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Mechanism of Dibucaine-Induced Apoptosis in Promyelocytic Leukemia Cells (HL-60)

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ABSTRACT. Dibucaine, a local anesthetic, inhibited the growth of promyelocytic leukemia cells (HL-60) without inducing arrest of the cell cycle and differentiation to granulocytes. Typical DNA fragmentation and DNA ladder formation were induced in a concentration- and time-dependent manner. The half-maximal concentration of dibucaine required to induce apoptosis was 100 μM. These effects were prevented completely by the pan-caspase inhibitor z-Val-Ala-Asp-(OMe)-fluoromethylketone (z-VAD-fmk), thereby implicating the cysteine aspartase (caspase) cascade in the process. Dibucaine activated various caspases, such as caspase-3, -6, -8, and -9 (-like) activities, but not caspase-1 (-like) activity, and induced mitochondrial membrane depolarization and the release of cytochrome *c* (Cyt.c) from mitochondria into the cytosol. Processing of pro-caspase-3, -8, and -9 by dibucaine was confirmed by western blot analysis. Bid, a death agonist member of the Bcl-2 family, was processed by caspases following exposure of cells to dibucaine. However, 100 μM dibucaine scarcely inhibited oxidative phosphorylation, but it induced membrane permeability transition in isolated rat liver mitochondria. Taken together, these data suggest that dibucaine induced apoptosis of HL-60 cells through activation of the caspase cascade in conjunction with Cyt.c release induced by a processed product of Bid and depolarization of the mitochondrial membrane potential. BIOCHEM PHARMACOL **60**;7:905–915, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; Bid; caspase activation; cytochrome *c*; dibucaine; membrane permeability transition

Apoptosis is an active form of cell death observed under a variety of physiological and pathological conditions [1]. The mechanism of apoptosis is now partially understood on the basis of cell biology, and several lines of evidence indicate that cell death occurs by cross-linking of the Fas receptor [2] and the release of Cyt.c** through the formation of the Apaf complex [3, 4]. These reactions result in the activation of caspase-3 and are supposed to cleave directly the so-called death substrates, such as the lamins, fodorin, poly(ADP-ribose) polymerase, and the inhibitor of caspase-activated DNase [5, 6].

Anesthetics have been shown to interact with phospholipids in biological membranes and to affect the biological functions of neuronal as well as non-neuronal cells [7, 8]. It is also known that dibucaine is incorporated into the inner layer of cell membranes and affects the activities of various enzymes located in cellular membranes, such as PKC and phospholipase A_2 [9–11].

Various drugs and agonists induce apoptosis through signal transduction. Thus, apoptosis might be affected by anesthetics through membrane modulation. Recently, it has been reported that dibucaine induces apoptosis of cultured neuroblastoma cells [12]. However, the molecular mechanism of apoptosis induced by local anesthetics has not been well analyzed. Thus, it would be interesting to know whether dibucaine has any effects on apoptotic induction of non-neuronal cells. To investigate the molecular mechanism of dibucaine-induced apoptosis, we examined the effects of dibucaine on differentiation, DNA fragmentation, DNA ladder formation, activation of various caspases, MPT, and the release of Cyt.c from mitochondria. In this paper, we report that dibucaine-induced cell death caused DNA laddering and fragmentation, and activated caspases, which appear to be associated with the release of Cyt.c.

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** *Abbreviations*: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; Ac-YVAD-MCA, acetyl-Tyr-Val-Ala-Asp-MCA; Ac-VEID-MCA, acetyl-Val-Glu-Ile-Asp-MCA; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-MCA; Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-MCA; Apaf, apoptotic protease-activating factor; caspase, cysteine aspartase; CsA, cyclosporin A; Cyt.c, cytochrome *c*; MCA, 4-methyl-coumaryl-7-amide; MPT, membrane permeability transition; NSE, nonspecific esterase; PKC, protein kinase C; and zVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone.

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MATERIALS AND METHODS

Chemicals

RNase A and proteinase K were obtained from the Sigma Chemical Co. Ac-DEVD-MCA as a substrate for caspase-3/7, Ac-YVAD-MCA for caspase-1/4, Ac-VEID-MCA for caspase-6, Ac-IETD-MCA for caspase-8, Ac-LEHD-MCA for caspase-9, and *z*-VAD-fmk were obtained from the Peptide Institute. Dibucaine, tetracaine, lidocaine, and procaine were obtained from Sigma. 3,3′-Dipropylthiadicarbocyanine iodide [diS-C3-(5)] was obtained from the Kankoshikiso Research Institute. Anti-Cyt.c antibody was obtained from PharMingen. Anti-caspase-3, -8, and -9 and anti-Bid antibodies were obtained from Santa Cruz Biotechnology. All other chemicals were of analytical grade and were obtained from Nacalai Tesque.

Cell Line

The HL-60 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were grown in a humidified incubator at 37° under a 5% CO_2/95% air atmosphere and used for assays during the exponential phase of growth. Cell counts were performed routinely to maintain a low-density population and assayed for viability by the trypan blue exclusion method. HL-60 cells (2.25×10^5 cells) generally were treated in 1.5 mL of RPMI medium containing 10% fetal bovine serum and various reagents and then were incubated in an O_2/CO_2 culture incubator (BNP-110, Tabai Espec Corp.). Before adding the reagents, preincubation was normally performed for at least 1 hr [13].

Isolation of Mitochondria

Rat liver mitochondria were isolated by the method of Hogeboom [14] using sucrose density gradient centrifugation, as described in a previous paper [15].

Subcellular Fractionation of HL-60 Cells

After harvesting, HL-60 cells ($\sim 10^7$) were resuspended in 50 μL of ice-cold buffer A [250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride] and homogenized with a Teflon homogenizer. The homogenate was centrifuged at 750 *g* for 10 min at 4°. Then the supernatant was centrifuged at 10,000 *g* for 15 min at 4°. The resulting pellet was resuspended in buffer A and represented the mitochondrial fraction (M). The supernatant was centrifuged further at 100,000 *g* for 60 min at 4°. The final supernatant represented the cytosolic fraction (S). Aliquots (20 μg) were used for western blot analysis of Cyt.c.

Treatment with Dibucaine and Evaluation of Cell Differentiation

The HL-60 cells were treated with various local anesthetics including dibucaine for 4, 8, 12, and 24 hr at 37°. After incubation, the cells were centrifuged onto slides by the Cytospin System (Kubota, Inc.). The differentiation of cells into granulocytes was evaluated by staining for NSE activity as described in a previous paper [13].

