Leishmania, a unicellular trypanosomatid protozoan parasite, causes a wide range of human diseases ranging from the localized self-healing cutaneous lesions to fatal visceral leishmaniasis. However, it undergoes a process of programmed cell death during treatment with the topoisomerase I poison camptothecin (CPT). The present study shows that CPT-induced formation of reactive oxygen species increases the level of cytosolic calcium through the release of calcium ions from intracellular stores as well as by influx of extracellular calcium. Elevation of cytosolic calcium is responsible for depolarization of mitochondrial membrane potential (ΔΨm), which is followed by a significant decrease in intracellular pH levels. CPT-induced oxidative stress also causes impairment of the Na+/K+-ATPase pump and subsequently decreases the intracellular K+ level in leishmanial cells. A decrease in both intracellular pH and K+ levels propagates the apoptotic process through activation of caspase 3-like proteases by rapid formation of cytochrome c-mediated apoptotic complex. In addition to caspase-like protease activation, a lower level of intracellular K+ also enhances the activation of apoptotic nucleases at the late stage of apoptosis. This suggests that the physiological level of pH and K+ are inhibitory for apoptotic DNA fragmentation and caspase-like protease activation in leishmanial cells. Moreover, unlike mammalian cells, the intracellular ATP level gradually decreases with an increase in the number of apoptotic cells after the loss of ΔΨm. Taken together, the elucidation of biochemical events, which tightly regulate the process of growth arrest and death of Leishmania donovani promastigotes, allows us to define a more comprehensive view of cell death during treatment with CPT.

DNA topoisomerases are ubiquitous enzymes that catalyze the breakage and rejoining of DNA strands to permit topological changes in DNA (1). They are classified into two types. Type I topoisomerase breaks and rejoins one strand of duplex DNA, whereas type II topoisomerase breaks and rejoins both strands of DNA by using ATP as cofactor (2). These enzymes play a pivotal role in the maintenance of genome integrity and are essential for many chromosomal functions including DNA replication, recombination, transcription, and chromosome segregation (3). Other than the orderly synthesis of nucleic acids, these enzymes also have been identified as the molecular targets for numerous clinically important antibacterial and antitumor agents like fluorouracil, etoposide, and camptothecin (CPT) (1), etc. (2, 3).

CPT, an inhibitor of DNA topoisomerase I, has been widely used to induce apoptosis under experimental conditions and is in phase III clinical trials for colon cancer (4, 5). Poisoning of topoisomerase I by CPT causes protein-linked single strand breaks, but the breaks are by themselves not sufficient for cell death. The collision between the DNA replication fork with CPT-stabilized topoisomerase I-DNA covalent complex is thought to be responsible for cell killing (6). The double strand breaks resulting from the fork arrest are repaired very slowly and lead to prolonged S phase or G2 arrest of the cell cycle, followed by apoptosis (7).

Apoptosis, a physiological mode of cell death, results from the action of a genetically encoded suicide program that leads to series of characteristic morphological and biochemical changes (8). These changes include activation of caspases, cell shrinkage, chromatin condensation, and nucleosomal degradation (9). But the most significant event in apoptosis is mitochondrial dysfunction, which was shown to be involved in an early phase of apoptosis in a variety of cells upon induction of a number of stimuli including tumor necrosis factor (10), glucocorticoids (11), ceramides (12), and oxidative stress (13, 14).

As compared with necrosis, apoptosis is an energy-dependent process requiring functional mitochondria. Without the supply of ATP, cell cannot transmit apoptotic death signals from the cytoplasms to the nucleus (15). Moreover, changes in the intracellular concentration of cations are responsible for alterations in cell volume, which is one of the most striking morphological changes during the process of apoptosis (16). But very little is known about the effects of these ionic changes on the activity of underlying apoptotic machineries, including caspases and nucleases.

