DNA fragmentation in permeabilized cells and nuclei

The role of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent endodeoxyribonuclease

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Permeabilized mammalian cells and isolated nuclei were used to study various aspects of DNA replication and repair. The present paper describes a progressive fragmentation of parental DNA in human lymphoblastoid cells that were permeabilized with *L*- α -lysophosphatidylcholine or with saponin and incubated at 37 °C in a DNA-synthesis mixture. The formation of DNA single-strand breaks (measured by alkaline elution) was linear with the time of incubation and was temperature-dependent. It was prevented by deleting Mg^{2+} or both Mg^{2+} and Ca^{2+} from the incubation mixture, or by the addition of EDTA. It was increased by deleting the components necessary for DNA synthesis, and by substituting Mn^{2+} for Mg^{2+} and Ca^{2+} . DNA strand breaks also accumulated in isolated nuclei incubated in a DNA synthesis mixture, but not when Mg^{2+} was omitted. These results suggest that DNA fragmentation in permeabilized cells and nuclei was due to an activation of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent endodeoxyribonucleases. The integrity of template DNA needs to be ascertained when the conditions for measuring DNA synthesis in permeabilized cells or in nuclei are formulated.

INTRODUCTION

Permeabilized mammalian cells permit studies of various aspects of DNA replication and repair that are not possible with intact cells. A number of methods of permeabilizing cells have been developed. Two such highly successful methods use *L*- α -lysophosphatidylcholine (Miller *et al.*, 1978, 1979) and saponin (Smollen & Stoehr, 1985; Kaufmann & Briley, 1987) as permeabilizing agents. Using *L*- α -lysophosphatidylcholine-permeabilized cells, Miller *et al.* (1978, 1979) showed that permeabilized cells replicated DNA in a normal semi-conservative manner for at least 2 h and initially at the same rates as intact cells. More recently, Pawlak *et al.* (1986) reported that incorporation of labelled nucleotides into nucleic acids in cells permeabilized by this method was not consistent with semi-conservative DNA replication and RNA transcription, and suggested that DNA in permeabilized cells was impaired in its function as a template. We found that parental DNA underwent progressive fragmentation after cells were permeabilized and incubated in the DNA-synthesis mixture (Li & Kaminskas, 1987a). We thought that this fragmentation occurred because of a disruption of structures that are necessary to maintain the integrity of DNA or because of a release of DNA-degrading enzymes. Here we report that DNA fragmentation was not due to the permeabilization, but rather to the presence of Mg^{2+} and Ca^{2+} in the DNA-synthesis mixture. Parental DNA in isolated nuclei also underwent fragmentation, which was Mg^{2+} -dependent. These findings suggest that DNA fragmentation is probably due to the activation of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent endodeoxyribonucleases.

EXPERIMENTAL

Materials and cell cultivation

L- α -Lysophosphatidylcholine (Sigma type I), saponin, phosphoenolpyruvate and nucleoside triphosphates were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The sources of $[2\text{-}^{14}\text{C}]\text{thymidine}$, other chemicals, serum, growth media and filters were as previously described (Li & Kaminskas, 1987b).

L- α -Lysophosphatidylcholine was dissolved in buffer A (150 mM-sucrose/80 mM-KCl/35 mM-Hepes/7.5 mM- K_2HPO_4 /4 mM- MgCl_2 /1 mM- CaCl_2 , pH 7.4) (Miller *et al.*, 1978) minus MgCl_2 and CaCl_2 , and saponin was dissolved in buffer K (100 mM-KCl/20 mM-NaCl/30 mM-Hepes, pH 7.4, with or without 1 mM-EGTA) (Smollen & Stoehr, 1985) on the day of experiment. Human lymphoblastoid RB4580 cells (Tarone *et al.*, 1984) and cultured Ehrlich ascites-tumour cells (Li & Kaminskas, 1987b) were cultivated as previously described. For all experiments, cells were seeded at 3.5×10^5 /ml and grown in the presence of $[2\text{-}^{14}\text{C}]\text{thymidine}$ (0.01 $\mu\text{Ci}/\text{ml}$) for 48 h and then in fresh medium without the label for 16 h. For each experiment, $(10\text{--}12) \times 10^6$ cells were collected by centrifugation, resuspended in buffer A (or in buffer K in saponin experiments) or in buffer A without Mg^{2+} and Ca^{2+} and distributed into conical 1.5 ml polyethylene tubes such that each tube contained approx. 1.5×10^6 cells. The cells were then collected by centrifugation in an Eppendorf microcentrifuge. After the residual liquid in the tube had been carefully removed with lintless tissue, the tubes containing the cell pellets were kept in an ice/water bath until permeabilization.

