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Apoptosis Induced by Calcein Acetoxymethyl Ester in the Human Histiocytic Lymphoma Cell Line U-937 GTB

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ABSTRACT. Effects of calcein acetoxymethyl ester (calcein/AM) on macromolecular synthesis, mitochondrial membrane potential, and mode of death were studied in U-937 GTB lymphoma cells. This was accomplished by measurements of ^{14}C-labeled thymidine and leucine incorporation, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and caspase-3 activity measurements, TdT-mediated dUTP nick end labeling (TUNEL) staining, morphology, and a newly developed assay of apoptosis detection, the microculture kinetic assay (MiCK). This assay, based on absorbance measurements of cells, has been reported to reflect morphological changes in apoptosis. At 2.5 μg/mL, rapid inhibition of DNA and protein synthesis resembling that of the known inhibitors, aphidicholin and cycloheximide, was observed. Decreased mitochondrial membrane potential was evident after 1 hr of exposure and was followed by an increase in caspase-3 activity, while at 6 hr 30% of cells appeared positive with TUNEL staining. After 12 hr of exposure, viability was less than 5% as judged by morphological examination. In the MiCK assay, calcein (2.5 μg/mL) gave a rapid rise in absorbance after 3.5 hr of exposure with a peak at 5 hr, indicating maximum extent of apoptosis at that time. This was similar to the pattern generated for etoposide and doxorubicin. The results indicate that calcein, similar to cytotoxic drugs, induces a strong apoptotic response within hours of exposure. BIOCHEM PHARMACOL **60**;12:1751–1759, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. calcein/AM; calcein; apoptosis; U-937 GTB; MiCK assay; Cytostar-T® plate

We and others have previously reported on the feasibility of calcein/AM as a probe for detection of drug efflux related to Pgp§ and the MRP [1–3], both of which belong to the ATP-binding cassette (ABC) superfamily of transmembrane transporters. Calcein/AM, the acetoxymethyl ester of calcein, is commercially available from Molecular Probes Inc. Besides its application as an MDR-detecting probe, calcein/AM is also used as a viability probe in various cytotoxicity assays [4]. It is non-fluorescent and lipophilic enough to diffuse over the cytoplasmic membrane, and once inside it, is rapidly hydrolyzed by intracellular esterases into fluorescent calcein. Since calcein is hydrophilic and only retained in cells with intact cell membranes, it thus discriminates living cells from dead. However, previous studies have also revealed that calcein/AM itself can induce cytotoxicity at low concentration [5], already detectable after only a few hours of incubation, without immediate loss of membrane integrity. In the histiocytic lymphoma cell line U-937 GTB and the myeloma cell line RPMI 8226/S, almost complete cell death was evident after 12–24 hr exposure of calcein/AM at a concentration of 2.5 μg/mL (approximately 2.5 μM). The cytotoxic activity of calcein/AM was also investigated in fresh tumor cell samples of patients with both hematological and solid malignancies. Calcein/AM at 2.5 μg/mL produced a cell kill greater than 50% in 94% of the hematological samples and in 63% of the solid tumor samples [6], making it an interesting candidate for further evaluation as a new potential anticancer agent.

In the present study, we characterized the morphological and temporal aspects of calcein/AM-induced cell death. This is of importance in the further evaluation of its potential as an anticancer agent and its use in cellular research. We also indicate applications of two newly described methods, the Cytostar-T® plate and the MiCK assay. In the following article, we refer to the ester and free acid form as calcein/AM.

MATERIALS AND METHODS

Cell Line and Maintenance

The histiocytic lymphoma cell line U-937 GTB, kindly provided by Prof. K. Nilsson, Dept. of Genetics and Pathology, Uppsala, Sweden, was grown under standard cell

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§ *Abbreviations:* CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; calcein/AM, calcein acetoxymethyl ester; ara-C, cytarabine; cisp, cisplatinum; DFF, DNA fragmentation factor; DOX, doxorubicin; VP-16, etoposide; FMCA, fluorometric microculture cytotoxicity assay; MGG, May–Grunewald–Giemsa; MiCK, microculture kinetic assay; $\Delta\Psi_m$, mitochondrial membrane potential; MRP, multidrug resistance-associated protein; taxol, paclitaxel; Pgp, P-glycoprotein 170; topo, topotecan; VCR, vincristine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide; and TUNEL, TdT-mediated dUTP nick end labeling.

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culture conditions in RPMI-1640 medium (Sigma), supplemented with glutamine (Sigma), penicillin (Sigma), streptomycin (Sigma), and 10% (v/v) heat-inactivated fetal bovine serum (Sigma), in 75-mL flasks (Nunc). Growth and morphology were checked twice a week. Cell concentrations used are specified in each experiment.

Drugs and Reagents

Calcein/AM and JC-1 were purchased from Molecular Probes Inc. and dissolved in DMSO (calcein/AM) or methanol (JC-1) to 1 mg/mL and stored in aliquots in −70° until further use. VP-16, VCR, DOX, taxol, topo, ara-C, and cisp were purchased from the local hospital pharmacy and dissolved according to instructions from the manufacturers. Aphidicolin (Sigma) dissolved in sterile water served as positive control for inhibition of DNA synthesis, whereas cycloheximide (Sigma) dissolved in PBS served as a positive control for inhibition of protein synthesis. CCCP (Sigma), an ionophore and uncoupler of oxidative phosphorylation that served as a positive control for disruption of $\Delta\Psi_m$, was dissolved in DMSO. Fresh preparations were made for each experiment. All other chemicals used were of analytical grade.

Measurement of DNA and Protein Synthesis

Protein and DNA synthesis were measured in a Cytostar-T® plate, (*in situ* mRNA Cytostar-T® assay kit, Amersham International plc.), a pre-made 96-well scintillating microtiter plate with scintillation fluid molded into the bottom of the plate. Cells in direct contact with the bottom of the wells are close enough to the scintillation fluid to generate a detectable signal [7, 8]. Cells were suspended in fresh medium containing either 111 nCi/mL of [^{14}C]thymidine (Amersham CFA.532, 56 mCi/mmol, 50 μCi) or 222 nCi/mL of [^{14}C]leucine (Amersham CFB.183, >300 mCi/mmol, 50 μCi), yielding a final radioactivity in the wells of approximately 20 and 40 nCi, respectively. Cell suspension (180 μL) containing 50,000 cells was added to each well. Blank wells received medium containing only isotope. Radioactivity was measured with a computer-controlled Wallac 1450 MicroBeta® trilux liquid scintillation counter (Wallac OY.) using MicroBeta® Windows Workstation software (Wallac). The plate was read in the MicroBeta® counter immediately after addition of the cell suspension and at different time points up to 72 hr. Between measurements, the plate was stored in a regular cell-cultivating incubator. During measurement, the plate was covered with a plate sealer to inhibit microbiological contamination.

Drugs (test wells) and PBS (blank and untreated control wells) were added in duplicate (20 μL/well) 2 hr after cell seeding when the measured radioactivity in cell-containing wells was at least double compared to baseline. The concentration used for standard cytotoxic drugs was that giving less than 10% viability after 72 hr of continuous exposure, using the FMCA [9, 10].

Measurement of Mitochondrial Membrane Potential

Changes in $\Delta\Psi_m$ were assessed using JC-1, a lipophilic cation that accumulates in mitochondria. JC-1 shows green fluorescence at low concentrations [11], but inside mitochondria at concentrations above 0.1 μg/mL forms J-aggregates and produces red fluorescence [11].

U-937 GTB cells were diluted in RPMI-1640 medium to a concentration of 500,000 cells/mL in polypropylene test tubes. Calcein/AM at final concentrations of 0.5 and 2.5 μg/mL was then added, while control tubes received only PBS. The samples were incubated in a standard incubator and at 1, 3, 6, and 24 hr, 500,000 cells were collected and dissolved in 1 mL of PBS followed by addition of 10.1 μL (50 μg/mL) of JC-1, yielding a final concentration of 0.5 μg/mL. Incubation for 10 min was followed by centrifugation (200 *g*, 5 min). The cells were resuspended in fresh PBS and 200 μL/well was dispensed into a V-shaped 96-well microtiter plate. The red fluorescence of JC-1 was detected using a spectrofluorometer (Fluoroscan II, Labsystems OY), with excitation and emission wavelengths set to 540 and 585 nm, respectively. At excitation wavelengths 540 nm and above, no interfering fluorescence of calcein was detected.

Measurement of Caspase-3 Activity

Caspase-3 activity was measured using the commercially available ApoAlert® CPP32/caspase-3 colorimetric assay kit (Clontech Labs, Inc.). Caspase-3 is a cysteinyl protease that activates DFF, which in turn activates the nucleases responsible for degradation of the nuclei: caspase-3 is thought to be a key enzyme in apoptosis control [12, 13]. U-937 GTB cells were incubated in RPMI-1640 medium at a density of 250,000 cells/mL in separate 450-mL cell-cultivating bottles (Nunc) without (negative control) or with calcein/AM 2.5 μg/mL or 15 μg/mL VP-16 (positive control). At various time points, 2 million cells were collected in duplicates and dispensed into polypropylene tubes, and the following caspase-3 assay was performed according to the kit manual. Briefly, supernatants from lysed and centrifuged cells were transferred to a flat-bottomed microtiter plate to which reaction buffer and substrate were added. After 60 min of incubation at 37°, absorbance at 405 nm was measured in an absorbance spectrophotometer (SpectraMAX plus, Molecular Devices Inc.).

TUNEL and Morphology

The TUNEL assay, *in situ* cell death detection kit, fluorescein (Boehringer Mannheim GmbH), was used according to kit instructions. The assay is proposed to detect DNA strand breaks, a key biochemical event for apoptosis. Terminal deoxynucleotidyl transferase incorporates fluorescein-labeled dUTP to free 3′-OH termini, and an increase in nuclear fluorescence is observed in cells with fragmented DNA.

