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B Cell Receptor-Stimulated Mitochondrial Phospholipase A\textsubscript{2} Activation and Resultant Disruption of Mitochondrial Membrane Potential Correlate with the Induction of Apoptosis in WEHI-231 B Cells\textsuperscript{1}

Elad Katz,* Maureen R. Deehan,* Sandra Seatter,* Caroline Lord,* Roger D. Sturrock,† and Margaret M. Harnett\textsuperscript{2,*}

Cross-linking of the Ag receptors on the immature B cell lymphoma, WEHI-231, leads to growth arrest and apoptosis. We now show that although commitment to such B cell receptor (BCR)-mediated apoptosis correlates with mitochondrial phospholipase A\textsubscript{2} activity, disruption of mitochondrial function, and ATP depletion, it is executed independently of caspase activation. First, we demonstrate a pivotal role for mitochondrial function in determining B cell fate by showing up-regulation of cytosolic phospholipase A\textsubscript{2} expression, induction of mitochondrial phospholipid A\textsubscript{2} activity, arachidonic acid-mediated collapse of mitochondrial transmembrane inner potential ($\Delta\psi_{\text{mt}}$) and depletion of cellular ATP under conditions of apoptotic, but not proliferative, signaling via the BCR. Importantly, disruption of $\Delta\psi_{\text{mt}}$, ATP depletion, and apoptosis can be prevented by rescue signals via CD40 or by $\Delta\psi_{\text{mt}}$ stabilizers such as antimycin or oligomycin. