Cell Tissue Res (2006) 324: 255–266
DOI 10.1007/s00441-005-0095-y

REGULAR ARTICLE

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Apoptosis-inducing factor of a cytotoxic T cell line: involvement of a secretory phospholipase A_2

Received: 8 September 2005 / Accepted: 7 October 2005 / Published online: 27 January 2006

Abstract Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) kill target cells by the granule-exocytosis pathway and by the engagement of molecules belonging to the tumor necrosis factor family. The involvement of secretory phospholipase A_2 ($sPLA_2$) in the cytotoxic process has been proposed in NK cells. However, its molecular identity and intracellular localization remain unknown, and its mechanism of action is poorly understood. Here, we have readdressed this issue by studying the cytotoxic activity of whole cell extracts of a CTL line. We observed that inactivation of the perforin-granzyme pathway at 37°C in the presence of 1 mM Ca^{2+} enhanced the ability of CTL extracts to induce apoptosis. This potentiation of cell death was Ca^{2+}-dependent, thermo-resistant, and inhibited by 4-bromophenacyl bromide and scalaradial (two inhibitors of $sPLA_2$). The involvement of an $sPLA_2$ was confirmed by blocking the pro-apoptotic activity of the Ca^{2+}-treated cell extract with an anti-$sPLA_2$ polyclonal antibody. By cell fractionation assays, we showed that the pro-apoptotic $sPLA_2$ was localized in the cytoplasmic fraction but not in perforin-rich granules or plasma membrane fractions. Western blotting analysis revealed the presence of four distinct bands of 56, 29.5, 21, and 15 kDa. The highest molecular weight band was consistent with the expression of a group III sPLA2. Taken together, these data indicate that an apoptosis-inducing $sPLA_2$ is expressed in the cytosol of a CTL cell line and suggest that it plays an effector role in CTL-mediated cytotoxicity.

Keywords Phospholipase A2 · Apoptosis · Cytotoxic T lymphocytes · Cell death · Perforin · Mouse (Swiss-Webster, C57 BL/6 rp $55^{-/-}$)

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Programa de Núcleos de Excelência (PRONEX–CNPq).

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Introduction

The mechanism through which natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) kill their target cells involves the exocytosis of perforin- and granzyme-containing granules and the engagement of death receptors such as Fas-L (Barry and Bleackley 2002). However, additional cell-death mechanisms may be present in cytotoxic lymphocytes, as CTLs from double knockout mice for perforin and Fas-L can eliminate tumors in vivo (Lee et al. 2004), and target cells derived from *lpr* mice can be killed by perforin knock-out CTLs (Ahmed et al. 1997).

Mammalian phospholipase A_2 (PLA_2) comprises a large group of enzymes that are involved in several cell functions including the processing of pro-inflammatory mediators, cell proliferation, and apoptosis (Capper and Marshall 2001; Cummings et al. 2000; Taketo and Sonoshita 2002). Some PLA_2 are Ca^{2+}-independent, whereas others vary in their Ca^{2+} requirement. Secretory PLA_2 ($sPLA_2$) are released from the cells and are activated only in the presence of millimolar concentrations of Ca^{2+}, whereas cytosolic PLA_2 ($cPLA_2$) remain inside the cells and require micromolar amounts of Ca^{2+} (Six and Dennis 2000).

The involvement of phospholipases in lymphocyte-mediated killing was initially suggested by Frye and Friou

(1975), Hoffman et al. (1981), and Deem et al. (1987). Later, the engagement of CD16 and NKR–P1A in NK cells was shown to trigger the release of an, as yet, unidentified $sPLA_2$ whose activity was important to NK cytotoxic activity (Milella et al. 1997, 1999; Cifone et al. 1993, 1997). However, whether this $sPLA_2$ has a direct cytotoxic effect on the target cell or even if its molecular target is at the effector or the target cell membrane remains unclear. Although the release of $sPLA_2$ occurs simultaneously with the exocytosis of perforin- and granzyme-containing granules, its precise intracellular localization has not been established.

Here, we have addressed this issue by analyzing the cytotoxic activity present in whole cell extracts and subcellular fractions derived from the CTL line R8 (CTLL–R8). We have demonstrated not only Ca^{2+}-dependent, perforin-granzyme-independent, and Fas-L-independent pro-apoptotic activity, but also the presence of an $sPLA_2$, possibly a group III $sPLA_2$, in CTLL–R8 cells. In addition, we have shown that this $sPLA_2$ is present in the cytoplasm but not in the perforin-containing granules of the cells.

Materials and methods

Materials RPMI 1640 medium, alpha minimal essential medium (alpha-MEM), D-MEM, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco/BRL (Grand Island, N.Y. USA); ethidium bromide was obtained from United States Biochemical (Cleveland, Ohio, USA); phosphate-buffered saline (PBS), HEPES, benzamidine, arachidonic acid (AA), 4-bromophenacyl-bromide (pBPB), dimethyl sulfoxide (DMSO), 2-mercaptoethanol, and Triton X-100 were obtained from Sigma–Aldrich (St. Louis, Mo., USA); $CaCl_2$, NaCl, $MgCl_2$, KCl, sodium citrate, methanol, acetic acid, and chloroform were obtained from Reagen (Rio de Janeiro, RJ, Brazil); EDTA was obtained from Amersham Pharmacia Biotech (São Paulo, SP, Brazil); 5,5′-dithiobis-(2-nitrobenzoic acid), p-nitrophenyl phosphate, N-a-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), Boc-AAD-S-Bzl, and Z-AAD-CMK were obtained from Calbiochem (San Diego, Calif., USA); 1-acyl-2-hydroxy-sn-glycero-3-phosphate (lysophosphatidic acid, LPA) was obtained from Avanti (Alabaster, Ala., USA); thioglycollate medium was obtained from Difco (Detroit, Mich., USA); 12-epi-scalaradial (SCA) was obtained from Alexis Biochemicals (Woburn, Mass., USA); human-recombinant interleukin-2 (IL-2) was obtained from Cetus (Emeryville, Calif., USA); the Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, Calif., USA); the recombinant mature-mouse soluble tumor necrosis factor receptor 1 (TNFR1)/Fc chimera (mouse TNFR1/TNFR SF1A/Fc chimera) and mouse soluble Fas receptor/Fc chimera (mouse Fas/TNFRSF6/Fc Chimera) were purchased from R&D Systems (Minneapolis, Minn., USA); fluorescein isothiocyanate (FITC)-conjugated annexin-V was obtained from Dr. Gustavo P. Amarantes Mendes (Dep. of Immunology-ICB/USP, São Paulo, SP, Brazil).

Animals Female Swiss–Webster mice (8–12 weeks old) weighing approximately 30 g were purchased from the animal facilities of the Federal University of Rio de Janeiro (Rio de Janeiro, RJ, Brazil). C57 BL/6 mice deficient for TNFR1 (C57 BL/6 rp $55^{-/-}$; Pfeffer et al. 1993) were originally purchased from Jackson Laboratories (Bar Harbor, Me., USA) and kindly donated by Dr. L. Q. Vieira (Federal University of Minas Gerais - UFMG, Brazil). Animal handling was performed according to the guidelines for the use of animals in scientific experiments as issued by the Carlos Chagas Filho Biophysics Institute of the Federal University of Rio de Janeiro.