Cell permeabilization and preparations of nuclei

The cell pellets were suspended in 0.1 ml of L- α -lysophosphatidylcholine solution (0.15 mg/ml in buffer A without Mg^{2+} and Ca^{2+}), mixed three times briefly with a vortex mixer and incubated at 4 °C for 2 min (Miller *et al.*, 1978, 1979). In saponin experiments the cell pellets were suspended in 0.1 ml of saponin solution (0.1 mg/ml in buffer K), mixed and incubated at room temperature for 15 min (Smollen & Stoehr, 1985; Kaufmann & Briley, 1987). The extent of cell permeabilization was 90–100 % as determined by the uptake of Trypan Blue. Permeabilized cells were then incubated in 1.5 ml of pre-warmed DNA synthesis mixture, or of buffer solutions as specified, in a 37 °C water bath with gentle agitation. The DNA-synthesis mixture contained, in addition to buffer A, 1.5 mM-ATP, 0.15 mM each of CTP, GTP, UTP, 0.25 mM each of dATP, dCTP, dGTP and dTTP, and 10 mM-phosphoenolpyruvate with pH adjusted to 7.4 (Miller *et al.*, 1978). In experiments where Mn^{2+} was included in the incubation mixture, K_2HPO_4 in buffer A was omitted in order to avoid precipitate formation. Incubations were terminated by placing the tubes in an ice/water bath. Nuclei were prepared by washing cells in a hypo-osmotic buffer (1 mM-Tris/HCl (pH 7.6)/25 mM-KCl/0.9 mM-MgCl₂/0.9 mM-CaCl₂/0.14 mM-spermidine) at 4 °C, resuspension in the same buffer and homogenization with a Dounce glass homogenizer until more than 95 % nuclei were obtained (about eight strokes), and then washing three times in the hypo-osmotic buffer at 4 °C (Matsumoto *et al.*, 1988). The DNA synthesis mixture for nuclei contained 0.2 mM each of dATP, dCTP, dGTP and dTTP, 5 mM-ATP, 100 mM-KCl, 4 mM-MgCl₂, 1 mM-EDTA, 2 mM-2-mercaptoethanol and 40 mM-Tris/HCl, pH 7.8 (Matsumoto *et al.*, 1988).

Alkaline elution of DNA

The procedure was that of Kohn *et al.* (1981), except that cells were lysed with 2.5 ml of lysis solution containing proteinase K at 0.5 mg/ml; eluates were collected in six 5 ml fractions over 15 h; and the 0.4 M-NaOH wash (5 ml) was neutralized with 0.13 ml of 6 M-HCl before its radioactivity was determined (Li & Kaminskas, 1987a). Relative elution values, which are proportional to the frequency of DNA single-strand breaks (SSB), were obtained from:

$$\text{Relative elution value} = \log R_0 - \log R$$

where R_0 and R represent fractions of DNA retained on the filter in the control sample and in the experimental sample respectively after 20 ml (or as stated) of eluates were collected (Fornace & Little, 1977).

