appears possible that some of the DNA fragmentation observed in cells exposed to oxidants is due to Ca\textsuperscript{2+}-mediated activation of this constitutive endonuclease activity rather than to direct oxidant damage to the DNA.

The complexity of the intact nuclear system precludes a definitive interpretation of the site(s) of Ca\textsuperscript{2+} dependency. Bach and Carafoli (15) have studied the calmodulin content of rat liver nuclei and found that calmodulin is present in different nuclear subfractions. Five nuclear calmodulin-binding proteins were identified, and a calmodulin-dependent inhibition of protein phosphorylation was detected. However, the biological functions of these proteins are not known. Earlier studies suggested the involvement of calmodulin in DNA repair and gene expression (23, 24). We showed previously that glucocorticoid-induced ATP fragmentation in thymocytes is mediated by calmodulin (5). Our recent studies of Ca\textsuperscript{2+} accumulation by nuclei (34) show that calmodulin is also involved in ATP-dependent Ca\textsuperscript{2+} uptake by nuclei. The role of calmodulin in DNA fragmentation is probably in mediating the Ca\textsuperscript{2+} accumulation, although calmodulin may also confer Ca\textsuperscript{2+} sensitivity to the endonuclease or to a regulatory system for the endonuclease.

In conclusion, the current study shows that rat liver nuclei contain a DNA endonuclease that is stimulated by submicromolar concentrations of Ca\textsuperscript{2+} when nuclei are incubated with NAD\textsuperscript{+} and ATP. The process involves calmodulin and poly(ADP-ribose)ylation and results in rapid and extensive cleavage of chromatin into oligonucleosome-length fragments. The activity and characteristics of this process are appropriate to account for the extensive DNA fragmentation that occurs under certain toxicological conditions in hepatocytes, and it is probably analogous to the activity which mediates thymocyte programmed cell death. The relationship of this activity to other DNA endonucleases and the nature of its normal physiological function are subjects requiring further investigation.

REFERENCES

Calcium-activated DNA Fragmentation