Cell culture Thioglycollate-elicited macrophages were obtained from the intraperitoneal cavity of Swiss-Webster mice, collected 4 days after thioglycollate injection as described (Coutinho-Silva and Persechini 1997). In brief, cells were washed three times in PBS and either kept on ice at a concentration of 2×10^5 cells/ml until used or plated in 24-well culture dishes. In the latter case, cells were first added to the culture dishes in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 g/l sodium bicarbonate, 0.3 mg/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were then removed after 2 h, and the macrophages were kept for 4 days under the same culture conditions. The cytotoxic T lymphocytes murine cell line CTLL–R8 was maintained in alpha-MEM supplemented with 10% heat-inactivated fetal bovine serum, 10% rat-spleen-conditioned medium, 2 g/l sodium bicarbonate, 0.3 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin as described (Palladino et al. 1983). HCT–8 and K562 cell lines were maintained in D-MEM supplemented with 10% fetal bovine serum, 2 g/l sodium bicarbonate, 20 mM HEPES, 0.3 mg/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were kept at 37°C in a humidified atmosphere containing 5% CO_2. Lymphokine-activated killer (LAK) cells were prepared as described (Gunji et al. 1989; Ojcius et al. 1991). Spleens from Swiss-Webster mice (8–12 weeks old) were removed, and cell suspensions were prepared by gently crushing spleens against a nylon screen in D-MEM. Red cells were lysed in ACK buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; sterile-filtered) and washed tree times in serum-free D-MEM. T cells were isolated by nylon-wool column fractionation. More than 83% of the cells were found to be $CD3^+$ and less than 1% $CD14^+$ by flow cytometry. Cells were then cultured for 72 h in complete medium (D-MEM, 10% heat-inactivated fetal bovine serum, 2 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/l L-glutamine, and 18 U/ml recombinant human IL–2 at 37°C in 5% CO_2. After 3 days, medium and non-adherent cells were removed, and adherent cells were harvested by placing the culture flasks at 4°C for 1 h with PBS containing 1 mM EDTA.

Crude cell extracts and cellular fractionation CTLL-R8 cells were detached from culture flasks by incubation in cold PBS containing 1 mM EDTA for 30 min and washed

twice in cold PBS-EDTA. Crude CTLL-R8 extracts were obtained by three cycles of freezing and thawing. The pellet was then suspended in cold PBS, and cell debris was removed by centrifugation at 1,000*g* for 45 s. The supernatant was then frozen at −20°C until use. Cellular fractionation was performed as described (Young et al. 1986). In brief, cells were suspended in relaxation buffer (5 mM NaCl, 130 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM and EDTA, pH 6.5), disrupted by N_2 cavitation (400 psi, 30 min), centrifuged at 1,000*g* for 5 min to isolate the nuclei (nuclear or N fraction), and subjected to medium-speed centrifugation (20,000*g* for 40 min); this produced an organelle-rich pellet (O fraction) and a supernatant containing soluble components and low-density vesicles (microsomal/cytosolic or MC fraction). The MC fractions were further submitted to high-speed centrifugation (100,000*g*, 2 h) and separated into an MC pellet and an MC supernatant fraction. In some experiments, the post-nuclear supernatants were submitted to a discontinuous Percoll gradient (1.07–1.04–1.02 g/ml, 20,000*g* for 40 min). Samples were collected and assayed for hemolytic activity in order to obtain purified perforin-rich granules.

Hemolytic assays Sheep red blood cells (SRBC) were used to quantify Ca^{2+}-dependent hemolytic activity as described (Young et al. 1986); 1 hemolytic unit (HU) corresponded to the dose required to lyse 50% of 2×10^7 SRBC. Unless otherwise specified all samples used to induce cell death were derived from cell extracts containing 10 HU/200 μl before inactivation of perforin (this correspond to $2–5\times10^6$ cells and 5–10 μg protein).

Enzymatic activity and protein determination assays The activity of granzymes A and B was measured as described (Krähenbühl et al. 1988; Odake et al. 1991). Alkaline phosphatase (AP) activity was assayed as described by Modolell and Munder 1994). Protein content was determined by the Bradford method (Bradford 1976).

Lipid extraction The MC fraction was submitted to lipid extraction in chloroform-methanol-HCl (200:100:0.75 v/v; Horwitz and Perlman 1987). The aqueous phase (upper phase) and the interface (non-soluble material) were removed and stored in ice. The organic phase (lower phase) containing the lipids was washed twice with chloroform:methanol:0.6 N HCl (3:48:47 v/v) and dried under N_2. The hydrophilic phase and the interface were dried in a Speed–Vac centrifuge (Savant, Albertville, Minn., USA). The three samples were suspended in a final volume corresponding to 3×10^6 cells/ml in relaxation buffer and stored at −20°C until use.

Analysis of cell death The induction of cell death was performed by incubating thioglycollate-elicited intraperitoneal cells (2×10^5 cells in 200 μl) for 3 h at 37°C in the presence of CTLL-R8-derived samples or control solutions in a polypropylene micro-centrifuge tube. The tube was then centrifuged at 5,600*g* for 1 min. The supernatant was collected, and the pellet was gently resuspended in 250 μl apoptosis buffer (50 μg/ml ethidium bromide, 0.1% sodium citrate, 0.1% TritonX-100). The DNA content was determined by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, Calif., USA), and apoptosis was estimated by quantifying the presence of hypodiploid nuclei (Shan et al. 1998). At least 5,000 events were collected per sample. Early apoptotic events were determined by incubating thioglycollate-elicited intraperitoneal cells (2×10^5 cells in 200 μl) for 1 h at 37°C in the presence of MC supernatant fractions derived from CTLL-R8 and by counting annexin-V^+ cells by flow cytometry. In brief, cells were suspended in 100 μl annexin buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) and incubated for 20 min with FITC-labeled annexin-V (1:500) on ice. Cells were then washed in annexin buffer and, following the addition of ethidium bromide at a final concentration of 1 μg/ml, immediately analyzed by flow cytometry. Only FITC-positive ethidium-bromide-negative cells were counted as apoptotic cells.

Cell morphology was evaluated by phase-contrast microscopy, and nuclear morphology was evaluated by acridine orange (AO, 10 μM) fluorescence. Culture dishes containing 4-day-old macrophage cultures were gently washed once in pre-warmed D-MEM buffered with 20 mM HEPES without serum, and aliquots of MC supernatant fraction derived from CTLL-R8 were added to the culture dishes at a concentration of 10 HU/200 μl cell equivalents. Cells were then kept at 37°C for 3 h. The dishes were washed with PBS buffer twice and observed in an inverted Axiovert 100 microscope coupled to an AxionVision 4.3 image system (Carl Zeiss, Germay).

Cell lysis (necrosis) was determined by measuring lactate dehydrogenase (LDH) activity in the supernatants collected as above, by using a commercially available assay kit according to the maker's instructions (CytoTox, Promega, Madison, Wis., USA; Rizzardi et al. 1996). Control lysis was obtained by using supernatants of untreated cells (0%) and cells treated with 0.1% Triton X-100 (100%). The reading of each well was subtracted from the value obtained in a control well containing all reagents but the target cells. Thus, measurements of the LDH present in the effector cell extracts were not taken into account.