Assay for Mitochondrial Swelling, Membrane Potential, and Oxidative Phosphorylation

For the measurement of mitochondrial swelling, mitochondria (0.1 mg protein/mL) were incubated in a medium consisting of 10 mM Tris–HCl buffer (pH 7.4) and 0.15 M KCl at 25°. Absorbance changes at 540 nm were recorded by a dual-beam spectrophotometer (Shimadzu UV-3000) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer [15]. The membrane potential of mitochondria was measured in a medium consisting of 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.4), 0.1 μg/mL of diS-C3- (5), and mitochondria (30 μg protein/mL); the fluorescence intensity at 670 nm was recorded in a fluorescence spectrophotometer (Hitachi 650-10LC) with excitation at 622 nm [16]. Oxidative phosphorylation of mitochondria was assayed polarographically in a medium consisting of 0.25 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 2 mM phosphate buffer (pH 7.4), and 5 mM succinate as substrate at 25° [16].

Assay for Mitochondrial Membrane Potential in HL-60 Cells

HL-60 cells (1.5×10^5 cells/mL) were incubated with medium alone or with 50 μM dibucaine for various periods, and the mitochondrial membrane potential in the cells was measured by the method of Salvioli *et al.* [17]. The cells were washed with PBS, stained with 2 ng/mL of JC-1 for 15 min at room temperature in PBS, and subjected to fluorescence-activated cell sorting in a FACSCalibur flow cytometer (Becton Dickinson).

Discrimination of Apoptotic Cells by Flow Cytometry and Sorting

The numbers of cells in each phase of the cell cycle and in apoptosis were determined by use of a FACScan flow cytometer after staining the DNA with propidium iodide as described in a previous paper [18]. Cells (2×10^6) treated with dibucaine were fixed in 200 μL of 70% ethanol at 4° for 4 hr, and then incubated in 40 μL of phosphate–citrate buffer (0.2 M Na_2HPO_4 and 0.1 M citrate) at room temperature for 30 min. Following treatment with 0.1 mg/mL of RNase A in 100 μL of PBS at 37° for 30 min, the cells were stained with 50 μg/mL of propidium iodide in 1 mL of PBS at 37° for 30 min in the dark. Analytic flow

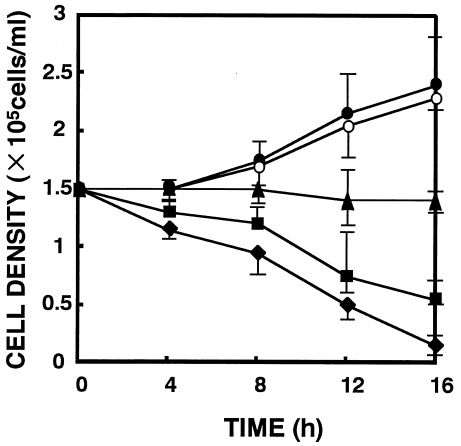

FIG. 1. Effect of dibucaine on cell growth of HL-60 cells. The cells were treated with 0 (●), 50 (○), 100 (▲), 150 (■), and 200 μM (♦) dibucaine under the experimental conditions described in Materials and Methods. At the indicated times, the viable cell numbers were determined by trypan blue dye exclusion. Data are the means ± SD from three independent experiments.

cytometric measurements were performed using a FACScan flow cytometer with argon laser excitation at 488 nm (Becton Dickinson), and fluorescence (FL2; DNA) was detected through a 564–606 nm band-pass filter. Twenty thousand cells were analyzed in each sample.

Analysis of DNA Fragmentation

The extent of DNA fragmentation was determined spectrophotometrically by a method using diphenylamine [13]. After incubation of HL-60 cells with different concentrations of dibucaine, cells were lysed in 150 μL of lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100] on ice for 20 min. The lysate was centrifuged at 13,000 *g* at 4° for 20 min to separate intact and fragmented chromatin. Both the pellet and the supernatant were precipitated at 4° for over 30 min with 6% perchloric acid. The precipitates were sedimented at 13,000 *g* for 20 min at 4°. The DNA precipitates were heated at 70° for 20 min in 50 μL of 6% perchloric acid, and were mixed with 100 μL of diphenylamine solution [1.5% (w/v) diphenylamine, 1.5% sulfuric acid, and 0.01% acetaldehyde in glacial acetic acid]. After overnight incubation at 30° in the dark, both O.D. values were measured at 600 nm, and the percentage of DNA fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA.

The laddering pattern of the DNA fragmentation was detected by agarose gel electrophoresis. Briefly, the lysate was treated with 400 μg/mL of RNase A at 37° for 1 hr and 400 μg/mL of proteinase K at 37° for 1 hr. The DNA was precipitated with an equal volume of isopropanol, and then was electrophoresed at 100 V through a 2% agarose gel containing 0.1 μg/mL of ethidium bromide in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). DNA bands were visualized under ultraviolet illumination and photographed on Polaroid type 667 (3000 ASA) film 38.

Western Blot Analysis

Cell lysates were prepared as described elsewhere [19]. Cell lysates (~10^7) were dissolved in SDS–sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue] and boiled at 100° for 5 min. Then the samples were subjected to SDS–PAGE. After transfer of proteins in the gel to an Immobilon filter (Millipore Co.), the filter was incubated with primary antibody (1:1000 dilution for Cyt.c and caspase-3, 1:100 dilution for caspase-8 and -9, 1:500 dilution for Bid) and then with horseradish peroxidase-linked secondary antibody (1:2000 dilution for Cyt.c, 1:5000 dilution for caspase-3, -6, and -9, 1:50,000 dilution for Bid) and analyzed by using an ECLplus kit (Amersham Co.).