U-937 GTB cells at 250,000/mL were incubated in RPMI-1640 medium with PBS, calcein/AM at 2.5 μg/mL, or VP-16 at 15 μg/mL. At 0, 1, 3, 6, 12, and 24 hr, cytospin slides were prepared using a Cytospin® 3 centrifuge (500 rpm, 5 min) (Shandon Scientific Ltd.) and stored at −20° until further use. The slides were either TUNEL- or MGG-stained and prior to analysis of the TUNEL staining, preparations were mounted with Vectashield® antifade mounting solution (Vector Laboratories Inc.) and covered with a coverslip. The preparations were analyzed with a computer-controlled Zeiss LSM 410 inverted laser scan microscope (Carl Zeiss Jena GmbH), using a 40× oil-immersion lens, laser excitation at 488 nm, and an emission bandpass-filter of 515–525 nm. For pictures, an average of 16 scans was employed and attenuation was set to avoid photobleaching. Cells were judged TUNEL-positive, i.e. apoptotic, if nuclei were fragmented and brightly fluorescent, and TUNEL-negative if only dim fluorescence was observed throughout the whole cell, which in fact could only be detected if brightness and contrast on the confocal microscope were increased considerably. Micrographs of conventional morphology (MGG stain) were taken using a Nikon ECLIPSE E400 microscope and a personal computer-based video image analysis system (Bergströms Instrument). Morphological criteria for apoptotic cells were fragmented nuclei but intact cytoplasmic membranes.

MiCK Assay

The MiCK assay was originally described and developed by V. D. Kravtsov [14] and is based on measurements of absorbance. Upon cell death either by apoptosis or necrosis, absorbance changes compared to untreated control cells. The characteristic features of apoptosis, i.e. cell shrinkage, membrane blebbing, and chromatin condensation, give rise to an initial steeper slope than untreated control, whereas the following decrease in absorbance is probably due to postnecrotic degradation of the cells [15]. In control wells, absorbance slowly increases while the cells proliferate, whereas agents that induce rapid necrosis, i.e. ethanol and hydrogen peroxide at high concentrations, give a flat fading curve compared to control [15].

Flat-bottomed 96-well microtiter plates (Nunc) were prepared in advance with 20 μL drug solution, in triplicate wells, at 10 times the desired concentrations, while untreated control wells received only PBS. The plates were then stored in −70° until further use. Cells were washed and resuspended in complete RPMI-1640 medium without phenol red. Cell concentration was empirically derived in separate experiments, as described by Kravtsov [15].

Two computer-controlled absorbance spectrophotometers were employed, both capable of incubation at 37°. For the DIAS (Dynatech Deutschland GmbH), a cell concentration of 555,000 cells/mL was employed and for the SpectraMAX Plus (Molecular Devices Inc.), 1.1 million cells/mL were used. Cell suspension (180 μL) was seeded into each well and to avoid evaporation 50 μL of sterile mineral oil was carefully layered on top of each well. Plates were then incubated in a standard cell incubator for 30 min for equilibration, followed by absorbance measurement every 14 min at 600 nm for 48 or 72 hr. Data from the readings were imported to Microsoft Excel (Microsoft) spreadsheets and processed in a custom-made macro.

RESULTS

DNA Synthesis

To investigate the role of DNA synthesis in the induction of apoptosis, [^{14}C]thymidine incorporation into U-937 GTB cells incubated with calcein was studied using the Cytostar-T® plate. Calcein/AM at 2.5 μg/mL completely inhibited thymidine incorporation after 3 hr, but a slight inhibition could in fact be detected after 1 hr (Fig. 1A). For calcein/AM at 0.5 and 0.1 μg/mL, inhibition was detected from 10 hr. In untreated control cells, thymidine incorporation increased linearly until approximately 40 hr and then leveled off (not shown). Compared to untreated control, ara-C and aphidicholin completely inhibited thymidine incorporation within the first hour of incubation. For VCR, cisp, and taxol, the inhibitory effects on thymidine incorporation were less pronounced (Fig. 1B). VP-16, DOX, and topo showed a similar pattern of inhibition as calcein/AM at 2.5 μg/mL.

Protein Synthesis

Next, we investigated the role of protein synthesis in the induction of apoptosis. [^{14}C]Leucine incorporation was studied in cells incubated with calcein using the Cytostar-T® plate. Leucine incorporation increased almost linearly throughout the 72-hr period, whereas in wells containing cycloheximide at 10 μg/mL, the protein synthesis was abruptly shut off during the first hour of incubation (Fig. 2A). For calcein/AM (2.5 μg/mL), the protein synthesis was shut off after approximately 3 hr, but in wells containing 0.5 and 0.1 μg/mL of calcein/AM, a clear decrease in leucine incorporation was not detected until approximately 20 and 44 hr, respectively. The standard cytotoxic drugs, VP-16, cisp, DOX, VCR, ara-C, topo, and taxol, clearly attenuated leucine incorporation to varying degrees from 10 hr onward (Fig. 2B).

Mitochondrial Membrane Potential

Depolarization of the inner mitochondrial membrane is reported to be an early key event for initiation of the apoptotic cascade and was quantified by measuring the generation of red fluorescence of JC-1 using a microtiter-plate-based spectrophotometer technique. After the first hour of calcein/AM incubation, JC-1 fluorescence decreased to 82% of untreated control (Table 1), followed by a further decline after 3 hr and recovery after 6 hr to approximately 90% of untreated control. After 24 hr, the fluorescence signal dropped to 21% of untreated control. At