Anti-PLA_2 serum and affinity purification Rabbit anti-PLA_2 antiserum obtained against a purified *Crotalus durissus terrificus* sPLA_2 was used in some experiments (Hanashiro et al. 1978). Affinity-purified anti-PLA_2 immunoglobulin was obtained by applying ammonium-sulfate-precipitated proteins from the immune serum to a BrCN-activated Sepharose B4 column (Pharmacia, São Paulo, SP, Brazil) bound to purified *Crotalus durissus terrificus* PLA_2. The non-adsorbed protein fraction was washed out with 50 mM TRIS-HCl pH 7.0, and the adsorbed protein was eluted with 3.5 M KSCN in PBS. The elution process was monitored at 280 nm, and purification was confirmed by an enzyme-linked immunosorbent assay against purified PLA_2.

Western blotting CTLL-R8 extracts containing 40 μg protein were prepared as described above, run on 8%–25%

SDS–polyacrylamide gels, transferred into a nitrocellulose membrane, and stained with AP-conjugated anti-rabbit IgG (BD-PharMingen) following standard protocols. The sample contained 2-mercaptoethanol (2.5%) and SDS (10%).

Statistical analysis Unless otherwise specified, each experiment was performed at least three times in triplicate. Data were analyzed by using the Prisma program (GraphPad Software, version 3.0) and applying the Tukey test. Differences were considered significant at $P<0.05$ (*) and $P<0.01$ (**).

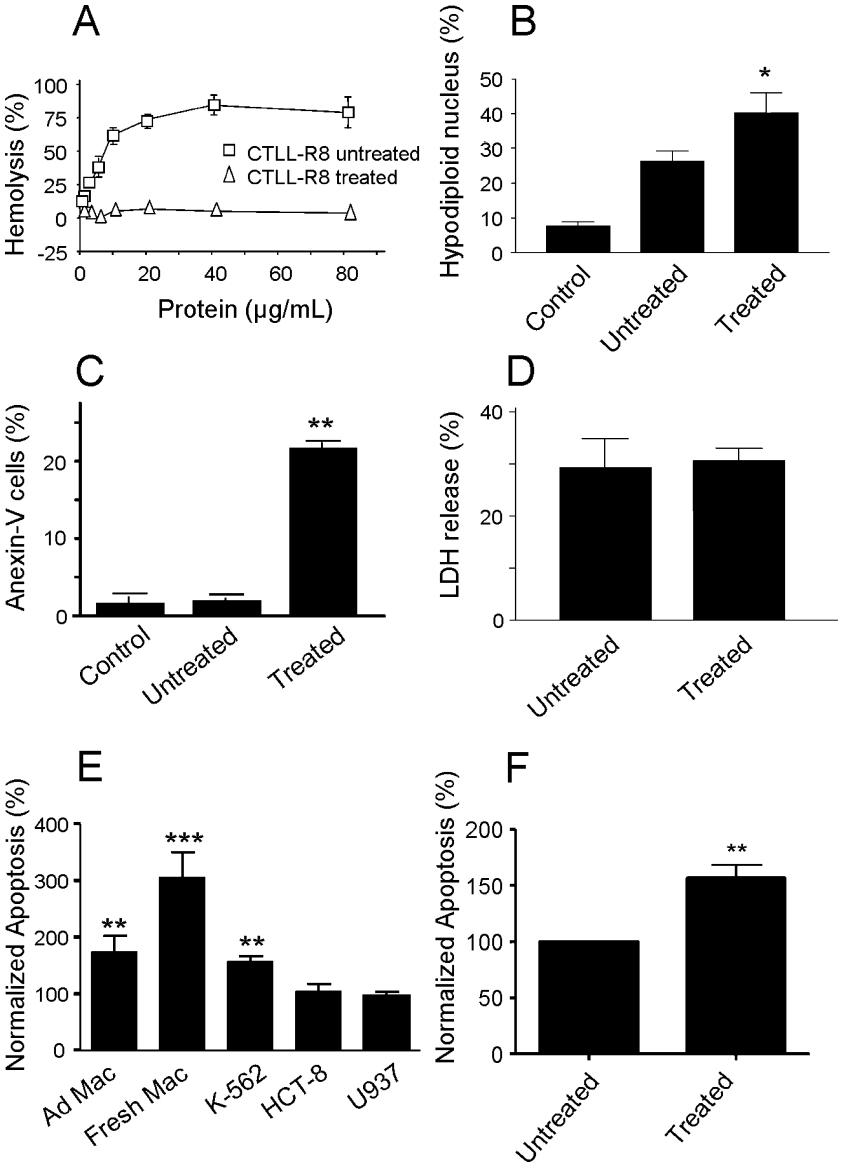

Fig. 1 Enhancement of apoptosis by treated cell extracts. CTLL-R8 cell extracts (**a**–**e**) were first submitted to hemolytic activity assay (**a**) and then samples corresponding to 10 HU (1 HU is defined as the amount of extract required to induce 50% hemoglobin release) were tested for their ability to induce apoptosis and LDH release under various conditions (**b**–**e**, *bars* mean ± standard error of at least three experiments performed in triplicate). **a** Various amounts of crude CTLL-R8 extract were incubated with sheep red blood cells for 20 min at 37°C in the presence of 1 mM Ca^{2+}, and the release of hemoglobin was measured. Cell extracts were used before (*open squares*) or after (*open triangles*) treatment with 1 mM Ca^{2+} at 37°C for 30 min. **b** Apoptosis was evaluated by the percentage of hypodiploid nuclei induced by untreated and treated CTLL-R8 extracts. Target cells (freshly isolated intraperitoneal cells) were incubated for 3 h with 10 HU untreated crude cell extract or the same quantity of treated cell extracts prepared as in **a**. Control cells were incubated under the same conditions with the addition of buffer only. **c** Apoptosis was evaluated by the percentage of FITC-labeled annexin-V binding. Target cells (freshly isolated intraperitoneal cells) were incubated for 1 h as in **b**. **d** LDH release was measured in the supernatants of the same samples used in **b**. **e** Apoptosis (hypodiploid nuclei) was evaluated as in **b** by using adherent macrophages (*Ad Mac*), freshly isolated intraperitoneal cells (*Fresh Mac*), and K562 and U937 cell lines as targets. In each case, the apoptosis induced by untreated extract was taken as the 100% reference value. **f** LAK cell extracts (10 µg protein/assay) were prepared in the same way as CTLL-R8 extracts. Apoptosis (hypodiploid nuclei) was evaluated as in **e** by using freshly isolated intraperitoneal cells as targets. *$P<0.05$, **$P<0.01$ when results obtained with treated extracts were compared with untreated extracts or as indicated

Results

Perforin and Fas-L-independent enhancement of apoptosis In order to investigate the possible existence of perforin-independent cytolytic activity, we first obtained crude cell extracts by repeatedly freezing and thawing CTLL-R8 cell pellets in ice-cold Ca^{2+}-free salt solution and then adding Ca^{2+} to a final concentration of 1 mM, followed by incubation at 37°C for 30 min; this procedure inactivated perforin-dependent hemolytic activity (Podack et al. 1985). Indeed, the extracts obtained in the Ca^{2+}-free solution were rich in Ca^{2+}-dependent hemolytic activity, whereas the extracts treated in the presence of 1 mM Ca^{2+} at 37°C displayed no hemolytic activity (Fig. 1a). The induction of cell death by both Ca^{2+}/37°C-treated and -untreated extracts was evaluated by incubating freshly isolated thioglycollate-elicited intraperitoneal murine cells for 3 h (Fig. 1b,d) or 1 h (Fig. 1c) at 37°C in complete culture medium. Unexpectedly, the Ca^{2+}-treated extract exhibited enhanced induction of apoptosis as measured either by the presence of hypodiploid nuclei (Fig. 1b) or by labeling with annexin-V (Fig. 1c). In contrast, both treated and untreated extracts induced similar levels of LDH release (Fig. 1c). The CTLL-R8 extract also displayed apoptosis-enhancing activity against adherent intraperitoneal macrophages and K562 cells, but not against HCT-8 and U937 cells (Fig. 1e). In addition, cell extracts obtained from LAK cells possessed a similar apoptosis-inducing activity to the CTLL-R8 cell extract (Fig. 1f), whereas extracts obtained from intraperitoneal murine cells and K562 cells did not have any significant apoptosis-inducing activity either before or after similar Ca^{2+}/37°C treatment (data not shown). The enhancement of apoptosis was more evident in cell extracts treated for a period of 30–60 min and was absent in extracts treated for 2 h or more (data not shown).