** The abbreviations used are: CPT, camptothecin; NAC, N-acetylcysteine; BHT, butylated hydroxy toluene; FFA, flufenamic acid; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide m-chlorophenylhydrazone; fmk, fluoromethyl ketone; AFC, 7-amino-4-trifluoromethyl coumarin; Fura 2AM, fura-2 acetoxymethyl ester; PBB-F-AM, potassium binding benzofuran isothipate acetoxymethyl ester; PMSF, phenylmethysulfonyl fluoride; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)]-1-propanesulfonic acid; DTT, dithiothreitol; PBS, phosphate-buffered saline; TG, thapsigargin; ROS, reactive oxygen species; MOPS, 4-morpholinepropanesulfonic acid; PI, propidium iodide.
Camptothecin-induced Imbalance in Cation Homeostasis

Camptothecin is responsible for the propagation of apoptosis. Isomerase I poison CPT appears to be an essential event in altering concentrations and alterations in pH homeostasis by the topoisomerase I poison CPT.

Leishmaniasis, one of the dreaded protozoon diseases threatening mankind, does not have enough combative measures. CPT has been shown to inhibit type I DNA topoisomerase of *Leishmania donovani* promastigotes and leads to apoptosis. To characterize the cellular events associated with apoptosis, we have found that CPT-induced oxidative stress causes depolarization of mitochondrial membrane potential. This is followed by the activation of caspase-like proteases inside leishmanial cells after the release of cytochrome c into the cytosol. But the molecular mechanisms connecting ion fluxes to the apoptotic machinery are still unknown, and these aspects remain to be investigated for the apoptotic cell death in unicellular parasites like *L. donovani* during treatment with CPT.

Here we have dissected the mechanism of action of CPT by analyzing the nuclear, mitochondrial, and cytosolic changes associated with apoptosis of leishmanial cells. In the present study we show that changes in the level of both cytosolic cations (calcium and potassium) and alterations in the level of mitochondrial membrane potential and activity of caspase-like proteases and endonucleases. Taken together, our results provide the first insight into the mechanistic pathway of apoptosis in leishmanial cells where a decrease in cytosolic K⁺ ion concentrations and alterations in pH homeostasis by the topoisomerase I poison CPT appears to be an essential event responsible for the propagation of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture and Maintenance—** *Leishmania* strain AG 83 promastigotes were grown at 22 °C in M199 liquid media supplemented with 10% fetal calf serum.

**Drug Solutions—** CPT was dissolved in 100% Me₂SO at 20 mM concentration and stored at −20 °C. BHT, NAC, FFA, and verapamil were dissolved in 100% Me₂SO at 50 mM concentration and stored at −20 °C.

**Measurement of ROS Level—** Intracellular ROS level was measured in treated and untreated cells as described by Mukherjee et al. (27). Briefly, cells (2 × 10⁶) after different treatments were washed and resuspended in 500 μl of medium 199 and were loaded with the cell-permeant probe 5-(and -6)-chloromethyl-2,7'-dichlorodihydro-fluorescein diacetate acetyl ester. It is a nonpolar compound and is hydrolyzed within the cell to form a nonfluorescent derivative, which in the presence of a proper oxidant is converted to a fluorescent product. Fluorescence was measured through a spectrophotometer by using 507 nm as excitation and 530 nm as emission wavelengths. For all measurements basal fluorescence was subtracted.

**Intracellular Ca²⁺ Measurement—** Intracellular Ca²⁺ concentration was measured with the fluorescent probe Fura 2 AM as described by Sarkar and Bhaduri (22). Briefly, cells with differently treated groups were harvested and washed twice with wash buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 5.5 mM glucose, 1 mM CaCl₂, and 50 mM MOPS, pH 7.4. Cells were then suspended in the same buffer containing 15% sucrose and incubated with Fura 2-AM (5 μM) at 27 °C for 1 h with mild shaking. Then cells were pelleted down and after two subsequent washing steps were suspended in the same wash buffer. Fluorescence was measured for the dye-treated and washed cells at 340 and 380 nm excitation and at 500 nm as emission wavelength. Fura 2 fluorescence of the cells after adding 10 mM CaCl₂ to the above-treated cells.