RESULTS

The following experiments were performed with human lymphoblastoid cell line RB4580; the main findings were confirmed by using cultured Ehrlich ascites-tumour cells. When cells prelabelled with [¹⁴C]thymidine were permeabilized with L- α -lysophosphatidylcholine and incubated in the DNA-synthesis mixture at 37 °C, there was a progressive formation of SSB in parental DNA. Fig. 1(a) shows alkaline elution profiles of parallel cultures of permeabilized cells after 15, 30 and 60 min of incubation in the DNA-synthesis mixture. When relation elution values were plotted, the increase in the frequencies of SSB was approximately linear with time of incubation (Fig. 1b). The formation of SSB was temperature-dependent. When permeabilized cells were incubated in the DNA synthesis mixture at 4 °C for 60 min, SSB formation did not occur.

Fig. 1. Time courses of SSB formation in permeabilized cells

(a) Alkaline elution patterns of intact cells (○) and of permeabilized cells that were incubated in parallel cultures in the DNA-synthesis mixture at 37 °C for 0 (●), 15 (▲), 30 (▼) and 60 min (■). (b) A plot of relative elutions, calculated from fractions of DNA retained on the filter after 5 ml of elution as a function of time of incubation in the DNA-synthesis mixture. Symbols indicate different experiments. (c) Alkaline elution patterns of intact cells (○), of permeabilized cells that were incubated in the DNA-synthesis mixture without Ca^{2+} or Mg^{2+} for 0 (●), 15 (▲), 30 (▼) and 60 min (■), and of permeabilized cells incubated in the DNA-synthesis mixture with Ca^{2+} and Mg^{2+} for 15 min (△).

Fig. 2. Effects of Ca^{2+} , Mg^{2+} , Mn^{2+} , EDTA and nucleotides on SSB formation in permeabilized cells

(a) Alkaline-elution patterns of permeabilized cells incubated for 15 min in phosphate-free buffer A without Ca^{2+} or Mg^{2+} (○), without Mg^{2+} (▽), without Ca^{2+} (●), with both Ca^{2+} and Mg^{2+} (▼) and with Mn^{2+} only (■). (b) Alkaline-elution patterns of permeabilized cells incubated for 15 min in buffer A (■), in buffer A with 5 (▲), 10 (▼), 15 or 20 (●) mM-EDTA, in buffer A without Ca^{2+} and Mg^{2+} (□), and of intact cells (○). (c) Alkaline-elution patterns of permeabilized cells incubated for 15 min in buffer A (○, ●), in buffer A plus 1.5 mM-ATP and 10 mM-phosphoenolpyruvate (▽, ▼), in buffer A plus 0.15 mM-ribonucleotides and 0.25 mM-deoxyribonucleotides (△, ▲) and in DNA-synthesis mixture (■). Open symbols indicate omission of Ca^{2+} and Mg^{2+} ; closed symbols indicate the presence of Ca^{2+} and Mg^{2+} .

The various components of the DNA-synthesis mixture were examined for their effect on the formation of SSB. SSB were decreased by 94 % (range 85–100 % in 12 expts.), when Mg^{2+} and Ca^{2+} were omitted from the DNA-synthesis mixture. Fig. 1(c) shows the alkaline-elution patterns of permeabilized cells that were incubated in the DNA-synthesis mixture without Mg^{2+} and Ca^{2+} for 15, 30 and 60 min at 37 °C in parallel cultures. Thus the formation of SSB in permeabilized cells was not due to the permeabilization, but rather to the presence of Ca^{2+} and of Mg^{2+} in the DNA-synthesis mixture.

The omission of Mg^{2+} affected the formation of SSB to a far greater extent than the omission of Ca^{2+} . When Mg^{2+} was omitted from the incubation mixtures (DNA-synthesis mixture or buffer A), the frequencies of SSB were decreased by over 90 % (seven expts., Fig. 2a). The same results were obtained even when the Ca^{2+} concentration was increased from 1 to 4 mM. When Ca^{2+} was omitted from the incubation mixtures, the frequencies of SSB were reduced by approx. 40 % (range 11–59 % in seven expts.) (Fig. 2a). Thus Ca^{2+} potentiated the Mg^{2+} effect, but to a variable extent. The addition of 5 mM-EDTA to buffer A reduced the frequency of SSB and the addition of 10–20 mM-EDTA completely prevented SSB formation (Fig. 2b).