Cell morphology was evaluated by incubating adherent macrophages for 3 h in the presence or absence of untreated or treated CTLL-R8 extracts (Fig. 2). When compared with control cells (Fig. 2a,c), cells exposed to Ca^{2+}/37°C-treated extracts shrank and displayed nuclear fragmentation (Fig. 2b,d).

In order to evaluate whether the enhancement of apoptosis described above was dependent on granzyme activity, we compared the effects of cell extracts prepared in the presence or absence of either Z-AAD-CMK, an inhibitor of granzyme B (Gorak-Stolinska et al. 2001), or benzamidine, a non-specific inhibitor of trypsin-like enzymes. As shown in Fig. 3a, these drugs had no significant effect on the induction of cell death, whereas in control experiments, they inhibited granzyme B (cleavage of Boc-AAD-Bzl) and the trypsin-like (cleavage of BLT) specific activities of CTLL-R8 extracts, respectively (data not shown).

We next investigated the involvement of the membrane molecules Fas-L and TNFα in the apoptosis induced by Ca^{2+}/37°C-treated CTLL-R8 extracts. Addition of soluble forms of either TNFR1 or Fas during the 30-min period of Ca^{2+}/37°C treatment did not induce any significant changes in the enhancement of apoptosis (Fig. 3b). In addition, a significant enhancement of apoptosis was observed using macrophages derived from $TNFR1^{-/-}$ knockout mice as target cells (Fig. 3c).

Requirement for Ca^{2+} and temperature We subsequently examined the effect of Ca^{2+} concentration in the treatment of crude extracts of CTLL-R8 by incubating aliquots at

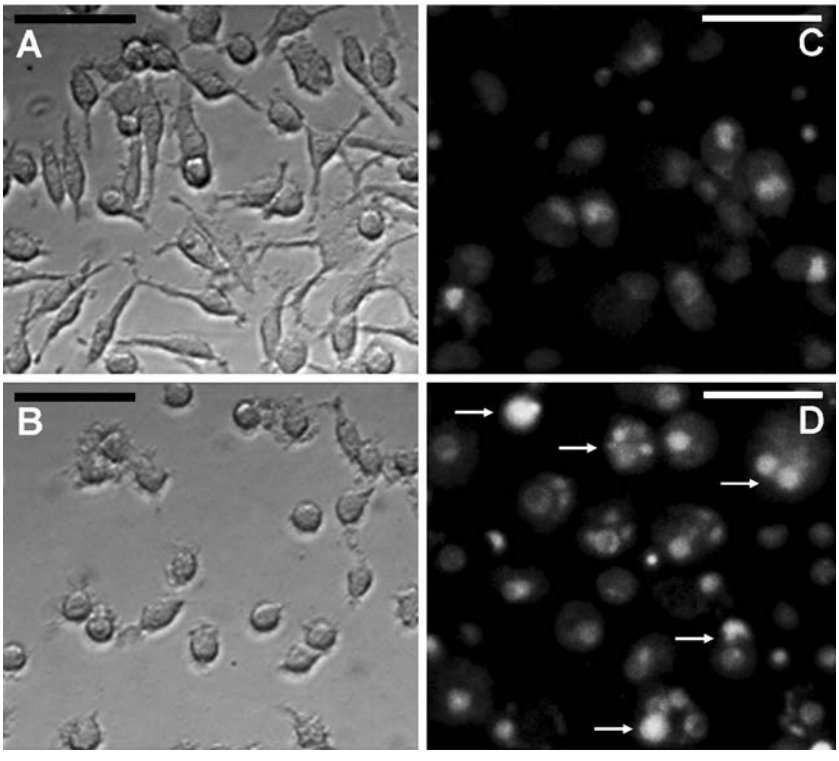

Fig. 2 Alterations in cell morphology accompanying cell death. Adherent macrophages were incubated for 3 h in the absence or presence of MC fractions of CTLL-R8 cells in a cell equivalent dose of 10 HU/200 µl and observed under phase-contrast microscopy. **a**, **c** Control cultures. **b**, **d** MC fractions pre-treated with 1 mM Ca^{2+} at 37°C for 30 min or with 10 µM acridine orange and observed under a fluorescence microscope (*arrows* fragmented nuclei). *Bars* 50 µm

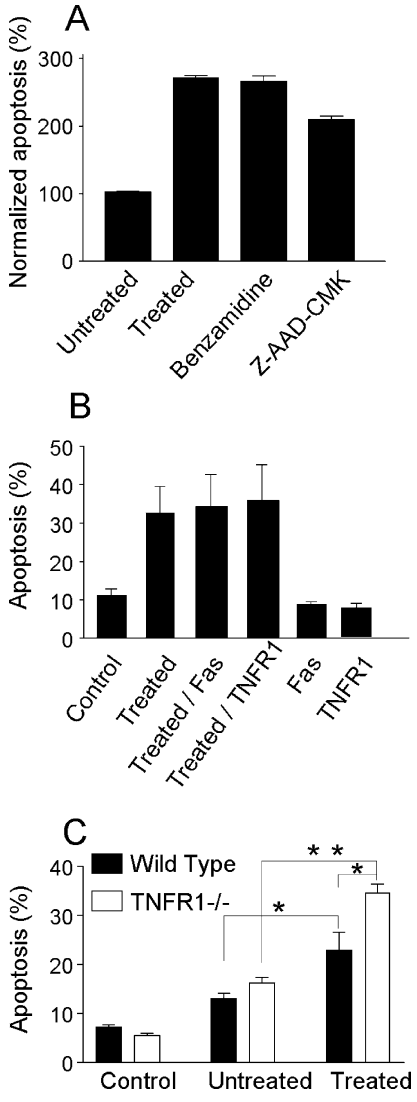

Fig. 3 Perforin and Fas-L-independent enhancement of apoptosis. CTLL-R8 cell extracts equivalent to 10 HU were treated with 1 mM Ca^{2+} at 37°C for 30 min or left untreated and tested for their ability to induce apoptosis (% hypodiploid nuclei) in freshly isolated intraperitoneal cells after 3 h. Control cells were incubated under the same conditions with the addition of buffer only (*bars* mean ± standard error of three experiments performed in triplicate). **a** Apoptosis induced by extracts treated either in the absence or presence of serine-protease inhibitor benzamidine (1 mM) and granzyme-B inhibitor Z-AAD-CMK (1 mM). **b** Crude cell extracts (10 HU) were incubated either in the absence or presence of 1.5 μg/ml soluble TNFR1 or 0.04 μg/ml soluble Fas receptor during the treatment step (30 min, 37°C, 1 mM Ca^{2+}). Apoptosis induced in target cells was measured after 3 h. Neither TNFR1 nor Fas receptor alone induced any significant apoptosis when added to target cells at the same final concentration (*Fas*, *TNFR1*). **c** Apoptosis induced by untreated and treated extracts in cells obtained from either wild-type (*filled bars*) or $TNFR1^{-/-}$ knockout mice (*open bars*). **P*<0.05, ***P*<0.01 when results obtained with treated extracts were compared with untreated extracts or as indicated. In **b**, there were no statistically significant differences (*P*>0.05) between treated, treated/Fas, and treated/TNFR1 (not shown)