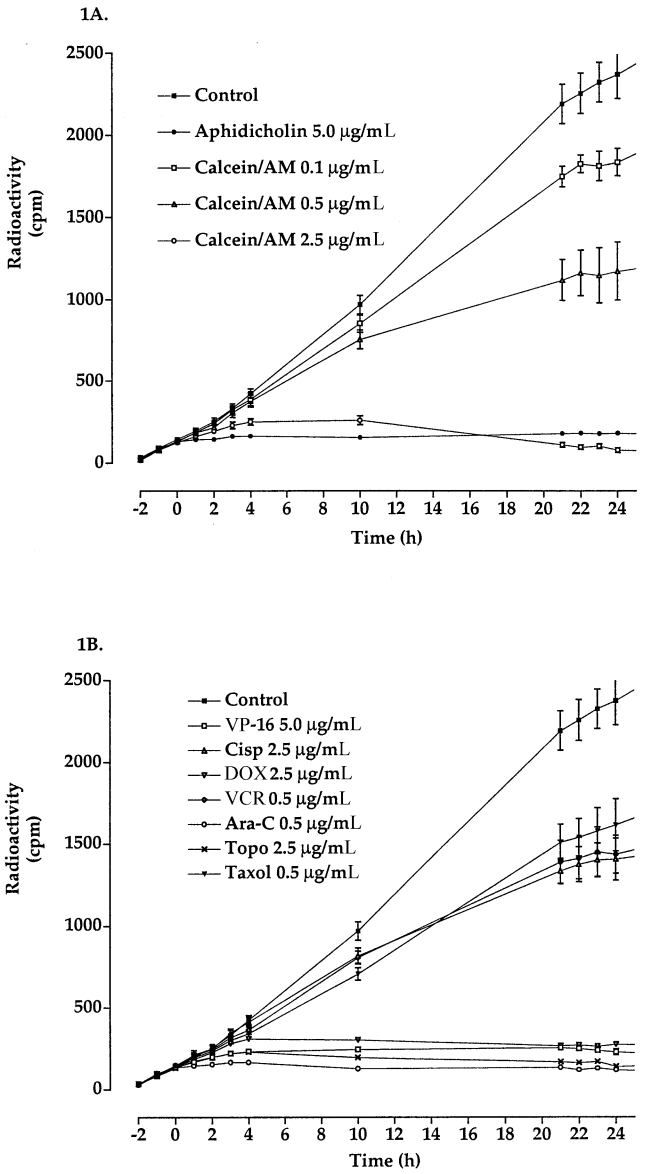

FIG. 1. [^{14}C]Thymidine incorporation assayed in the Cytostar-T® plate. (A) Calcein/AM- and aphidicholin-induced inhibition compared to untreated control. (B) Standard cytotoxic drugs shown as a comparison. The graphs show the first 24 hr and are expressed as means of 4 consecutive experiments ± SEM.

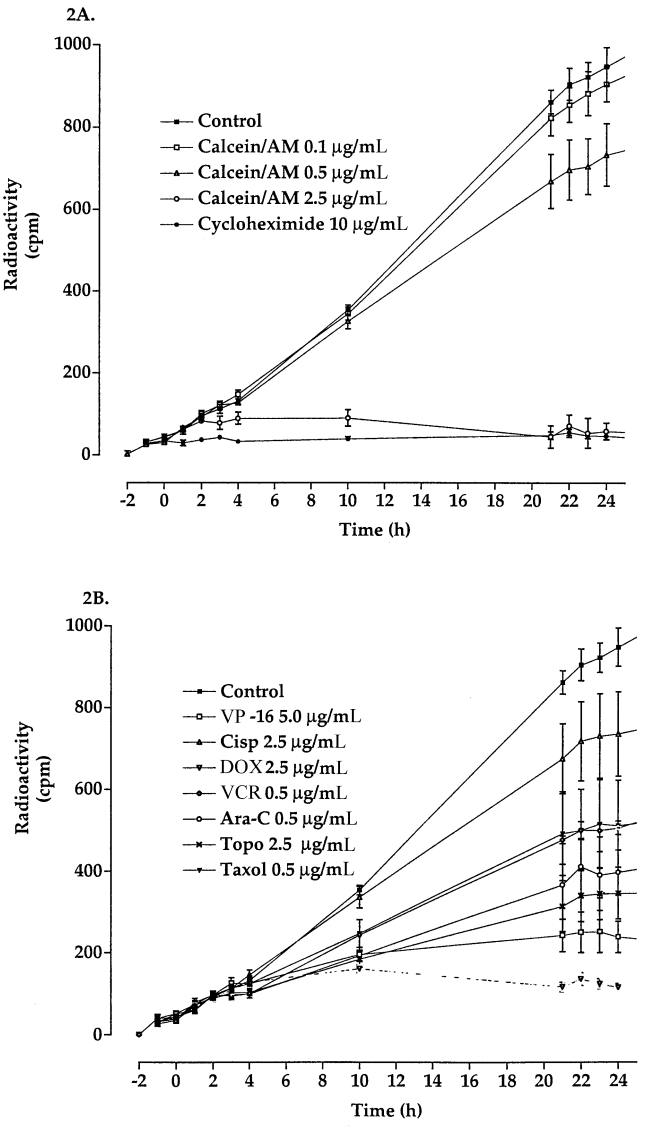

FIG. 2. [^{14}C]Leucine incorporation assayed in the Cytostar-T® plate. (A) Calcein/AM- and cycloheximide-induced inhibition compared to untreated control. Standard cytotoxic drugs shown as a comparison. The graphs show the first 24 hr and are expressed as means of 4 consecutive experiments ± SEM.

this time point, viability was less than 5% as judged by the trypan blue exclusion test. Cells incubated with CCCP (10 μM) also exhibited a decrease in JC-1 fluorescence to approximately 40% of untreated control, one that was noted already after 1 hr and was consistent throughout the whole 24-hr period. Viability after 24 hr of incubation with CCCP was above 95% as judged by trypan blue exclusion, although approximately 10% of the cells were judged to be apoptotic on MGG-stained cytospin slides.