**Measurement of Intracellular K⁺ Level—** Intracellular K⁺ level was measured in both treated and untreated cells using PBFI-AM as the cell-permeant probe (23). Briefly, cells (2 × 10⁶) after different treatments were harvested and were washed twice with wash buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 5.5 mM glucose, and 50 mM MOPS, pH 7.4. Cells were then suspended in the same buffer containing 15% sucrose and incubated with PBFI-AM (5 μM) at 27 °C for 1 h with mild shaking. Then cells were pelleted down and after two subsequent washing steps were suspended in the same wash buffer. Fluorescence was measured for the dye-treated and washed cells at 340 and 380 nm excitation and at 500 nm as emission wavelength. PBFI fluorescence of the cells after adding 4 mM EGTA, 30 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100 simultaneously to the sample. *F*ₘₐₓ corresponds to the maximum fluorescence of the cells after adding 10 mM CaCl₂ to the above-treated cells.

**Assay for Na⁺–K⁺-ATPase Activity—** The plasma membrane of *L. donovani* promastigotes has a characteristic structure that provides strength to the membrane against hypotonic shock. Unsealed ghosts devoid of flagella were prepared according to Mukherjee et al. (25). Briefly, 0.5 g of treated and untreated cells were collected at mid-log phase and suspended in 25 ml of 5 mM Tris-HCl, pH 7.4, containing 0.5 mM PMSF. Cells suspensions were kept at 4 °C and were mixed by mild vortexing to detach flagella from the cell body. Formation of unsealed ghosts was confirmed by total leakage of the marker cytoplasmic enzymes (26).

Aliquots of each unsealed ghost (about 5 mg protein/ml) were incubated in a medium containing 3 mM ATP, 4 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 110 mM Tris-HCl, pH 7.4, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 15 μg/ml bovine serum albumin, 120 mM NaCl, 30 mM KCl, and 75 nml of [γ-³²P]ATP (0.15 μCi, specific activity 3000 Ci/ nmol) to a total volume of 150 μl. After 45 min of incubation at 37 °C, the reaction was stopped by adding trichloroacetic acid to a final concentration of 8%. A 150-μl suspension of 50% activated charcoal in water and 10 μl of 100 mM KH₂PO₄ were added to the above suspension. After mild agitation for 10 min, charcoal was precipitated by centrifugation. The process was repeated two more times, and finally 100 μl of the supernatant was transferred to a scintillation counter.

**Measurement of Mitochondrial Membrane Potential—** The fluorescence of the mitosensor reagent (BD Biosciences) is considered as an indicator of relative mitochondrial energy state. When mitochondrial membrane depolarizes, the dye remains as a monomer (emission 530 nm, green fluorescence), but during normal or higher membrane potential, the dye remains as an aggregate (red fluorescence, emission 590 nm) (20). Briefly, cells after different treatments were harvested and washed with 1× PBS. Cells were then incubated at 37 °C in a 5% CO₂ incubator for 1 h with a final concentration of mitosensor reagent (BD Biosciences) at 5 μg/ml (according to the manufacturer’s protocol). Cells were then analyzed by flow cytometry as well as by fluorescence measurements. The ratio of the reading at 590 nm to the reading at 530 nm (590/530 ratio) was considered as the relative ΔΨₘ value.

**Measurement of ATP Level—** The ATP content was determined by the luciferin-luciferase method (27). The assay is based on the requirement of luciferase for ATP in producing light (emission maximum 560 nm at pH 7.8). Briefly, cells (2 × 10⁶) after different treatments were harvested and resuspended in 1× PBS. An aliquot of this cell suspension was assayed for ATP using the Sigma chemical luciferase ATP assay kit. The amount of ATP in experimental samples was calculated from a standard curve with ATP and expressed as nmol/10⁶ cells.

**Intracellular pH Measurements—** Intracellular pH was measured with the fluorescent probe BCECF as described by Mukherjee et al. (25). Briefly, cells were harvested and washed two times with wash buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 5.5 mM glucose, and 50 mM MOPS, pH 7.4. Cells were then suspended in the same buffer containing 15% sucrose and incubated with BCECF-AM (10 μM) at 27 °C for 1 h with mild shaking. The cells were pelleted and after two

\[ \text{[Ca}^{2+}] = K_D(F - F_{\text{min}})/(F_{\text{max}} - F) \]  

where *K*D is the dissociation constant of the calcium-bound Fura 2 complex, and *F*ₘₐₓ represents the minimum fluorescence of the cells after adding 4 mM EGTA, 30 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100 simultaneously to the sample. *F*ₘₐₓ corresponds to the maximum fluorescence of the cells after adding 10 mM CaCl₂ to the above-treated cells.