37°C for 30 min in PBS containing exogenous Ca^{2+} at concentrations ranging from 0 to 5 mM. After this period, target cells were then incubated for 3 h with these CTLL-R8 extracts in complete culture medium assayed for apoptosis. As shown in Fig. 4a, apoptosis was induced only by cell extracts pre-treated in the presence of Ca^{2+} at concentrations above 0.1 mM and saturated at 1 mM. The addition of up to 5 mM Mg^{2+} to the buffer containing 1 mM Ca^{2+} did not change the enhancement of apoptosis (data not shown). Induction of apoptosis decreased when the treatment step was performed in the absence of any added divalent cations (second bar in Fig. 4a) and was abolished by the addition of either EGTA or EDTA (last two bars, right, in Fig. 4a).

To characterize further the mechanism involved in the enhancement of apoptosis, crude extracts of CTLL-R8 were submitted to different temperatures in the presence of 1 mM Ca^{2+} (Fig. 4b,c). Enhancement of apoptosis was observed when the extracts were treated for 30 min at 37–90°C but not at 0–25°C (Fig. 4b). This phenomenon was significantly greater when extracts that were previously treated at 37°C were further submitted to an additional 5-min incubation at 98°C (Fig. 4c). In contrast to apoptosis, the cell death measured by the release of LDH was completely abolished by treatment at 98°C (Fig. 4c).

The resistance to high temperature prompted us to investigate the effect of proteinase K (PK) digestion. Crude CTLL-R8 extracts were submitted to 50 μg/ml PK for 15 min either before or after the Ca^{2+}/37°C treatment. No significant difference was observed in apoptosis induction by treated extracts (data not shown). However, the extracts digested by PK alone, in the absence of Ca^{2+}, lost both their hemolytic and BLT activities (data not shown), indicating that proteolysis was effective.

Involvement of an $sPLA_2$ in the enhancement of apoptosis

The experiments described above led us to hypothesize the involvement of an $sPLA_2$. These enzymes possess six to nine disulfide bonds that make them resistant to thermal denaturation and protease attack (Six and Dennis 2000; Capper and Marshall 2001). The $sPLA_2$ are Ca^{2+}-dependent in the millimolar range, and some snake $sPLA_2$ are thermoresistant for up to 240 min at 80°C (Fuly et al. 1997). To address this hypothesis, we treated the extracts at 37°C for 30 min in the presence of either pBPB or SCA, inhibitors of $sPLA_2$ (Deem et al. 1987; Thommesen et al. 1998). Both drugs inhibited the enhancement of apoptosis without affecting the release of LDH (Fig. 5a). These effects clearly showed the specific action of these drugs on the CTLL-R8 extracts since neither the addition of the solvent (DMSO) nor the drugs alone significantly affected the basal levels of apoptosis and LDH release (data not shown). Moreover, the addition of pBPB directly to the target cells during the 3-h period did not significantly affect the induction of apoptosis by the Ca^{2+}/37°C-treated CTLL-R8 extracts (Fig. 5b). These results were consistent with the hypothesis that an $sPLA_2$ present in CTLL-R8 extracts reacted with a substrate present in the same extract, inducing the release of an apoptosis-inducing factor. Alternatively, this putative $sPLA_2$ could itself have been activated and might have directly acted as the apoptosis-inducing factor. To characterize further the involvement of an $sPLA_2$, we investigated the effects of AA

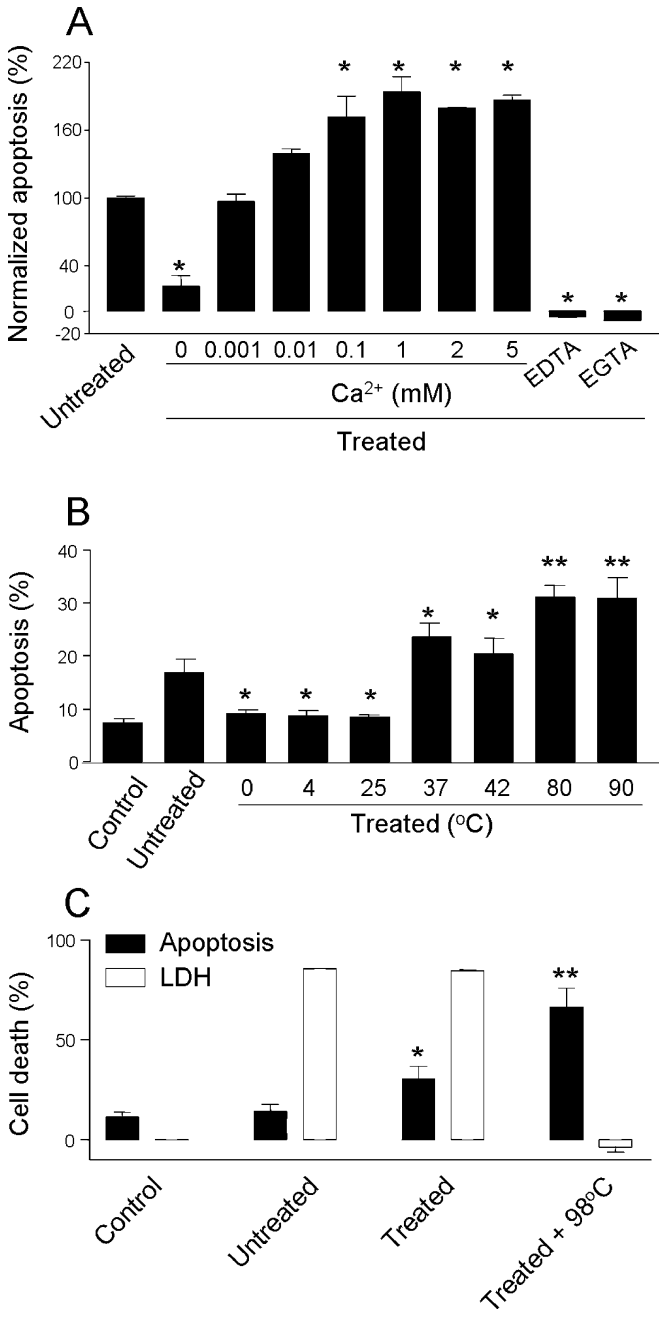

Fig. 4 Ca^{2+} and temperature dependence of the enhancement in apoptosis. Apoptosis and LDH release by freshly isolated intraperitoneal cells were assayed after a 3-h incubation at 37°C with 10 HU equivalents of CTLL-R8 cell extracts prepared under several conditions (*bars* mean ± standard error of three experiments performed in triplicate). **a** Cell extracts were treated at 37°C for 30 min in PBS containing 0, 0.001, 0.01, 0.1, 1, 2, or 5 mM Ca^{2+} (obtained by the addition of $CaCl_2$), or 0 mM Ca^{2+} plus 1 mM EGTA or 1 mM EDTA. **b** Cell extracts were either untreated or treated for 30 min with 1 mM Ca^{2+} at 0, 4, 25, 37, 42, 80, and 90°C in PBS. **c** Cell extracts were either untreated or treated for 30 min with 1 mM Ca^{2+} at 37°C in PBS, followed by an additional period of 5 min at 98°C. Results obtained from untreated samples were used as references for statistical comparisons. **P*<0.05, ***P*<0.01

and LPA, two metabolites of $sPLA_2$, on target cell apoptosis and showed that AA but not LPA could induce apoptosis in macrophages (Fig. 5c).