Caspase-3 Activity

Activation of caspase-3 is described to be a secondary event to mitochondrial membrane depolarization and a key enzyme in controlling various nucleases responsible for degradation of the nuclei. Determination of active caspase-3 was performed by an absorption spectrometry technique. After 1 hr, there was no increase in absorbance in calcein/

TABLE 1. Relative mitochondrial membrane potential in U-937 GTB cells treated with calcein/AM

	1 hr	3 hr	6 hr	24 hr
Control	100	100	100	100
Calcein/AM	82.0 (2.0)	74.3 (5.3)	90.3 (2.9)	23.3 (1.2)
CCCP	31.3 (4.9)	29.8 (6.4)	39.3 (4.9)	36.3 (4.5)

Mitochondrial membrane potential measurements after the indicated time points for untreated control, calcein/AM at 2.5 μg/mL, and CCCP at 10 μM serving as positive control. Results are presented as percent of untreated control. Means of 3 experiments (± SEM).

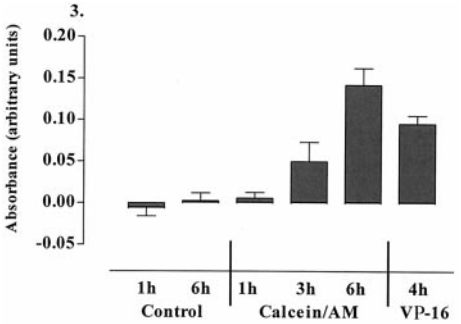

FIG. 3. Caspase-3 activity in U-937 GTB cells incubated with 2.5 μg calcein/AM/mL at indicated time points. VP-16 at 15 μg/mL is included as positive control. Untreated control showed no apparent caspase activity at any tested point of time. Means of 3 consecutive experiments ± SEM.

AM-treated cells, whereas after 3 hr and even more so after 6 hr the absorbance increased considerably (Fig. 3). The absorbance of calcein/AM-treated cells at 6 hr was even higher than that observed after incubation in VP-16 at 15 μg/mL for 4 hr. The absorbance generated from untreated wells serving as negative control did not differ from blank wells, indicating no detectable caspase-3 activity in non-induced cells.

TUNEL and Morphology

In order to compare the results from the biochemical experiments and the MiCK assay with the actual appearance of the cells, conventional morphology and TUNEL staining was performed. Throughout the observation period, between 1 and 3% of spontaneous apoptotic cells were observed in untreated control slides with TUNEL (Fig. 4A) and MGG staining (Fig. 5A). After 6 hr in 2.5 μg/mL of calcein/AM, the percentage of apoptotic cells was 30% (Figs. 4B and 5B). After 12-hr calcein/AM incubation, 20% of the cells showed apoptotic features, while remaining cells appeared postnecrotic, with faint stained nuclei and no detectable cytoplasmic membranes (not shown, see Table 2 Legend). At 24 hr, all cells appeared postnecrotic and pycnotic. VP-16 at 15 μg/mL induced positive staining in 20% and 80% of the cells at 2 and 4 hr, respectively (Figs. 4C and 5C).

MiCK Assay

The morphological changes cells show when undergoing apoptosis were monitored by measurements of absorbance over time using the automated MiCK assay. This was performed to compare the kinetics of the morphological changes and the various time points for the different biochemical assays performed. Calcein/AM at 2.5 μg/mL induced an increase in absorbance at approximately 3.5 hr with a peak after 5 hr (Fig. 6A). The effect of calcein/AM (0.5 μg/mL) was more modest, with only a slight increase being observed. MiCK curves of various standard cytotoxic