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subsequent washing steps were suspended in the same wash buffer. Fluorescence was measured for the dye-treated and washed cells in a Hitachi spectrofluorometer (model F4010) at 440 and 490 nm excitation and at 535 nm emission wavelengths. Calibration of the internal pH of the control promastigotes was done by incubating the BCCBF-loaded cells in various pH buffers in the presence of nigericin (1 μM). The value of pH in the experimental samples was calculated from the calibration curve.

Preparation of Cytoplasmic Extract—Cytoplasmic extracts were prepared both in treated and untreated cells according to Das et al. (28). Briefly, cells (2 × 10^7) after different treatments were harvested, suspended in cell extraction buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl_2, 1 mM EDTA, 1 mM DTT, 200 μM PMSF, 10 μg/ml leupeptin and pepstatin), and lysed by a process of freeze-thaw using nitrogen cavitation and a 37 °C water bath simultaneously. Then lysate was centrifuged at 10,000 × g for 1 h, and supernatants were used as a source of cytoplasmic extract. Protein was estimated by the Bio-Rad protein determination kit.

Determination of Caspase Activity—Cytosolic extracts from untreated leishmanial cells were adjusted to different pH values by mixing various concentrations of NaOH or HCl solutions. To initiate caspase-like protease activation, 10-μl aliquots of the extracts were incubated with cytochrome c (10 μM) and/or 1 mM dATP at 30 °C for 1 h. For caspase activity measurements, 4 μl of the mixture (30 μg of protein) was added to 100 μl in caspase buffer (final concentration 50 mM Tris-HCl, 50 mM KCl, 10% sucrose, 0.1% CHAPS, 10 mM DTT) together with 100 μM DEVD-AMC. AMC release was measured fluorometrically at 505 nm (29).

Isolation of Nuclear Fraction from Leishmanial Cells—Nuclear fractions were prepared from CPT-treated and untreated cells according to Chakrabarty et al. (30). Briefly, cells were suspended in a hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM benzamidine hydrochloride, and 5 mM DTT) and were homogenized. The homogenate was centrifuged for 20 min at 10,000 rpm in Sorvall RC5B centrifuge using an SS34 rotor. The pellet was washed with the same buffer and centrifuged as above. The pellet was washed in Tris-HCl, pH 7.5, and was used as a source for nuclear fraction.

Preparation of Nuclear Extract from Leishmanial Cells—To prepare nuclear extract from isolated nuclear fractions of CPT-treated leishmanial cells, nuclei were suspended in 400 mM NaCl, 1 mM EDTA, and 20 mM Tris, pH 7.5, and were ultracentrifuged at 1,650,000 × g at 4 °C for 1 h. Supernatants were used as a source of nuclear extract like other mammalian cells (31, 32).

HeLa Nuclei Assay—The nuclei from healthy growing HeLa cells were isolated according to Schwartzman and Cidlowski (31, 32). Briefly, cells were harvested and lysed in ice-cold 10 mM MgCl_2 and 0.25% Nonidet P-40. Nuclei were pelleted down and suspended in 50 mM Tris-HCl, pH 7.5. For the DNA fragmentation assay, 2 × 10^6 nuclei were suspended in CPT-treated nuclear extract of leishmanial cells and incubated at 37 °C for 3 h in the presence of 2 mM MgCl_2 and 1 mM CaCl_2. After incubation, 25 mM EDTA, 540 mM NaCl, and 0.5% SDS were added to these solutions. Then proteinase K was added to a final concentration of 0.5 mg/ml and was incubated at 55 °C for 1 h. This was followed by phenol/chloroform extraction. Then DNAs were precipitated, dissolved in TE buffer, and electrohoresed in 1.5% agarose gel.

Double Staining with Hoechst 33342 and Propidium Iodide—Condensation of genomic DNA is one of the characteristic nuclear phenotypes of apoptotic cells. To distinguish between apoptotic, necrotic, and viable cells, these were stained with Hoechst 33342 and PI according to Das et al. (28). Briefly, after fixing with 2% paraformaldehyde, cells were incubated with 0.2% Triton X-100 for 5 min for permeabilization, washed with 1× PBS, and incubated with Hoechst 33342 and PI for 10 min. The cells were visualized with a TCS-SP Leica confocal microscope. Healthy viable cells were stained only with Hoechst and were identified by dull blue nuclei, but apoptotic cells were identified with bright blue nuclei. PI only penetrates necrotic cells and stained the nuclei red. Total cells versus only bright blue cells were calculated, and data are expressed as percentage of apoptotic cells. It should be noted that 100 cells per group with identical morphology were calculated for each condition.

Statistical Analysis—Data are expressed as mean ± S.D. unless mentioned. Comparisons were made between different treatments using unpaired Student’s t test.
CPT-induced Oxidative Stress Causes Increase in Cytosolic Ca²⁺ Level—Many studies have shown that calcium flux is absolutely necessary not only for the activation of different proteases (35) but also for the appearance of phosphatidylserine on the outer leaflet of the plasma membrane (36) during apoptosis. We have shown by confocal microscopy and spectrofluorometric analysis that over the time course of drug treatment leishmanial cells showed a significant increase in intracellular calcium (Fig. 3). Control leishmanial cells maintained intracellular [Ca²⁺] at 85 nM, and the concentration increases with the increase in ROS formation of CPT-treated cells at different times to the extent of 140 nM (Fig. 3A). This was confirmed by a decrease in elevation of cellular Ca²⁺ level during treatment with different antioxidants like NAC and BHT separately prior to the treatment with CPT. The maximum intensity of Ca²⁺-bound Fura 2 dye was not further increased when TG was added to CPT-treated cells. The above observation clearly confirmed that CPT-induced oxidative stress causes damage in both selective and nonselective cation channels and provides definite reasons for the increase in Ca²⁺ in CPT-treated cells through influx of extracellular Ca²⁺ and release of Ca²⁺ from intracellular pools.

Increase in Cytosolic Calcium Results in Mitochondrial Depolarization—Dissipation of Δψₘ (mitochondrial transmembrane potential) is a characteristic feature of apoptosis (38). To determine the changes in the Δψₘ we used the BD Biosciences mitosensor reagent, which is a cationic dye that aggregates in the mitochondria of healthy cells. These aggregates fluoresce red at higher potentials, but at a lower potential this reagent cannot accumulate in the mitochondria and remains as monomers in the cytoplasm that fluoresces green. To confirm the sensitivity of the reagent to the change in mitochondrial mem-
FIG. 3. **Measurement of intracellular Ca\(^{2+}\) level, using Fura 2 AM as an indicator.** A, increase in Ca\(^{2+}\) level was measured after treatment with 5 μM CPT (■) compared with treatment with 0.2% Me2SO alone (●) and with NAC (□) and BHT (▲) prior to treatment with CPT. B, decrease in the elevation of intracellular Ca\(^{2+}\) level was observed during treatment with FFA (■) or verapamil (▲) separately prior to treatment with CPT compared with treatment with CPT (∆). But treatment with TG after treatment with CPT (□) had no effect in the elevation of intracellular Ca\(^{2+}\) level after treatment with CPT. Results are mean ± S.D. from three independent experiments. C, confocal microscopic analysis of the intensity of Ca\(^{2+}\)-bound Fura 2 in the cytosol of differently treated and untreated cells. Intensity of Ca\(^{2+}\)-bound Fura 2 in each cell is shown in the y axis in arbitrary units. The length of each parasite, which was scanned along the z axis, is shown in the x axis.
Intracellular Ca\(^{2+}\) are responsible for the loss of \(\Delta \Psi_m\), which subsequently decreases cellular ATP level. Moreover, a tight link exists between the intracellular ATP level and the number of apoptotic cells during treatment with CPT. As shown in Fig. 5B, a decrease in ATP level is consistent with an increase in the number of apoptotic cells that were detected by their condensed nuclei, which exhibited a bright blue fluorescence in the presence of Hoechst 33342 dye, but no red fluorescence of PI was observed in these apoptotic cells.