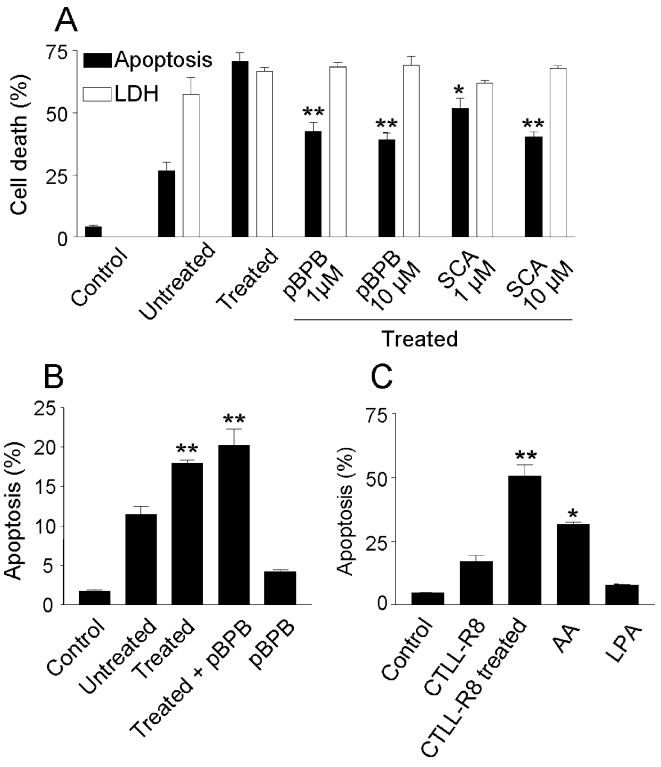

Fig. 5 Involvement of a secretory PLA_2 ($sPLA_2$) in the enhancement of apoptosis. Apoptosis and LDH release by freshly isolated intraperitoneal cells were assayed after 3 h incubation at 37°C with 10 HU/200 μl equivalents of CTLL-R8 cell extracts prepared under several conditions (*bars* mean ± standard error of three experiments performed in triplicate). **a** Crude extracts were treated at 37°C in the presence of 1 mM Ca^{2+} for 30 min in the presence or absence of 1 μM or 10 μM SCA or pBPB. **b** Cell extracts were treated (37°C/Ca^{2+}) as in **a** in the absence of drugs and added to target cells either in the absence (*treated*) or in the presence of 10 μM pBPB (*treated + pBPB*). The induction of apoptosis by pBPB alone is also shown (*pBPB*). **c** Target cells were incubated in the presence of untreated and treated (37°C/Ca^{2+}) CTLL-R8 extracts, 25 μM arachidonic acid (*AA*) and 10 μM lysophospholipid (*LPA*) for 3 h at 37°C. Cell death obtained by treated extract was used as the reference for statistical analysis in **a**. In **b** and **c**, apoptosis induced by untreated extracts was used as the reference. **P*<0.05, ***P*<0.01

Sub-cellular localization of the apoptosis-inducing factor

Cell death induced by CTLs and NK cells is thought to involve granule-contained and/or membrane-bound molecules. To investigate possible apoptosis-inducing activity in cellular fractions, CTLL-R8 cells were disrupted by N_2 cavitation and submitted to differential centrifugation to isolate fractions with nuclei (N) and organelles (O) and a supernatant fraction containing microsomes plus cytoplasm (MC). In some experiments the post-nuclear fraction was also submitted to a discontinuous Percoll density gradient in order to isolate perforin-containing cytolytic granules (G). Each fraction was submitted to protein dosage analysis, enzymatic assays for granzyme A and B and AP activity, and perforin detection by hemolytic activity. Protein was more concentrated in MC fractions, granzyme A and AP activities were present in both MC and O fractions, and granzyme B and hemolytic activity was present in O fractions and in purified granules (data not

shown). Each fraction was tested for its ability to induce cell death before and after treatment for 30 min at 37°C in the presence of 1 mM Ca^{2+}. Only MC fractions displayed a level of enhancement of apoptosis similar to the crude cell extract (Fig. 6a). We submitted MC fractions to ultracentrifugation at 100,000*g* and showed that the capacity to induce enhancement of apoptosis was present in the supernatant (Fig. 6b). The pro-apoptotic activity of this supernatant was also inhibited by pBPB and SCA (data not shown). Contrary to the induction of apoptosis, the induction of necrosis (LDH release) was significantly reduced by the Ca^{2+}/37°C treatment of MC fractions and absent in the MC supernatant (Fig. 6c). These results indicated that the pro-apoptotic factor that was enhanced by the Ca^{2+}/37°C treatment could be separated from the necrosis-inducing activity of the cell extracts and was concentrated in the high-speed supernatant of the MC fractions. To investigate further the localization of the pro-apoptotic activity, we performed a lipid extraction experiment with the MC fraction before Ca^{2+}/37°C treatment. This process yielded three samples: a hydrophilic fraction containing most of the proteins, a hydrophobic fraction containing total lipids, and an interface containing mostly amphiphilic molecules as previously described (Horwitz and Perlman 1987). These fractions were then treated for 30 min with 1 mM Ca^{2+} at 37°C and assayed for their ability to induce apoptosis. Only the interface sample displayed enhanced apoptosis-inducing activity after treatment (Fig. 6d) without significant induction of LDH release (data not shown). In addition, the hydrophobic phase plus the hydrophilic phase were unable to enhance the pro-apoptotic activity together (data not shown).