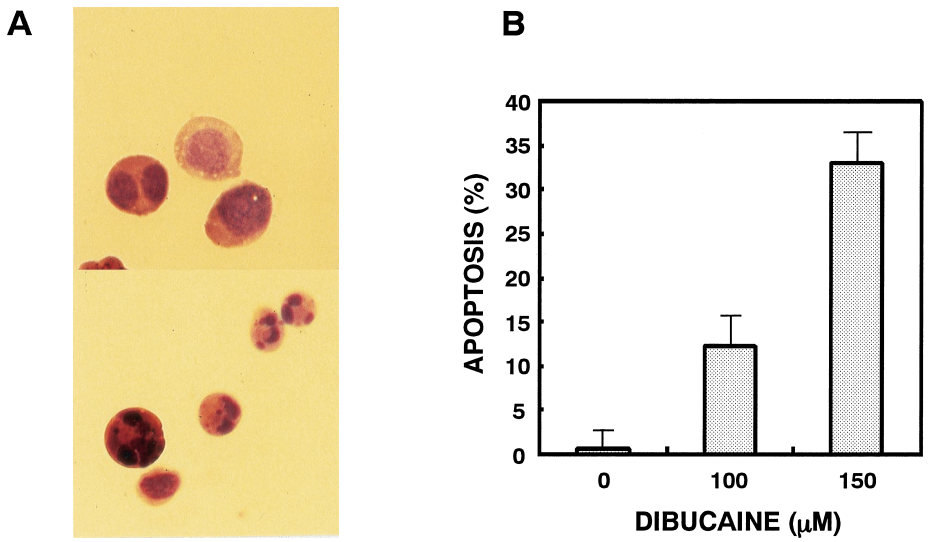

FIG. 2. Effect of dibucaine on morphological change and differentiation of HL-60 cells. (A) Cells were either treated with 100 μM dibucaine (bottom) or untreated (top) for 8 hr. The cells were fixed and stained for NSE activity. Similar results were obtained in three separate experiments. (B) Cells were treated with 0, 100, or 150 μM dibucaine for 8 hr. The percentage of cell death was determined by light microscopy of hematoxylin-stained cytospin preparations. Cells were scored as apoptotic when the cytoplasm was shrunken and the chromatin was condensed. Data are the means ± SD from three independent experiments.

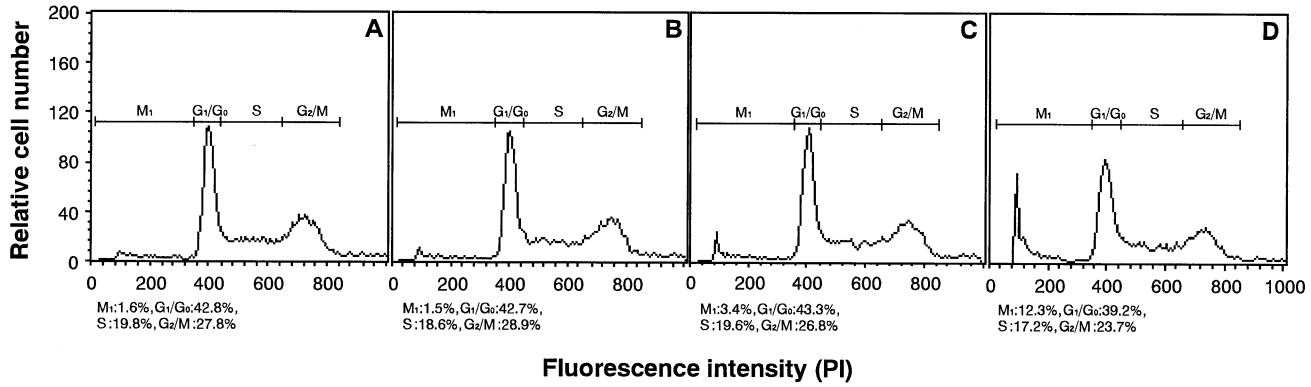

FIG. 3. DNA content frequency histograms of HL-60 cells: untreated (A) and following treatment with dibucaine (B–D). Cells treated with dibucaine were fixed in 200 μL of 70% ethanol at 4° for over 4 hr, suspended in phosphate–citrate buffer, and maintained at room temperature for 30 min prior to propidium iodide (PI) staining. Measurement by flow cytometry took place at 12 (B), 24 (C), and 48 (D) hr in the presence of 20 μM dibucaine. Similar results were obtained in three separate experiments.

Protein concentrations were determined by the method of Lowry *et al.* [20], using bovine serum albumin as a standard.

Assay for Caspase-Like Activity

Caspase-like activities were determined as described previously [13, 19, 21] using 10 μM concentrations of various caspase substrates, such as Ac-YVAD-MCA for caspase-1 (-like) activity, Ac-DEVD-MCA for caspase-3 (-like) activity, Ac-VEID-MCA for caspase-6 (-like) activity, Ac-IETD-MCA for caspase-8 (-like) activity, and Ac-LEHD-MCA for caspase-9 (-like) activity, and 20 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl and 5 mM DTT at 37° for 1 hr. The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured by a fluorospectrophotometer. The wavelengths for excitation and emission were 355 and 460 nm, respectively.

RESULTS

Effect of Dibucaine on Cell Growth and Morphological Change of HL-60 Cells

The doubling time of HL-60 cells was about 24 hr. Growth of HL-60 cells was inhibited by dibucaine in a concentration-dependent manner (Fig. 1). No cell growth was observed at a concentration of 100 μM dibucaine. More than 150 μM dibucaine decreased the number of cells during the time of cultivation. Apoptotic bodies were observed at 100 μM dibucaine after incubation for 8 hr, and the number of apoptotic cells increased as the concentration of dibucaine increased (Fig. 2). However, necrotic cell death was also observed at a concentration of more than 200 μM dibucaine. The growth-inhibited HL-60 cells were not differentiated into granulocytes as indicated by staining for NSE activity, a specific marker enzyme for granulocytes (Fig. 2A).

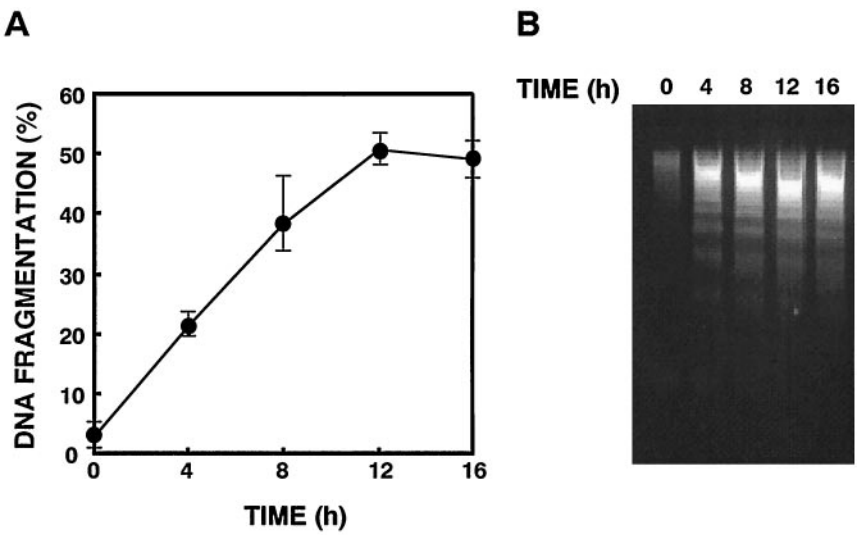

FIG. 4. Effect of dibucaine on DNA status in HL-60 cells. (A) Time course of changes in the percentage of DNA fragmented. The cells (2.25×10^5) were treated with 150 μM dibucaine for the indicated times. The percentage of fragmented DNA was determined by the diphenylamine method. Data are means ± SD from three independent experiments. (B) Time course of DNA ladder formation. The cells (2.25×10^5) were treated with 150 μM dibucaine, and DNA was extracted at the indicated times. DNA was subjected to 2% agarose gel electrophoresis. Similar patterns of gel electrophoresis were obtained in three separate experiments.