A fluorescent method was adopted to measure intracellular pH levels in leishmanial cells treated with or without CPT by using the dye BCECF. Leishmanial cells were loaded with acetethyl ester derivative of BCECF (BCECF-AM), a dye whose fluorescence emission is sensitive to pH variations. Cell samples were excited at the 440 and 490 nm wavelengths, and the emission was recorded at a single wavelength of 535 nm. The ratio of fluorescence excitation was used as a quantitative measure of the pH, independent of cell volume or dye concentration. The pH of untreated leishmanial cells was 7.4 ± 0.03. A significant decrease in the level of pH was observed to the extent of 7.1 immediately after loss of \(\Delta \Psi_m\), upon treatment with CPT for 2 h. This was further decreased to 6.8 ± 0.05 after 3 h of treatment with CPT (Fig. 5C). These experiments show that leishmanial cells treated with CPT become significantly more acidic than untreated cells. But treatment with NAC (20 mM), BHT (20 mM), and FFA (250 \(\mu\)M) separately prior to CPT treatment can prevent the decrease of intracellular pH.

**Fig. 4. Flow cytometry analysis of mitochondrial membrane potential.** Changes in mitochondrial membrane potential after treatment with CCCP, 0.2% Me\(_2\)SO alone (control), and 5 \(\mu\)M CPT for 2.5 and 5 h, respectively. This was also determined after treatment with FFA and verapamil separately prior to treatment with CPT. FL1 channel indicates green fluorescence intensity.

| Name of events | Value of mean green fluorescence intensity |
|----------------|-------------------------------------------|
| Control (0.2% Me\(_2\)SO) | 130.42 ± 4.91 |
| CPT (2.5 h) | 255.1 ± 8.21 |
| CPT (5.0 h) | 417.9 ± 7.25 |
| CCCP | 522.8 ± 5.39 |
| FFA + CPT | 154.91 ± 2.46 |
| Verapamil + CPT | 169.24 ± 3.02 |
| Control (0.2% Me\(_2\)SO) | 130.42 ± 4.91 |
| CPT (2.5 h) | 255.1 ± 8.21 |
| CPT (5.0 h) | 417.9 ± 7.25 |
| CCCP | 522.8 ± 5.39 |
| FFA + CPT | 154.91 ± 2.46 |
| Verapamil + CPT | 169.24 ± 3.02 |

**Table 1**

| Name of events | Value of mean green fluorescence intensity |
|----------------|-------------------------------------------|
| Control (0.2% Me\(_2\)SO) | 130.42 ± 4.91 |
| CPT (2.5 h) | 255.1 ± 8.21 |
| CPT (5.0 h) | 417.9 ± 7.25 |
| CCCP | 522.8 ± 5.39 |
| FFA + CPT | 154.91 ± 2.46 |
| Verapamil + CPT | 169.24 ± 3.02 |

**Decrease in Mitochondrial Membrane Potential Causes Depletion in Cytosolic ATP Level and a Decrease in Intracellular pH**—In CPT-treated leishmanial cells, disruption in the function of mitochondria caused reduced ATP generation. Here we have measured the ATP level in differently treated cells. As shown in Fig. 5A, there was a gradual fall in ATP level to the extent of 75% after 3.5 h of CPT treatment. Treatments with different antioxidants like NAC or BHT prior to the treatment with CPT prevent further a decrease in ATP level. Similar results were also obtained when cells were treated with selective Ca\(^{2+}\) channel inhibitor (verapamil) or with nonselective Ca\(^{2+}\) channel inhibitor (FFA) prior to treatment with CPT. This may be due to the fact that both an increase in ROS and

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**Fig. 5A. Effect of different treatments on the mitochondrial membrane potential of *L. donovani* as measured by fluorescence of BD mitoimperor reagent.**