Inhibition of apoptosis by an anti-sPLA2 antibody and Western blot In order to characterize further the involvement of an $sPLA_2$ in the induction of apoptosis, we investigated the effects of an affinity-purified rabbit polyclonal antibody prepared against PLA_2 from *Crotalus*

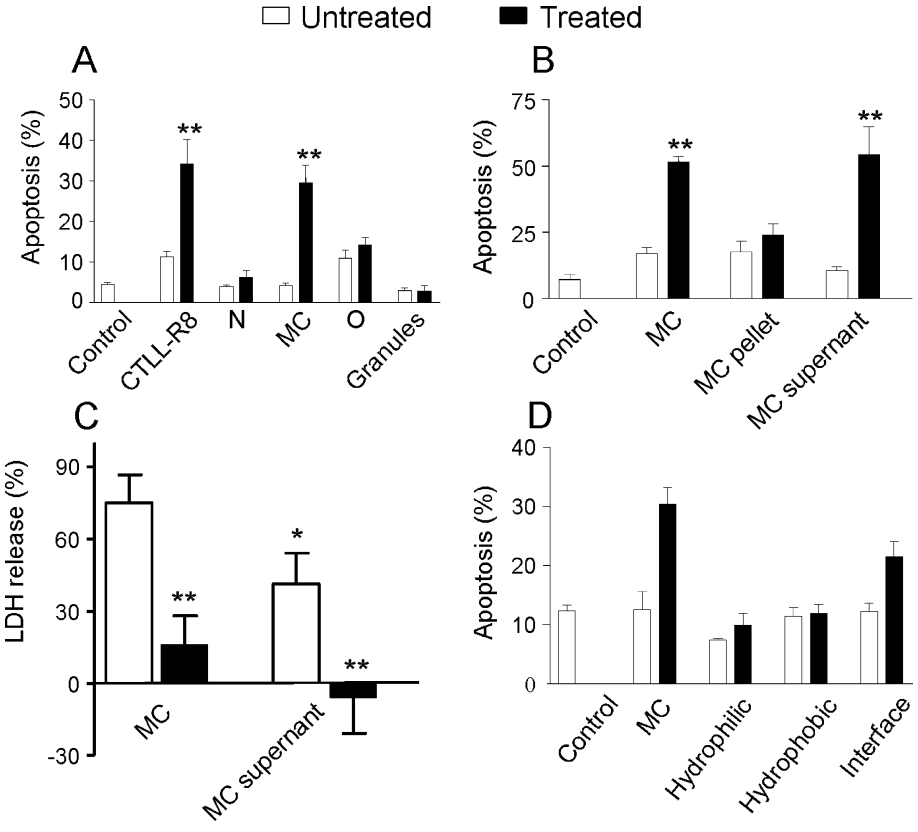

Fig. 6 Sub-cellular localization of the factor involved in the enhancement of apoptosis. CTLL-R8 cells were disrupted by N_2 cavitation and submitted to differential speed centrifugation and density separation in a discontinuous Percoll gradient. Each pellet was resuspended in the original volume of relaxation buffer (*CTLL-R8* whole cell suspension after N_2 cavitation, *N* nucleus fraction obtained after low-speed centrifugation, *MC* nucleus-free and organelle-free supernatant containing microsomes and cytoplasm, *O* pellet containing organelles, *Granules* hemolytic fraction obtained by separation on a discontinuous Percoll gradient). Each sample was used either untreated (*open bars*) or after treatment for 30 min at 37°C in the presence of 1 mM Ca^{2+} (*filled bars*). Each sample contained the equivalent of 10 HU (approximately 2–5×10^6 CTLL-R8 cells). Freshly isolated peritoneal cells were used as targets, and apoptosis and LDH release were measured after a 3-h incubation (*bars* in **a**–**c** three experiments performed in triplicate, *bars* in **d** mean ± standard error of two experiments performed in triplicate). **a** Apoptosis induced by the various cellular fractions. **b** MC samples were submitted to high-speed centrifugation (100,000*g* for 2 h at 4°C), and the pellet was suspended in the same volume of relaxation buffer. Apoptosis was determined as in **a**. **c** LDH release by MC pellet (*MC*) and MC supernatant. **d** The MC sample was submitted to a lipid-extraction procedure. Each of the three phases (hydrophobic, hydrophilic, and interface) was assayed for induction of apoptosis. Apoptosis induced by each untreated sample was used as the reference for statistical analysis. **P*<0.05, ***P*<0.01

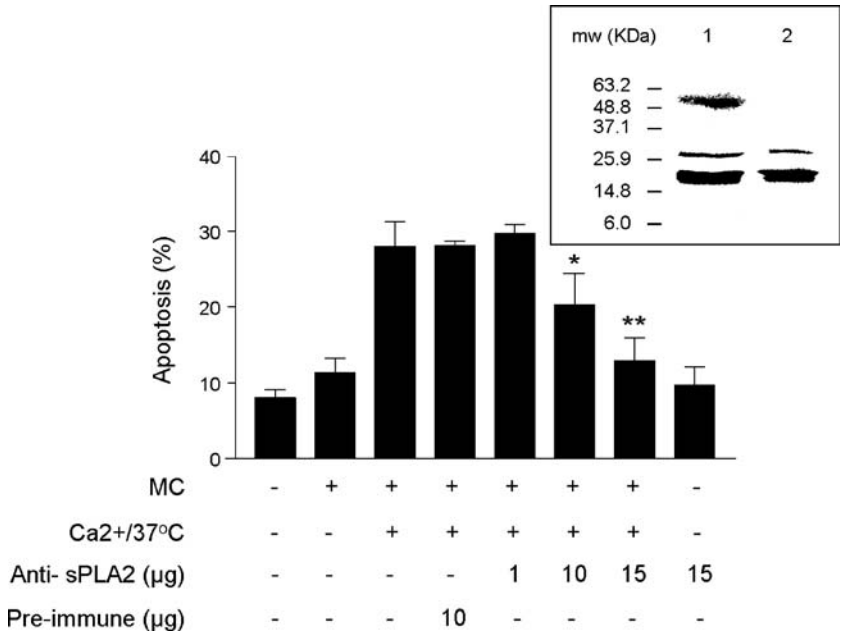

Fig. 7 Inhibition of apoptosis by an anti-sPLA2 antibody. MC fractions (equivalent to 2×10^6 cells) were treated at 37°C in the presence of 1 mM Ca^{2+} for 30 min and then kept for an additional 30 min in the presence or absence of the indicated amounts (microgram IgG in 100 µl of PBS) of an affinity-purified rabbit antibody prepared against an $sPLA_2$ from *Crotalus durissus terrificus*. Alternatively, pre-immune immunoglobulin was used as a substitute for the anti-$sPLA_2$ antibody as indicated. Target cells were then incubated for 3 h in the presence of untreated and treated MC fractions, and apoptosis was measured as the percentage of hypodiploid nuclei (*bars* mean ± standard error of three experiments performed in triplicate). Cell death obtained by treated MC fractions was used as the reference for statistical analysis of the effects of anti-$sPLA_2$ antibody, as indicated. **P*<0.05, ***P*<0.01. *Inset*: Western blot analysis of nuclei-free CTLL-R8 extract in a 8%–25% SDS–polyacylamide gel. Each lane was loaded with 40 µg protein mixed with sample buffer containing 2.5% 2-mercaptoethanol and 10% SDS (final concentration). *Lane 1* Sample was kept on ice until loading and was not heated, *lane 2* sample was heated at 98°C for 5 min before loading

durissus terrificus. Samples of Ca^{2+}/37°C-treated MC fractions were incubated either with the anti-PLA_2 antibody or with pre-immune immunoglobulin for 30 min and then added to the target cells. The anti-PLA_2 antibody displayed a dose-dependent inhibition of the apoptosis, an effect that was not mimicked by the pre-immune immunoglobulin (Fig. 7). A similar effect was observed by using the unpurified anti-PLA_2 serum (data not shown). In addition, Western blots with the same anti-PLA_2 antibody identified four bands (Fig. 7 inset). The 56-kDa band was only visible when the sample was not boiled in the presence of 2-mercaptoethanol (Fig. 7 inset, lane 1).