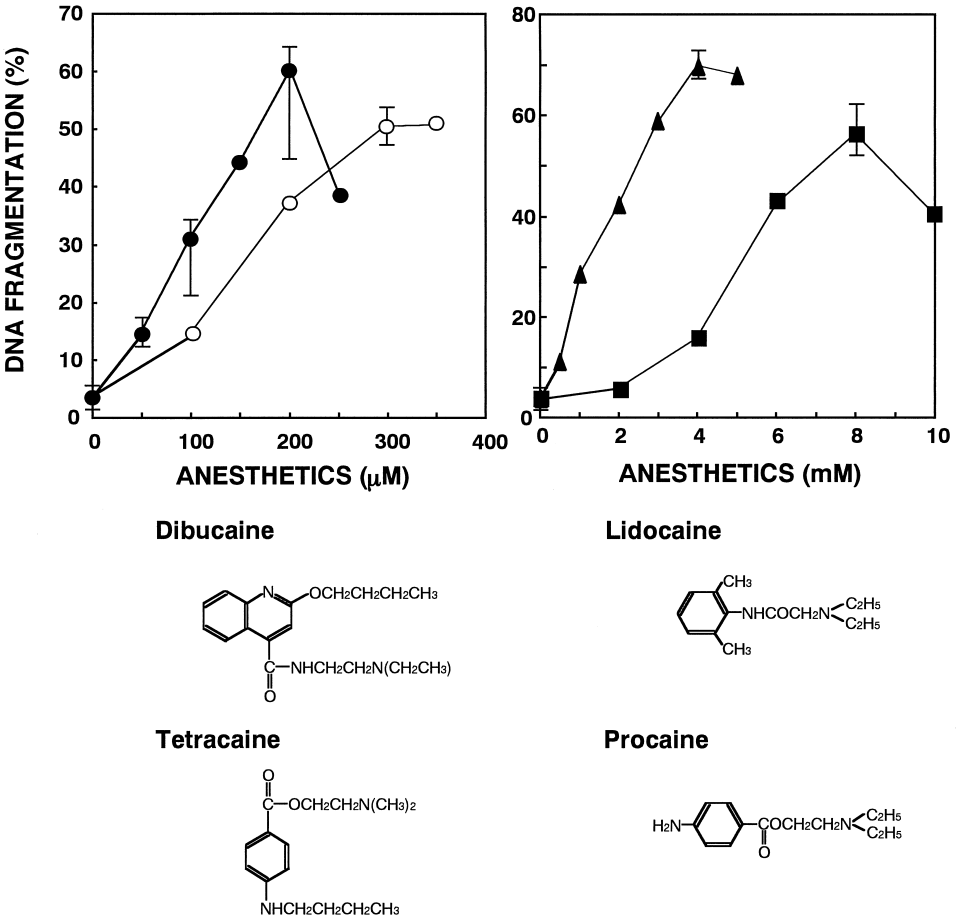

FIG. 5. Structures of various local anesthetics and their effects on DNA fragmentation of HL-60 cells. Experimental conditions were the same as indicated for Fig. 4A. Cells were incubated with various concentrations of local anesthetics [dibucaine (-●-), tetracaine (-○-), lidocaine (-▲-), and procaine (-■-)] for 24 hr. Data are the means ± SD from three independent experiments.

Relationship Between Cell Cycle and DNA Fragmentation after Dibucaine Treatment

Cells were quantitated by flow cytometry at 12, 24, and 48 hr after treatment with 20 μM dibucaine to confirm the state of apoptosis and to see if there was any arrest of the growth of the treated cells. This low concentration (20 μM) of dibucaine was used to assess the extent of the arrest of cell growth after long periods of treatment. Numbers of apoptotic cells were increased 24 and 48 hr after treatment with 20 μM dibucaine without the arrest of growth in any phase of the cell cycle (Fig. 3).

Effect of Anesthetics on DNA Fragmentation and DNA Laddering of HL-60 Cells

In agreement with the results obtained by Kim *et al.* [12] using a neuronal cell line, our experiments showed that apoptosis of HL-60 cells was induced by incubation with 150 μM dibucaine. DNA fragmentation and DNA ladder formation were induced in the cells in a time- and concentration-dependent manner (Figs. 4 and 5). These apoptosis-inducing activities of the anesthetics were in the following order of magnitude: dibucaine > tetracaine > lidocaine > procaine (Fig. 5). The concentrations required for half-maximal DNA fragmentation by dibucaine, tetracaine, lidocaine, and procaine were 100 μM, 150 μM, 1.5 mM, and 5 mM, respectively.

Effect of z-VAD-fmk on Dibucaine-Induced DNA Fragmentation of HL-60 Cells

Since the caspase proteases are important for the execution of apoptosis, the effect of a cell-permeable inhibitor of caspases, z-VAD-fmk [22], was tested on dibucaine-induced DNA fragmentation in HL-60 cells. Effective suppression of dibucaine-induced DNA fragmentation was observed in a concentration-dependent manner following pretreatment of cells with z-VAD-fmk (Fig. 6). These results imply that activation of caspases has an important role in dibucaine-induced apoptosis.