**Table 1**

| Name of events | Value of mean green fluorescence intensity |
|----------------|-------------------------------------------|
| Control (0.2% Me\(_2\)SO) | 130.42 ± 4.91 |
| CPT (2.5 h) | 255.1 ± 8.21 |
| CPT (5.0 h) | 417.9 ± 7.25 |
| CCCP | 522.8 ± 5.39 |
| FFA + CPT | 154.91 ± 2.46 |
| Verapamil + CPT | 169.24 ± 3.02 |

**FIG. 5A.** Flow cytometry analysis of mitochondrial membrane potential. Changes in mitochondrial membrane potential after treatment with CCCP, 0.2% Me\(_2\)SO alone (control), and 5 \(\mu\)M CPT for 2.5 and 5 h, respectively. This was also determined after treatment with FFA and verapamil separately prior to treatment with CPT. FL1 channel indicates green fluorescence intensity.
of pro-caspase 3-like proteases in cytosol, we prepared cytosolic extracts from untreated leishmanial cells, buffered them to various pH values, and then added cytochrome c in combination with dATP and 50 mM KCl in the assay mixture. Caspase activation induced by the combination of cytochrome c and dATP exhibited pH dependence, optimal activation occurring at pH 6.3–7.0. At pH 6.8, which represents the approximate cytosolic pH observed in cells undergoing apoptosis by CPT (Fig. 6C), the efficiency of cytochrome c/dATP-stimulated caspase activation was 1.8-fold higher than at physiological pH 7.4 (Fig. 6C). Moreover, the progress curve obtained at pH 6.8, 6.3, and 7.0 exhibited roughly similar slopes, but with a pH-
dependent delay in the time of onset of caspase activity following addition of cytochrome c and dATP. It indicates that lower pH accelerates the rate of formation of active apoptotic complex, as opposed to increasing the $V_{\text{max}}$ of cytochrome c and dATP-activated caspase 3-like proteases. So, the pH-dependent differences in cytochrome c/dATP-induced caspase activation were not due to faster substrate depletion as determined by evaluation of enzyme activities at different pH values (Fig. 6D).}

**DISCUSSION**

Apoptosis or programmed cell death can be activated in variety of cells through diverse signaling pathways. However, all apoptotic stimuli result in a highly conserved series of morphological and biochemical changes both in mammalian cells as well as in protozoan parasites, suggesting a common pathway distal to cell-specific events. But very little is known about the importance of cytosolic cations in the process of apoptosis particularly in the case of protozoan parasites like *L. donovani*.

In earlier studies we have found that CPT-induced formation of ROS inside leishmanial cells causes an increase in the level of lipid peroxidation (20). In general, the overall effect of lipid peroxidation is to decrease membrane fluidity, as well as in-
crease leakiness of the membrane leading to complete loss of membrane integrity (42). This in turn causes an increase in intracellular Ca\(^{2+}\) level inside cells, which is a common feature of apoptosis. The elevation in cytosolic Ca\(^{2+}\) causes cellular damage and death through disruption of the cytoskeletal networks and the action of Ca\(^{2+}\)-stimulated catabolic enzymes, such as protease, phospholipases, and endonucleases, which are involved in the nuclear apoptosis in mammalian cells (35). Here in the case of leishmanial cells, CPT-induced formation of ROS causes an increase in cytosolic Ca\(^{2+}\) due to opening of nonselective and L-type voltage-gated calcium channels and causes dysregulation of sarcoplasmic Ca\(^{2+}\)-ATPase channels. The excessive free cytosolic Ca\(^{2+}\) leads to uncoupling of mitochondrial oxidative phosphorylation and directs the cell to follow the executionary part of apoptosis.