We also tested the effect of 4 mM- Mn^{2+} , which has been reported to activate the (Ca^{2+} + Mg^{2+})-dependent endodeoxyribonuclease (Nakamura *et al.*, 1981) and also the Mg^{2+} -dependent endodeoxyribonuclease (Wang & Furth, 1977). The frequencies of SSB in cells incubated with Mn^{2+} were always greater (2–8-fold in five expts.) than those in cells incubated with Mg^{2+} or with Mg^{2+} and

Ca^{2+} (Fig. 2a). Neither Ca^{2+} nor Mg^{2+} potentiated the effect of Mn^{2+} .

The omission of all nucleoside triphosphates except ATP from the DNA-synthesis mixture increased the frequency of SSB by 40–50 % (Fig. 2c). A decrease in the ATP concentration from 1.5 to 0.15 mM and the omission of phosphoenolpyruvate from the DNA-synthesis mixture increased the frequency of SSB by 30–50 % (Fig. 2c). When all nucleoside triphosphates and phosphoenolpyruvate were omitted, the frequency of SSB was about 2.5-fold greater (2.2–3.0-fold greater, four expts.) than when cells were incubated in the DNA synthesis mixture (Fig. 2c).

The results with cells treated with saponin, which permeabilizes the cytoplasmic membrane but not the intracellular membranes (Smollen & Stoehr, 1985; Kaufmann & Briley, 1987), were similar to those with L- α -lysophosphatidylcholine-treated cells. The frequency of SSB after 15 min of incubation in buffer K with 1 mM- Ca^{2+} and 4 mM- Mg^{2+} was similar to that in lysophosphatidylcholine-permeabilized cells (Fig. 3); SSB were decreased by over 90 % when Mg^{2+} and Ca^{2+} were omitted; incubations with Mg^{2+} alone resulted in the same frequency of SSB as incubations with both Mg^{2+} and Ca^{2+} ; more SSB formed in the presence of 4 mM- Mn^{2+} than in the presence of 4 mM- Mg^{2+} , and the addition of EDTA decreased the formation of SSB.

The results with permeabilized cells suggested that SSB may also form in parental DNA of isolated nuclei when these are incubated in a DNA-synthesis mixture to measure replicative and repair DNA synthesis (Matsumoto *et al.*, 1988). As shown in Fig. 4, SSB accumulated in DNA of nuclei incubated in a DNA-synthesis mixture

Fig. 3. SSB formation in saponin-permeabilized cells

Alkaline-elution patterns are shown of cells permeabilized with saponin and incubated at 37 °C in buffer K for 0 (▽) and 15 min (▼), in buffer K with 1 mM- Ca^{2+} and 4 mM- Mg^{2+} for 0 (△) and 15 min (▲), of intact cells (○), and of cells permeabilized with $L\text{-}\alpha$ -lysophosphatidylcholine and incubated for 15 min in buffer K with Ca^{2+} and Mg^{2+} (●).

Fig. 4. SSB formation in nuclei

Alkaline-elution patterns are shown of nuclei incubated in a DNA-synthesis mixture for 15 min at 4° (▲), with Mg^{2+} omitted at 4 °C (△), with Mg^{2+} omitted at 37 °C (○), with Mg^{2+} present at 37 °C (●) and of cells permeabilized with $L\text{-}\alpha$ -lysophosphatidylcholine and incubated at 37 °C in the same DNA-synthesis mixture with Mg^{2+} omitted (□).

containing 4 mM- Mg^{2+} and 1 mM-EDTA. Omission of Mg^{2+} decreased SSB to the frequency found in nuclei incubated at 0 °C, which was higher than the frequency in permeabilized cells incubated without Mg^{2+} .