Discussion

We presented evidence that an $sPLA_2$ produced by a CTL line can induce target cell death. When treated by pre-incubation at 37°C for 30 min in the presence of Ca^{2+}, cell extracts derived from CTLL-R8 show an enhanced apoptosis-inducing activity compared with untreated extracts. This phenomenon is independent of the granule proteins (granzyme or perforin) and does not involve the Fas and TNF pathways. Perforin and granzyme involvement has been dicounted because of the inactivating hemolytic activity by Ca^{2+}/37°C treatment and by the use of a granzyme inhibitor (Fig. 3a). The participation of TNF and Fas-L has been discounted by using TNFR1 knockout mice, soluble TNFR1, and Fas (Fig. 3b,c). Moreover, cell fractionation experiments (Fig. 6) have shown that the apoptosis-inducing factor present in CTLL-R8 extracts is located in the membrane-free or soluble (100,000*g*) cell fraction, whereas Fas-L and perforin are located in membrane-bound organelles and in the plasma membrane (Barry and Bleackley 2002). The finding that the same pro-apoptotic activity is present in extracts of primary cultures of cytotoxic LAK cells indicates that the phenomenon that we are studying is not a particular property of the CTLL-R8 cell line used in most of our experiments.

The involvement of an $sPLA_2$ is suggested by the observation that apoptosis can be blocked by pBPB and SCA, two inhibitors of $sPLA_2$, and by a rabbit antibody raised against a PLA_2 of *Crotalus durissus terrificus*. The same antibody detects four bands in Western blots of CTLL-R8 cell extracts. The 56-kDa band disappears when the sample is boiled in the presence of 2-mercaptoethanol, suggesting that the antibody recognizes a structural epitope present in the $sPLA_2$.

The molecular identity of the CTLL-R8-derived $sPLA_2$ is still unclear. However, although most phospholipases have a molecular weight in the range 13–19 kDa, group III $sPLA_2$ is the only $sPLA_2$ so far described that has a similar molecular weight (55 kDa; Valentin et al. 2000; Six and Dennis 2000; Diaz and Arm 2003; Murakami and Kudo 2004). In addition, group III $sPLA_2$ has been shown to induce apoptosis in rat primary cortical neuronal cells

(DeCoster 2003). These data suggest that a group III $sPLA_2$ is expressed by CTLL-R8 and is involved in the induction of apoptosis by treated cell extracts. However, further experiments are necessary to confirm the molecular identity of the pro-apoptotic factor and the molecular processing triggered by the Ca^{2+}/37°C treatment.

In addition to having demonstrated the putative apoptosis-inducing $sPLA_2$, our data suggest that the crude cell extract displays pro-necrotic cytotoxic activity, as judged by the LDH-release experiments (e.g., Fig. 1d). However, the factor involved in this process differs from the putative $sPLA_2$, because it is not affected by the Ca^{2+}/37°C treatment (Fig. 1d), it is completely abrogated by heating at 98°C (Fig. 4c), it is insensitive to pBPB treatment (Fig. 5a), and it is not present in the 100,000*g* soluble fraction that contains most of the pro-apoptotic activity (Fig. 6c).

The possibility of the involvement of phospholipases in the killing mechanisms of CTLs and NK cells has been previously reported (Frye and Friou 1975; Hoffman et al. 1981; Carine and Hudig 1984; Namiuchi et al. 1984), but the effect was thought to occur in steps inside the effector cell at the beginning of the lytic process (Deem et al. 1987). Interestingly, although no evidence was presented indicating that an NK-cell-derived secretory PLA_2 could act as an effector molecule, an exogenously added PLA_2 acted at the target cell membrane and enhanced NK cell cytotoxicity (Deem et al. 1987). More recent data support the view that an NK-cell-derived $sPLA_2$ plays an important role in some intracellular events that precede granule exocytosis (Cifone et al. 1993; Milella et al. 1999; Whalen et al. 1999). However, none of these reports provide evidence of the direct involvement of this $sPLA_2$ as an effector molecule of cytotoxicity. Moreover, although the release of $sPLA_2$ occurs simultaneously with the exocytosis of granzyme-containing granules, its precise intracellular localization has not been established (Milella et al. 1997). Our data indicate that the pro-apoptotic activity lies in the organelle-free supernatant (MC fraction) and can be partitioned into the interface of the chloroform-methanol extract, implying that it has amphiphilic properties; this is consistent with the presence of a soluble molecule that also displays affinity for the lipid moiety, as expected for a phospholipase (Gelb et al. 2000). Further experiments are required in order to study its mechanism of secretion.

The above evidence, together with the data presented in this work, raises the possibility that NK cells and CTLs secrete one or more $sPLA_2$ that could induce cell death by apoptosis. The mechanism involved in this process is not as yet clear. One possibility is that this putative $sPLA_2$ bypasses normal pro-apoptotic stimuli and directly triggers a pre-existent intracellular pathway leading to cell death. This molecular strategy has a precedent in the case of granzyme B, which can act as a caspase (Barry and Bleackley 2002). However, although the involvement of some intracellular PLA_2 in the cascade of intracellular events triggered by death-inducing factors has been proposed (Capper and Marshall 2001; Shinzawa and Tsujimoto 2003; Cummings et al. 2000), this possibility seems unlikely as pBPB is able to inhibit apoptosis only if it is added before pre-treatment of cell extracts (Fig. 5b).

Another possibility is that the effector molecule is a product of PLA_2 enzymatic activity generated during the Ca^{2+}/37°C pre-treatment step. Our own data (Fig. 5c) and data from other groups indicating that AA (Koller et al. 1997; Whalen et al. 1999) and lyso-phospholipids (Masamune et al. 2001; Whalen et al. 1999) can induce cell death in some cells seem to support this possibility. However, the experiments performed with the phases obtained after the lipid extraction procedures (Fig. 6d) have demonstrated that the pro-apoptotic activity is concentrated in the non-lipid amphiphilic phase (interface) and does not change after reconstitution with the hydrophobic fraction. Therefore, a more reasonable hypothesis to explain our data is that the putative $sPLA_2$ requires a pre-activation, maturation, or aggregation step that is sensitive to pBPB and SCA, and that, after activation, it directly acts within the target cell to induce apoptosis through a non-enzymatic (pBPB-independent) mechanism, possibly via $sPLA_2$ receptors that are present in macrophages and other cells (Lambeau and Lazdunski 1999). The ability of the anti-$sPLA_2$ to inhibit apoptosis, even when added after the Ca^{2+}/37°C pre-treatment step, is consistent with this hypothesis. The requirement of a specific receptor would also explain why CTLL-R8 extracts do not induce apoptosis in some cell lines (Fig. 1e).

Cytotoxic lymphocytes and NK cells use multiple killing mechanisms that make them more efficient when facing the various escape mechanisms employed by viruses and tumor cells. The existence of a perforin/granzyme-independent and Fas-L-independent mechanism of cytotoxicity has previously been suggested (Lee et al. 2004; Ahmed et al. 1997). However, the contribution of secretory phospholipases to the overall mechanism of cell death remains to be clarified. Further developments in this field will require the molecular identification of the $sPLA_2$ characterized here and the development of more specific reagents.

Acknowledgements The authors wish to thank Drs. Russolina Zingale, Paulo Melo, Bruno L. Diaz, Bartira R. Bergmann, and Leda Q. Vieira for donating reagents, animals, and inhibitors, and Drs. João Viola, George dos Reis, Rodrigo C. Bisaggio, Robson Coutinho-Silva, André Fuly, Vera Koatz, Luciana Nogaroli, Cristiane M. Cruz, Miriam Werneck, and Camila Chaves Santos for helpful discussions.

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