Effect of Dibucaine on Various Caspase-Like Protease Activities in HL-60 Cells

Since DNA fragmentation induced by dibucaine was inhibited by z-VAD-fmk, the effect of dibucaine on the various caspase (-like) activities in HL-60 cells was examined to determine which caspases are involved in apoptosis. Caspase-3 and -6 (-like) but not -1 (-like) proteases were activated in a time-dependent manner by treatment with

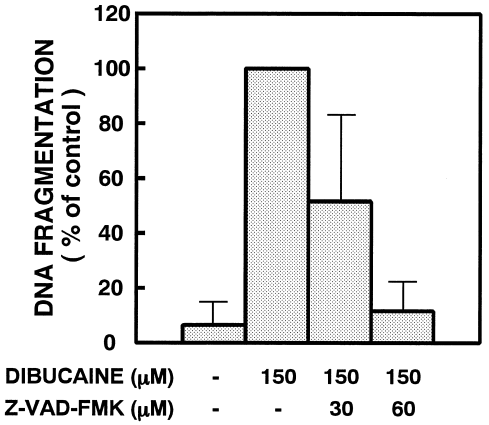

FIG. 6. Effect of a pan-caspase inhibitor on the dibucaine-induced DNA fragmentation of HL-60 cells. Experimental conditions were the same as described for Fig. 4A. The cells were pretreated for 1 hr with or without 30–60 μM *z*-VAD-fmk, followed by treatment with or without 150 μM dibucaine. After 8 hr, DNA fragmentation was determined by the diphenylamine method. Data are expressed as percent of control, and are the means ± SD from three independent experiments.

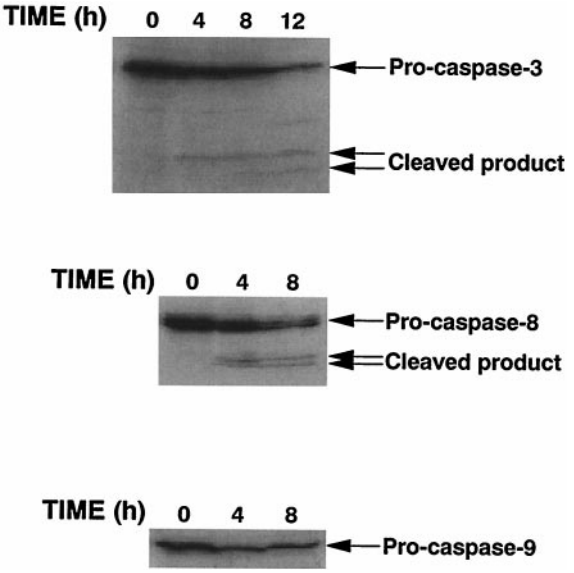

FIG. 8. Time-dependent cleavage of pro-caspase-3, -8, and -9 during dibucaine-induced apoptosis of HL-60 cells. The cells were treated with 150 μM dibucaine for the indicated periods. Total cell lysates (50 μg) were analyzed by immunoblotting. Similar results were obtained in three separate experiments.

150 μM dibucaine (Fig. 7A). Although the activities of caspase-8 and -9 (-like) proteases were lower than that of caspase-3 (-like) protease, they clearly were activated by dibucaine (Fig. 7B). It is known that caspase-3 and -6 function at late steps of the protease cascade and that activation of caspase-9 and -8 is linked with apoptosis through mitochondrial function and a receptor-mediated reaction, respectively [3, 4, 23, 24].

Cleavage of Pro-caspases in HL-60 Cells by Dibucaine

Since there is considerable redundancy in substrate utilization among caspases, in this experiment western blot analysis of pro-caspase and cleaved products was carried out to obtain direct evidence for the involvement of caspases in the process of dibucaine-induced apoptosis [25]. Caspase-3, -8, and -9 were cleaved after treatment of the cells with dibucaine (Fig. 8). These results suggest that the activation of caspase-3 might be coupled with Cyt.c release from mitochondria.

Release of Cyt.c from Mitochondria by Dibucaine

The release of Cyt.c from the mitochondrial intermembrane space appears to play an important role in the induction of apoptosis [3, 4] and occurs via MPT-induced dysfunction of mitochondria [26–28]. To demonstrate the involvement of mitochondrial MPT in the process of dibucaine-induced apoptosis, Cyt.c release from mitochondria into the cytosol during treatment with 150 μM dibucaine was analyzed by western blot analysis using anti-Cyt.c antibody. Both a significant increase in the amount of Cyt.c in the cytosol and a significant decrease in the mitochondria were detected after treatment with dibu-

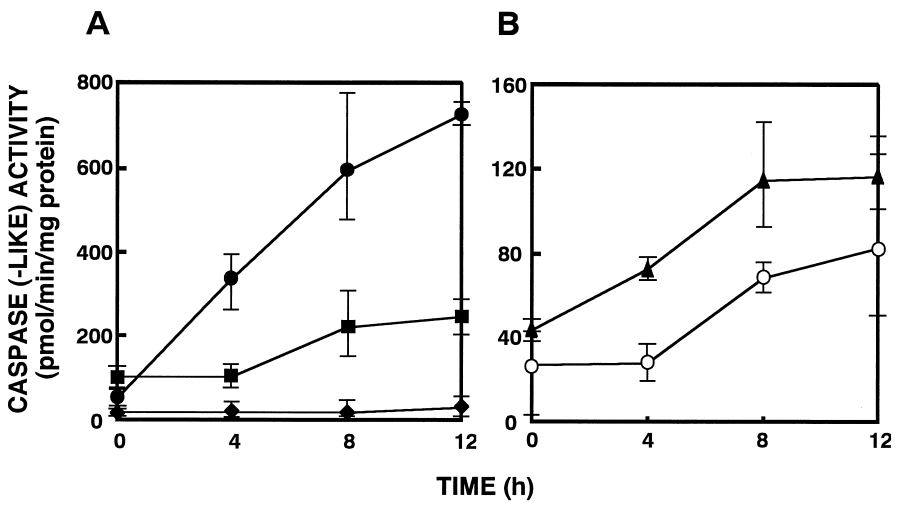

FIG. 7. Time course of changes in activities of caspase (-like) enzymes during dibucaine-induced apoptosis of HL-60 cells. The cells were treated with 150 μM dibucaine for the indicated periods. The cytosol of the cells (50 μL) was incubated with 10 μM fluorogenic peptide substrates: YVAD for caspase-1 (-like) activity (-◆-), DEVD for caspase-3 (-like) activity (-●-), VEID for caspase-6 (-like) activity (-■-) (A), IETD for caspase-8 (-like) (-▲-) activity, and LEHD for caspase-9 (-like) activity (-○-) (B). Data are the means ± SD from three independent experiments.