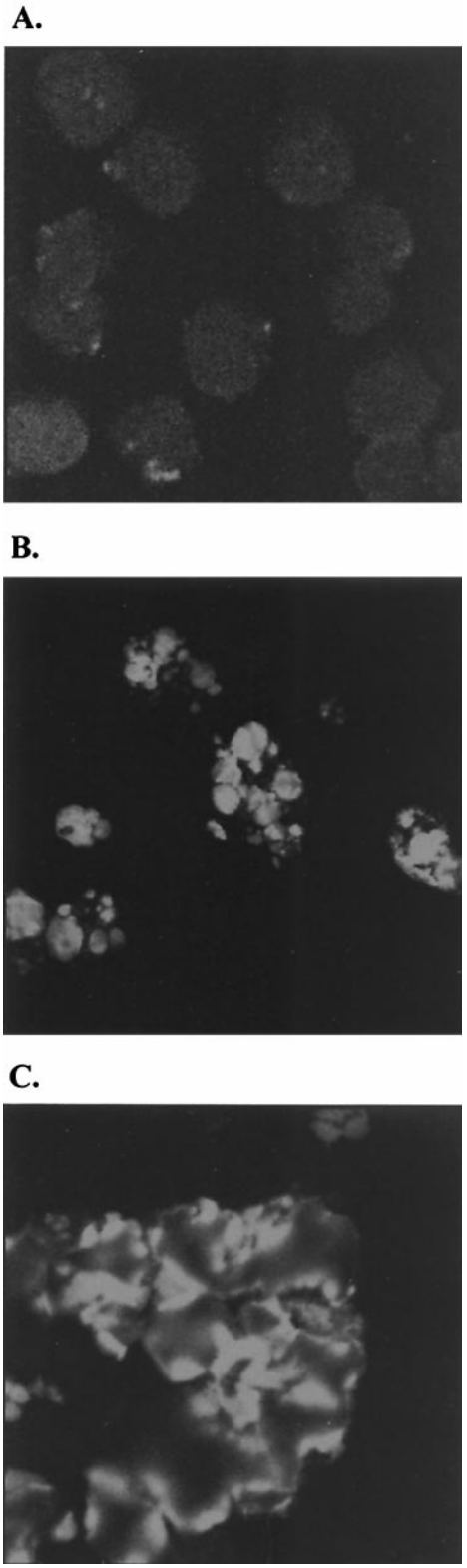

FIG. 4. TUNEL micrographs (40×) of U-937 GTB cells. (A) Untreated control 3 hr. (B) Incubated with calcein/AM at 2.5 μg/mL for 6 hr. (C) Incubated with VP-16 at 15 μg/mL for 4 hr. See Table 2 for percentage of apoptotic cells and the Materials and Methods section for apoptotic criteria. Pictures are from 1 out of 2–4 experiments.

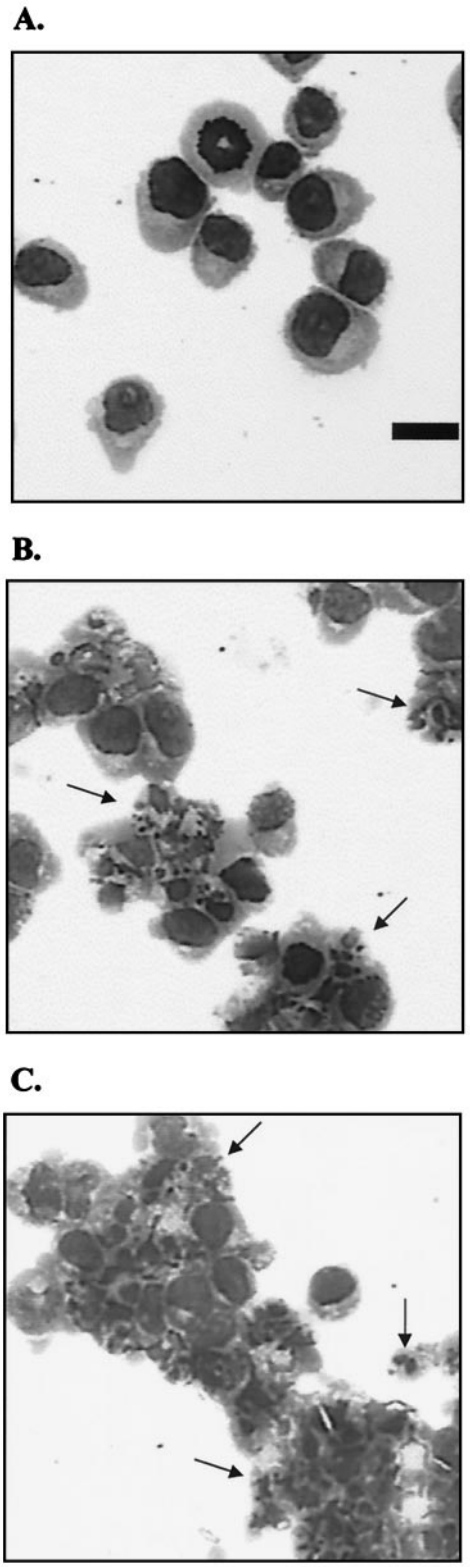

FIG. 5. MGG micrographs (40×) of U-937 GTB cells. (A) Untreated control 2 hr. Bar equals 20 μm. (B) Incubated with calcein/AM at 2.5 μg/mL for 6 hr. Apoptosis is seen in approximately 30% of the cells. (C) Incubated with VP-16 at 15 μg/mL for 4 hr, a majority of the cells show apoptotic features. They are condensed and the nuclei are fragmented, but cytoplasmic membranes are intact. Arrows indicate apoptotic cells. Pictures are from 1 out of 2–4 experiments.

TABLE 2. Percentage of apoptotic U-937 GTB cells after incubation with calcein/AM and VP-16

	1 hr	3 hr	6 hr	12 hr	24 hr
Control TUNEL	1	3	3	3	3
Control MGG	0	3	2	2	4
Calcein/AM TUNEL	1	7	30	20*	0*
Calcein/AM MGG	0	5	26†	25*	3*
		2 hr	4 hr		
VP-16 TUNEL		19	77		
VP-16 MGG		20	85		

Apoptotic cells judged by TUNEL and morphology after the indicated time periods of incubation in PBS (control), calcein/AM (2.5 μg/mL), or VP-16 (15 μg/mL). Results are presented as percent of totally counted cells. Two fields of vision (> 200 cells) were counted at 20× of magnification for each occasion.

*Indicates only brightly stained cells, the majority already having posted necrotic changes, i.e. "cell shadows" with faintly stained nuclei.

†At 6 hr, a majority of the nuclei in the non-apoptotic cells showed an incipient condensation of chromatin compared to the untreated control at the same time. Results are presented as 1 out of 2–4 experiments.

drugs are shown in Fig. 6, B–E. VP-16 (15 and 2.5 μg/mL) induced a rapid increase in absorbance noticed as early as 1.5 hr, whereas VP-16 at 0.5 μg/mL induced only minor changes in absorbance (Fig. 6B). DOX (2.5 μg/mL) gave rise to a steep increase in absorbance with a peak after 4 hr, while DOX at 0.5 μg/mL induced a slower increase (Fig. 6C). Cisp, 12.5 μg/mL, induced an increase in absorbance with a peak after 10 hr, whereas cisp at 2.5 μg/mL only induced a minor increase in absorbance initiated at approximately 16 hr (Fig. 6D). As a comparison, the necrosis-inducing agents Triton X-100 (0.1%) and ethanol (5%) induced no increase in absorbance (Fig. 6E).

DISCUSSION

The biochemistry of programmed cell death and the morphologic features of apoptosis have been reported in many publications in recent years and the agents that cause programmed cell death are numerous [13, 16]. The process of apoptosis has been divided into three major phases: the induction, effector, and degradation phases [13, 17]. During the induction (private) phase, various stimuli disrupt $\Delta\Psi_m$, most likely by different mechanisms of opening the permeability transition pore or the mega channel [18]. The uncoupling of mitochondria shifts the process into the effector phase, where it is believed that the release of cytochrome *c* and/or apoptosis-inducing factor activating caspase-3 [17, 19–21] is responsible for the initiation of the degradation phase. The caspase cascade probably activates other endonucleases to induce DNA fragmentation and karryorhexis in the presence of intact cytoplasmic plasma membrane [12, 17, 22, 23], the most prominent morphologic feature of apoptosis. In the present study, we used various techniques to characterize these phases for the effect of calcein/AM.

The earliest detectable cellular alteration observed in response to calcein/AM was a rapid and potent inhibition