DISCUSSION

The main finding of the present study is that the progressive fragmentation of parental DNA in permeabilized cells incubated in a DNA-synthesis mixture was due to the presence of Ca^{2+} and Mg^{2+} (Fig. 1). The fragmentation was prevented by the omission of Ca^{2+} and Mg^{2+} or by the addition of EDTA. The method of cell permeabilization (with $L\text{-}\alpha$ -lysophosphatidylcholine or with saponin) did not influence the fragmentation (Fig. 3); other methods of cell permeabilization most likely have a similar outcome. Kaufmann & Briley (1987) had noted substantial levels of 'non-specific' endodeoxyribonucleolytic activity in human fibroblasts permeabilized by hypo-osmotic shock or by saponin and incubated in a DNA-synthesis mixture. The results of the present study suggest that the fragmentation was due to the influence of bivalent cations into the nucleus and the consequent activation of endodeoxyribonucleases. The fragmentation of parental DNA of isolated nuclei (Fig. 4) is consistent with this hypothesis. These findings are not in conflict with the data of Vanderbilt *et al.* (1982), who described a stimulation and then an inhibition of endogenous nuclease activity, as the concentrations of Ca^{2+} and Mg^{2+} in the incubation buffer were increased from 0 to 40 mM and from 0 to 80 mM respectively (see Fig. 4 of Vanderbilt *et al.*, 1982). The inhibition at high ionic strengths resulted from the precipitation of chromatin.

The requirements of Ca^{2+} and Mg^{2+} for maximal rates of DNA synthesis in permeabilized cells and in nuclei (Miller *et al.*, 1978; Castellot *et al.*, 1978; Kaufmann & Briley, 1987) appear to be incompatible with the maintenance of the integrity of the DNA template. However, the results of the present study suggest that the maximal rates of DNA synthesis may be due in part to DNA damage by the activated ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent endonucleases and to the consequently increased rates of DNA repair. A challenge for future studies with permeabilized cells and isolated nuclei should be to devise conditions that both preserve the DNA template and permit DNA synthesis. The findings of the present study suggest that the integrity of DNA template be ascertained when the conditions for measuring DNA synthesis are devised.

The present work has no direct bearing on the controversy as to whether 'bona fide' DNA is synthesized by the permeabilized cells (Spyrou & Reichard, 1983; Pawlak *et al.*, 1986; Reddy *et al.*, 1986). However, it should be noted that template DNA in cells that were incubated in a DNA-synthesis mixture was far less fragmented than in cells incubated in buffer (Fig. 2c). The fragmentation-sparing effects of nucleoside triphosphates and of an ATP-generating system were additive (Fig. 2c). These findings suggest that DNA repair synthesis was taking place during the incubations. DNA fragmentation most likely sets limits to lengths of DNA replicated. Thus it may account for the observed declines of DNA synthesis with the time of incubation (Miller *et al.*, 1978; Matsu-moto *et al.*, 1988).

Alkaline-elution patterns of DNA of permeabilized

cells and of nuclei incubated in the DNA-synthesis mixture were similar to those of X-ray-irradiated cells (Kohn *et al.*, 1981), indicating the production of random SSB. This pattern of DNA fragmentation is characteristic of the action of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent endodeoxyribonuclease, which acts on DNA *in situ* (Hewish & Burgoyne, 1973; Strätling & Klingholz, 1981) and generates a continuum of fragment sizes (Strätling & Klingholz, 1981). Although the fragmentation occurred without added Ca^{2+} (Fig. 2a), the intranuclear concentrations of Ca^{2+} were presumably sufficient to activate the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent endodeoxyribonuclease upon the addition of Mg^{2+} . The possibility remains that other endodeoxyribonucleases, such as the Mg^{2+} -dependent endodeoxyribonucleases, were activated as well. However, the Mg^{2+} -dependent endodeoxyribonucleases do not attack native DNA *in situ* (Hewish & Burgoyne, 1973).

Activation of the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent endodeoxyribonuclease has been implicated in physiological cell death during development and aging and in various forms of cytotoxic cell death (Wyllie, 1987). One postulated mechanism of this activation is an increase in the intranuclear concentrations of Ca^{2+} (Wyllie, 1987). The results of the present study suggest that the intranuclear concentration of Mg^{2+} may be at least as important as that of Ca^{2+} .

We thank Karen Berchtold for preparing the typescript.

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Received 5 December 1988; accepted 5 January 1989