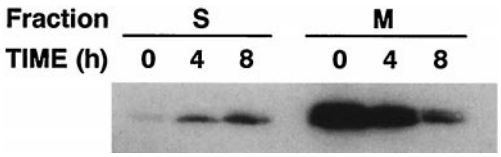

FIG. 9. Dibucaine-induced release of Cyt.c from mitochondria in HL-60 cells. The cells were treated with 150 μM dibucaine for the periods indicated, and Cyt.c in cytosolic (S) and mitochondrial (M) fractions was detected by western blotting with an anti-Cyt.c antibody. Similar results were obtained in three separate experiments.

caine at 37° for 4 and 8 hr (Fig. 9). The result indicates that the Apaf complex might be formed in the cytosol of HL-60 cells by treatment with dibucaine and contribute to the activation of caspase-3.

Depolarization of Mitochondrial Membrane in HL-60 Cells by Dibucaine

The MPT is a permeability increase of the inner mitochondrial membrane coupled with depolarization of the membrane and is most easily observed after matrix Ca^{2+} accumulation [26–28]. Thus, the effect of dibucaine on the mitochondrial membrane potential of HL-60 cells was examined by means of a FACS-Calibur flow cytometer using JC-1, a reliable fluorescent probe to assay mitochondrial membrane potential in intact cells. The membrane potential was depolarized in a manner that was dependent upon the time of incubation with 50 μM dibucaine (Fig. 10). These results suggest that dibucaine induced MPT of mitochondria and the release of Cyt.c.

Activation of Bid in HL-60 Cells by Dibucaine and Its Inhibition by z-VAD-fmk

Recently, it was found that Bid, a death agonist member of the Bcl-2 family, when cleaved by caspase-8 brought about the release of Cyt.c without inducing MPT [23, 24]. Therefore, we investigated whether or not Bid cleavage was implicated in Cyt.c release during the treatment of the cells with dibucaine. Bid was detected as a 22-kDa pro-form by immunoblot analysis and was cleaved to a 15-kDa fragment after treatment with dibucaine (Fig. 11A). The cleavage of the pre-form of Bid was prevented by z-VAD-fmk (Fig. 11B). The result indicates that Bid might be responsible for the release of Cyt.c during the dibucaine-induced apoptosis of HL-60 cells.

Effect of Dibucaine on the Swelling, Membrane Potential, and Oxidative Phosphorylation of Mitochondria

It has long been known that when mitochondria are overloaded with Ca^{2+}, it leads to mitochondrial swelling coupled with the opening of the MPT pore, which is sensitive to CsA. It also results in the release of Cyt.c [26–28]. It has been reported that anesthetics interact with phospholipids in biological membranes and affect their biological functions [7, 8] and some investigators have shown uncoupling of oxidative phosphorylation by dibucaine [29, 30], but another group showed no uncoupling by dibucaine [31]. Thus, the effects of dibucaine on oxidative phosphorylation, large-amplitude swelling, and Cyt.c release from isolated mitochondria were examined. Oxidative phosphorylation and the respiratory control ratio were decreased slightly by 100 μM dibucaine but were inhibited by high concentrations, such as 0.5 to 1 mM (Fig. 12). In agreement with this result, mitochondria were barely swollen by 100 μM dibucaine, but were swollen by 0.5 to 1 mM dibucaine in a CsA-insensitive manner (Fig. 13A). Under the same conditions, mitochondria were swollen by 200 μM Ca^{2+} in the presence of inorganic phosphate (P_i) in a CsA-sensitive manner (Fig. 13B). Small amounts of Cyt.c were released from mitochondria upon treatment with a high concentration of dibucaine (500 μM), and this release was insensitive to CsA (Fig. 13C). Consistent with these

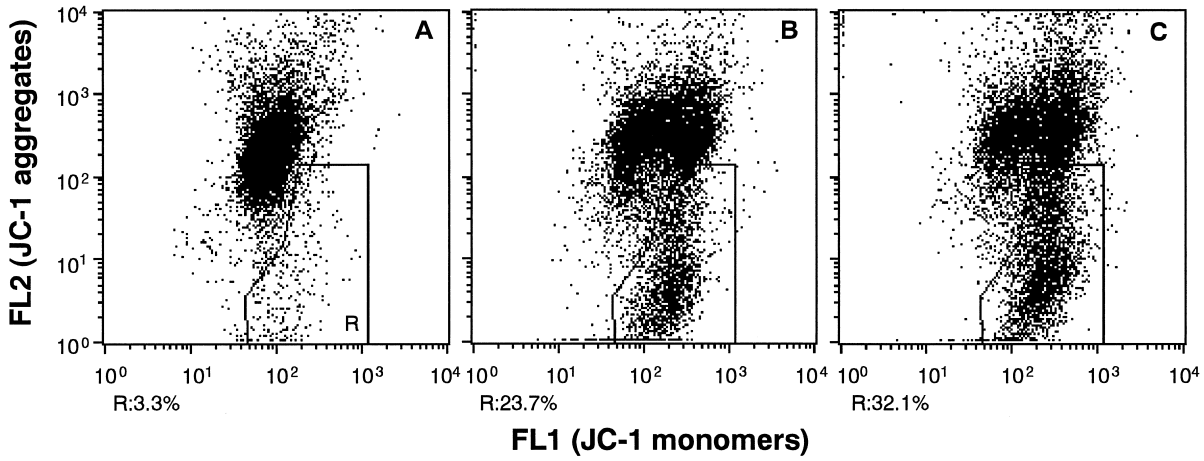

FIG. 10. Effect of dibucaine on mitochondrial membrane potential in HL-60 cells, untreated (A) and treated with dibucaine (B, C). Cells treated with dibucaine were stained with JC-1 in PBS. Measurement by flow cytometry took place at 16 (B) and 24 (C) hr in the presence of 50 μM dibucaine. R = the percentage of depolarized cells. Similar results were obtained in three separate experiments.

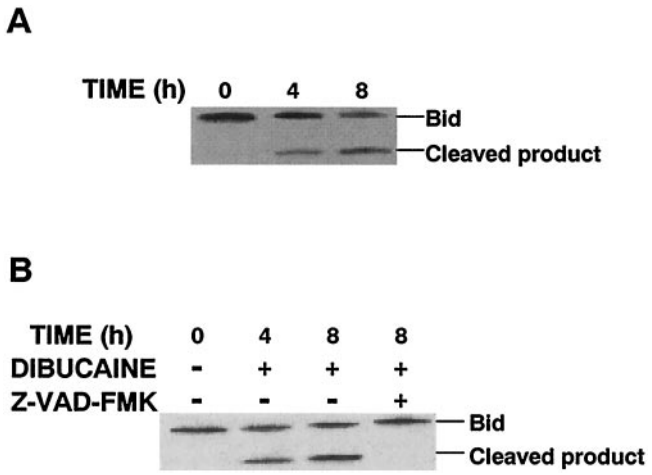

FIG. 11. Processing of Bid during incubation of HL-60 cells with dibucaine and its inhibition by z-VAD-fmk. (A) The cells were incubated with 150 μM dibucaine for the indicated period. Total cell lysates (25 μg) were analyzed by immunoblotting. (B) Cells were preincubated with z-VAD-fmk for 1 hr before adding dibucaine. Similar results were obtained in three separate experiments.

results, the membrane potential of isolated mitochondria was not depolarized by 100–500 μM dibucaine in the presence or absence of CsA (Fig. 14). These results indicate that a high concentration (1 mM) but not a low concentration (100 μM) of dibucaine may affect mitochondria directly.

DISCUSSION

The present experiments show that various local anesthetics induced apoptosis in HL-60 cells in a concentration- and time-dependent manner. The apoptosis-inducing activity of dibucaine was the strongest among these anesthetics. We also showed that the pan-caspase inhibitor z-VAD-fmk completely blocked dibucaine-induced DNA fragmentation and that dibucaine activated various caspase-like proteases. These results indicate that the typical death protease cascade mediates dibucaine-induced apoptosis of HL-60 cells. Accumulated evidence from recent research indicates that mitochondria-derived factors, such as Cyt.c, have an important role in the apoptosis of some cells [3, 4, 26–28, 32]. Cyt.c was released from mitochondria of HL-60 cells during the incubation with dibucaine. Accumulating evidence indicates that MPT coupled with depolarization of the membrane potential induces the release of Cyt.c [26–28]. In this experiment, we observed the depolarization of the mitochondrial membrane potential in HL-60 cells by treatment with dibucaine. However, no depolarization of membrane potential of isolated mitochondria by the same concentration of dibucaine was observed. These results indicate that a part of the Cyt.c might be released from mitochondria in the cells through MPT by the indirect action of dibucaine. In this context, it has been reported that a high concentration of dibucaine uncouples oxidative phosphorylation [29, 30]. We confirmed that high concentrations of dibucaine inhibited oxidative phosphorylation of isolated mitochondria and found that large-amplitude swelling was induced by high concentrations of dibucaine in a CsA-insensitive manner. In this case, however, the degree of swelling was very small and the release of Cyt.c very limited at a concentration of dibucaine sufficient to induce apoptosis. These observations also suggest that dibucaine may not interact directly with mitochondria in HL-60 cells to induce apoptosis. Recently, it has been shown that Cyt.c release occurs as a consequence of the processing of Bid by caspase-8 without opening of the MPT pore [23, 24, 26–28]. In the present experiments, we observed increased caspase-8 activity and the presence of an inactive pre-form of Bid in HL-60 cells. Dibucaine induced the cleavage of Bid and generated an active product of Bid, and this process was prevented completely by z-VAD-fmk. These results indicate that most of the Cyt.c release observed might be induced by the processed form of Bid through the activation of caspase-8.

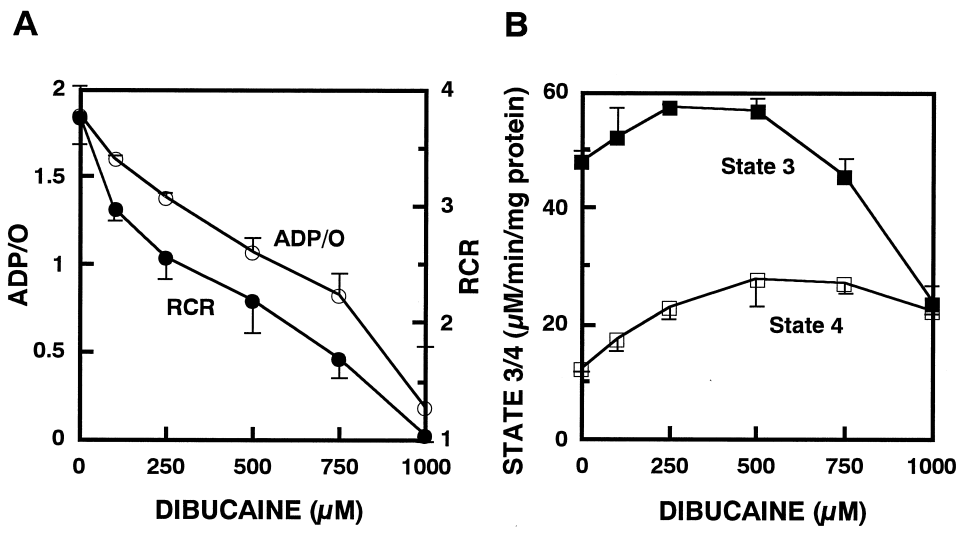

FIG. 12. Effect of dibucaine on oxidative phosphorylation of isolated rat liver mitochondria. Mitochondria (0.5 mg protein/mL) were incubated in medium comprising 0.25 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, and 2 mM phosphate buffer (pH 7.4) at 25°. The concentrations of succinate and ADP were 5 mM and 200 μM, respectively. Key: RCR, respiratory control ratio; state 3 and state 4, respiration in the presence or absence of ADP. Data are the means ± SD from three independent experiments.