Several studies (38) support the hypothesis that disruption of \(\Delta\Psi_m\), is an irreversible commitment of the cell to death. One of the consequences of this is the release of apoptogenic factors including cytochrome c into the cytosol of mammalian cells as well as in leishmanial cells after treatment with CPT (20, 43). But concerning the cellular bio-energy of leishmanial cells in apoptosis, it is observed that the ATP level gradually decreases as opposed to mammalian cells. This may be due to the fact that in higher eukaryotes not all mitochondria are responsible for the degradation of genomic DNA both in leishmanial cells and in HeLa cells. A, inhibition of apoptotic DNA degradation in leishmanial cells was analyzed in the presence of different concentrations of KCl. Lane 1, nuclei from control (untreated cell) incubated at 37 °C without CaCl\(_2\), MgCl\(_2\), or KCl in the assay mixture. Lanes 2–6, nuclei from untreated leishmanial cells were incubated in the presence of CaCl\(_2\), MgCl\(_2\), and different concentrations of KCl in the assay mixture. Lane 6, HeLa nuclei were incubated with nuclear extract prepared from VAD-fmk plus CPT-treated leishmanial cells on HeLa nuclei. Lane 1, untreated HeLa nuclei; lanes 2–5, HeLa nuclei were incubated with CPT-treated nuclear extract in the presence of CaCl\(_2\), MgCl\(_2\), and different concentrations of KCl in the assay mixture. Lane 6, HeLa nuclei were incubated with nuclear extract prepared from VAD-fmk plus CPT-treated cells. Pictures are representative from one of three similar results.

B. inhibitory effect of KCl was analyzed with nuclear extract prepared from CPT-treated leishmanial cells on HeLa nuclei. Lane 1, untreated HeLa nuclei; lanes 2–5, HeLa nuclei were incubated with CPT-treated nuclear extract in the presence of CaCl\(_2\), MgCl\(_2\), and different concentrations of KCl in the assay mixture. Lane 6, HeLa nuclei were incubated with nuclear extract prepared from VAD-fmk plus CPT-treated cells. Pictures are representative from one of three similar results.
Intracellular K⁺ is known to be one of the most important determinants for the maintenance of ionic balance, which is directly related to osmotic pressure inside cells (33). Most of the cells can achieve and maintain this osmotic balance through the continuous activity of an ATP-dependent Na⁺/K⁺-ATPase pump that exchanges 3Na⁺ for 2K⁺ against the electrochemical gradient (34). Here in case of leishmanial cells, CPT-induced oxidative stress and lipid peroxidation cause impairment of the Na⁺/K⁺-ATPase pump, which consequently decreases in intracellular K⁺ levels and facilitates apoptosis through the increase in the activity of these death enzymes. Collectively, it can be inferred that a decrease in intracellular pH level and [K⁺] are prerequisite events during apoptosis and are needed for the activation of caspase 3-like proteases in leishmanial cells during treatment with CPT.

Downstream of the caspase 3-like protease activation, nucleases become active and cause apoptotic DNA fragmentation in leishmanial cells during treatment with CPT. Evidence presented here also supports the fact that the nuclear extract from CPT-treated leishmanial cells causes degradation of HeLa nuclei but was suppressed at 150 mM concentration of K⁺. CPT-treated leishmanial cells causes degradation of HeLa nuclei but was suppressed at 150 mM concentration of K⁺. CPT-treated leishmanial cells causes degradation of HeLa nuclei but was suppressed at 150 mM concentration of K⁺.

Changes in the level of K⁺/H⁺-ATPase pump, which consequently decreases in intracellular K⁺ levels and facilitates apoptosis through the increase in the activity of these death enzymes. Collectively, it can be inferred that a decrease in intracellular pH level and [K⁺] are prerequisite events during apoptosis and are needed for the activation of caspase 3-like proteases in leishmanial cells during treatment with CPT.

In conclusion, our study demonstrates for the first time that apoptosis in leishmanial cells is orchestrated by the coordinated alterations in ion fluxes and subsequent activation of caspase 3-like proteases and endonucleases during treatment with CPT. Moreover, modification of pH by the topoisomerase I poison CPT likely represents an essential event responsible for the propagation of apoptosis and can act as a central regulator of the apoptotic machineries. So understanding the components and the steps involved in this intricate process provides the opportunities for discovering and evaluating molecular targets for drug designing, which now form a rational basis for development of improved therapy against leishmaniasis.

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