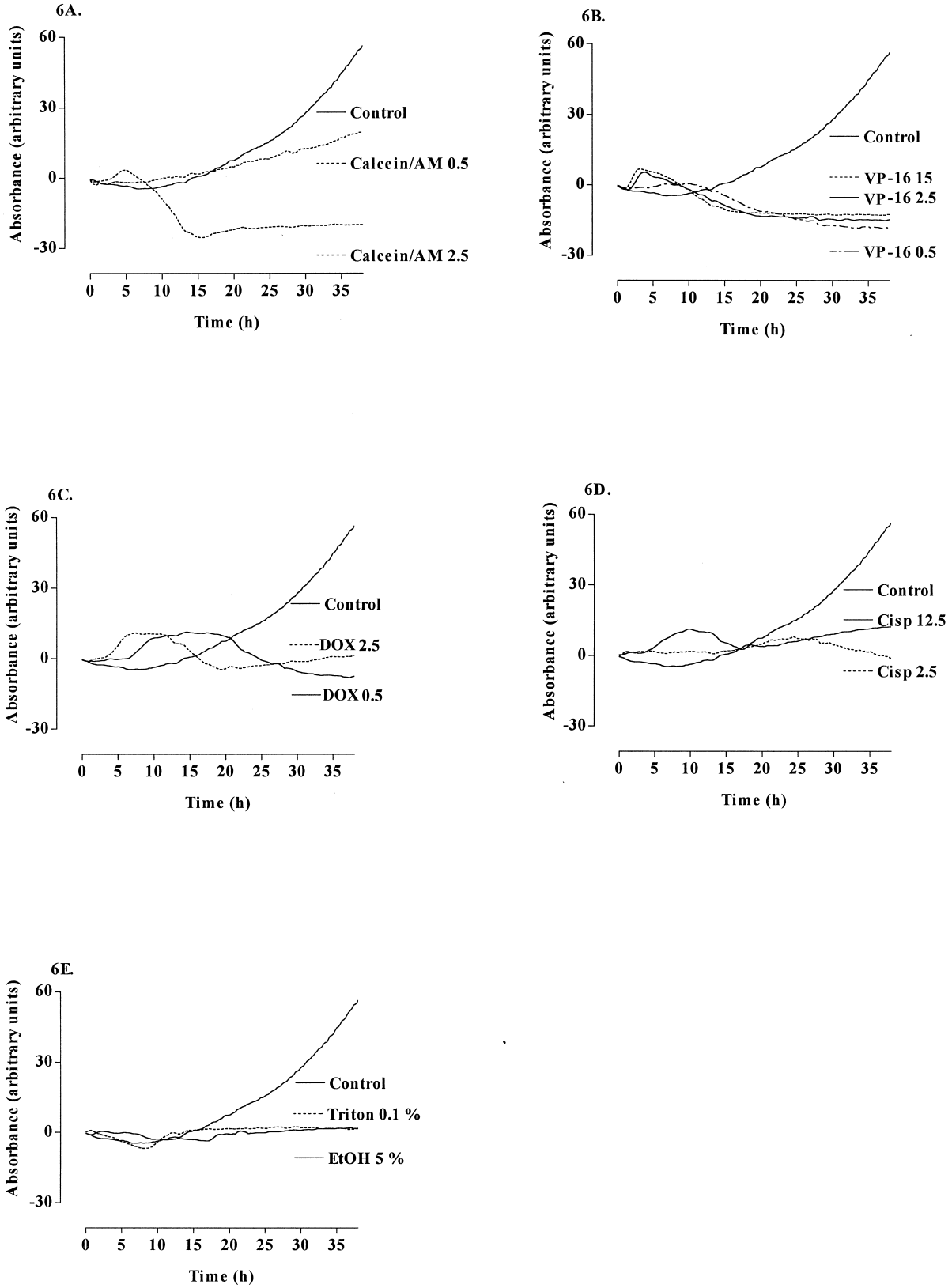

FIG. 6. MiCK assay. (A) Changes in absorbance displayed over time for calcein/AM 2.5 and 0.5 μg/mL compared to untreated control. The former induced an increased extinction at 3.5 hr and a peak after 5 hr, whereas the latter induced only a modest increase in absorbance. (B) VP-16 at 15 and 2.5 μg/mL induced a rapid increase in absorbance. (C) Dox (2.5 and 0.5 μg/mL) induced a curve similar to that of calcein/AM, but a slightly longer duration of the hyperbola was observed. (D) Cisp 12.5 and 2.5 μg/mL, where 12.5 μg/mL induced a rapid increase in absorbance compared to 2.5 μg/mL, which only induced a small increase in absorbance at approximately 16 hr. (E) Triton X-100 (0.1% v/v) and ethanol (5% v/v) as examples of necrosis-inducing agents. One typical experiment of 4 is presented in each panel.

of DNA and partial loss of $\Delta\Psi_m$. Within the first hour, inhibition of thymidine incorporation was initiated, whereas inhibition of leucine incorporation was detected after 3 hr. These data might indicate that cessation of protein synthesis is secondary to the inhibition of DNA synthesis. At 1 to 3 hr of calcein/AM exposure, no morphologic alterations of the cells were evident, but at 6 hr approximately 30% of the cells showed fragmented nuclei with a parallel increased frequency of TUNEL-positive cells. These early morphologic and biochemical changes were observed in the presence of an apparently intact plasma membrane integrity, a hallmark of apoptosis [22]. At 3 hr, there was also a calcein/AM-induced increase in caspase-3 activity well compatible with the notion of caspase-3 initiating and preceding cellular DNA fragmentation in the apoptotic cascade [24].

The MiCK assay was used to monitor the whole process of apoptosis over time and as a marker of early changes related to apoptosis, i.e. initial membrane blebbing and condensation of nucleus and cytoplasm. These morphologic changes are responsible for the increase in absorbance in the MiCK assay when studying apoptotic events [15, 25]. An initial increase above the absorbance of control cultures was observed already after 3.5 hr of calcein/AM exposure, with a peak signal at approximately 5 hr. At 12 hr, viability was low, with the curve showing a decline shortly followed by a plateau, typical of ongoing cell fragmentation and disintegration [15]. The temporal aspects of the calcein/AM-generated MiCK curve are thus well in accordance with the morphologic and biochemical measurements used to characterize the mode of calcein/AM-induced cell death. The MiCK assay appears to be a useful method for initial characterization of the mode of cell death and may guide the selection of suitable time points for additional morphologic and biochemical measurements. Taken together, these results strongly indicate that calcein/AM rapidly induces a classical form of apoptosis in the U-937 GTB cell line involving caspase-3 activation.

Caspase activation has been found to play a central role in the apoptotic response to a wide variety of stimuli [26]. Cytochrome *c* and/or apoptosis-inducing factor released from mitochondria subsequent to their depolarization is believed to be one of the ways whereby the caspase cascade is initiated [12, 19]. To investigate if this chain of events is also involved in response to calcein/AM, the fluorescent probe JC-1 was employed to follow changes in $\Delta\Psi_m$. JC-1 red fluorescence is specifically sensitive to changes in $\Delta\Psi_m$, whereas JC-1 green fluorescence is reflective of mitochondrial mass [27]. Since calcein exhibits a strong fluorescence with excitation and emission spectra of 494 and 517 nm, respectively, measurements of probes emitting light within the green spectra are not meaningful. Already at 1 hr, JC-1 red fluorescence was lowered to approximately 75–85% of control and remained stable at this level for up to 6 hr. These observations indicate at least an early partial depolarization of the mitochondrial membranes which is compatible with a role for mitochondrial triggering of the caspase cascade. However, not being able to measure the mitochondrial mass (JC-1 green fluorescence) could be a problem where proliferation of the mitochondria or a rapid swelling of the matrix occurs [22, 28, 29]. A larger decrease in $\Delta\Psi_m$ could therefore be masked by an increase in volume/mass either by proliferation or swelling. Since changes were noted within the first hour for both calcein/AM- and CCCP-treated cells, proliferation of the mitochondria is unlikely, but swelling of the matrix remains a distinct possibility and would lead to an overestimation of the actual $\Delta\Psi_m$. In this context, it is also interesting to note previously reported intracellular heterogeneity of $\Delta\Psi_m$, not only among different mitochondria but also at the level of one single mitochondrion [27, 30]. Partial disruption of $\Delta\Psi_m$, as observed in response to calcein/AM, may thus be part of the apoptotic cascade; on the other hand, viability in response to CCCP was close to 100% at 24 hr despite a substantial decrease in $\Delta\Psi_m$. This would indicate that early disruption of $\Delta\Psi_m$ alone is not sufficient for calcein/AM induction of apoptosis in the U-937 GTB cell line. Thus, the exact role of the mitochondria in calcein/AM-induced cell death is at present unclear.

In summary, these results are direct evidence for a rapid induction of apoptosis in response to calcein/AM; however, the precise mechanism of action remains to be investigated.

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