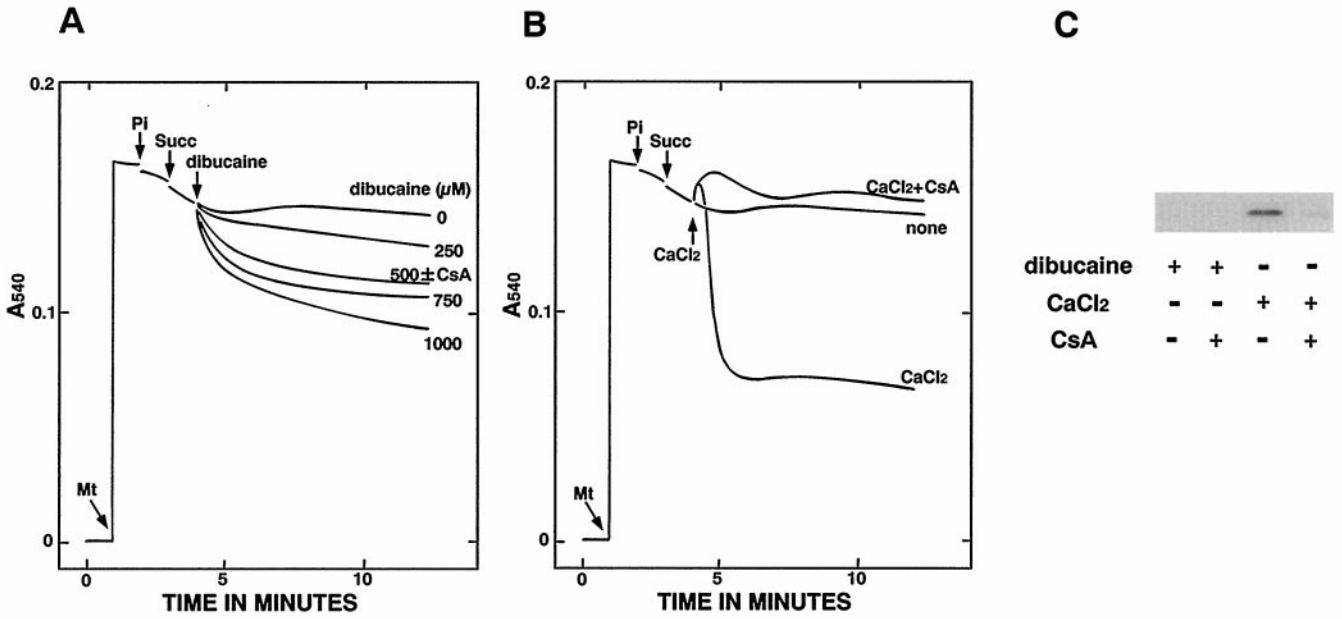

FIG. 13. Effect of dibucaine on MPT and the release of Cyt.c from isolated rat liver mitochondria. (A) Mitochondria (100 μg protein/mL) were incubated in a medium comprising 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.4) at 25°. Additions are indicated in the figure. Mitochondrial swelling was induced by 250, 500, 750, or 1000 μM dibucaine after the addition of 2 mM P_i and 5 mM succinate in the presence or absence of 1 μM CsA. (B) Effect of Ca^{2+} plus P_i on mitochondrial swelling. Incubation conditions were the same as for (A) except for the presence of 50 μM Ca^{2+}. (C) Cyt.c released from mitochondria was detected by western blotting using anti-Cyt.c antibody after treatment for 10 min at 25° with 500 μM dibucaine or 50 μM Ca^{2+} after the addition of 2 mM P_i and 5 mM succinate in the presence or absence of 1 μM CsA. Similar results were obtained in three separate experiments.

Recent studies have revealed that Cyt.c is released from brain mitochondria by an MPT-independent mechanism [33] and that caspase-8 was activated by a Fas-receptor-independent mechanism [34]. Furthermore, caspase-6 and -7 have been shown to trigger the release of Cyt.c without processing Bid [35]. Thus, the initiation of caspase activation and the mechanisms by which caspase-8 and -6 are activated by dibucaine also remain to be studied.

A number of reports have presented evidence that supports a role for PKC in the apoptotic process [11, 36]. Various kinds of local anesthetics inhibit PKC through lipid–protein interaction [37, 38]. With regard to a possible mechanism of apoptosis involving protein kinases, it was found recently that BAD (an Egl-1/Bad family protein) kinase phosphorylates Ser 112 and 136 of BAD and binds to the 14–3-3 protein in the cytoplasm [39]. The dephosphorylated BAD binds to the mitochondria and induces the release of Cyt.c. Thus, it is also possible that the inhibitory activity of dibucaine on PKC might be involved in Cyt.c release.

Many papers have reported the involvement of reactive oxygen species in the apoptosis of different cells induced by different ligands. Kim *et al.* [12] suggested that membrane damage and oxygen free radicals may be involved in the dibucaine-induced apoptosis of a neuroblastoma cell line. Thus, another possible course of apoptosis is the production

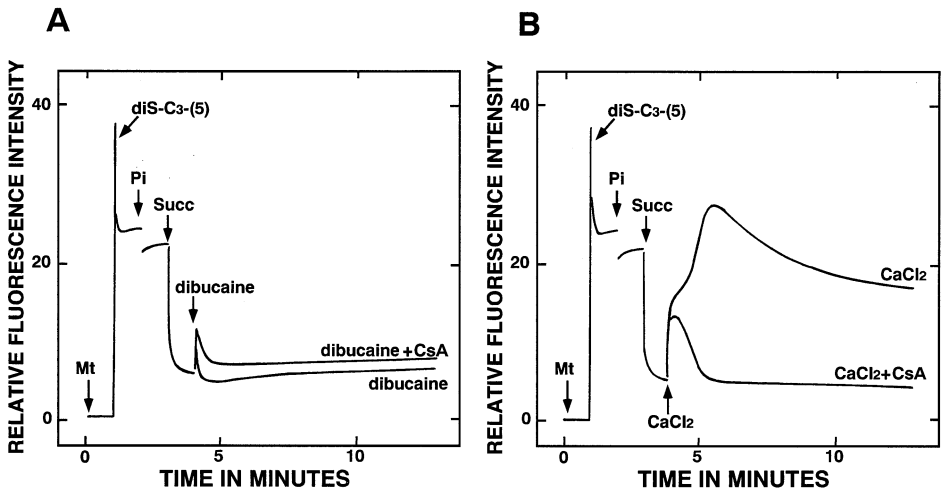

FIG. 14. Effect of Ca^{2+} and dibucaine on membrane potential of isolated rat liver mitochondria. Mitochondria (30 μg protein/mL) were incubated in 0.15 M KCl containing 10 mM Tris–HCl buffer (pH 7.4), 0.1 μg/mL of diS-C3- [5] in the presence or absence of 1 μM CsA. Then 2 mM P_i, 5 mM succinate, and 500 μM dibucaine (A) or 50 μM $CaCl_2$ (B) were added to the mixture. The mitochondrial membrane potential was assessed by fluorescence of diS-C3- [5]. Downward deflection indicates polarization of membrane potential. Similar results were obtained in three separate experiments.

of oxygen free radicals via oxidative pathways. Furthermore, Kim *et al.* showed that the level of intracellular calcium ions ($[Ca^{2+}]_i$) is increased by dibucaine. These results indicate that the increased level of $[Ca^{2+}]_i$ might be another cause of the release of Cyt.c from mitochondria.

Further studies are underway to establish whether dibucaine and other local anesthetics can induce apoptosis in other cell lines and in cells isolated from a tumor biopsy, and to elucidate the molecular mechanism of dibucaine-induced apoptosis.

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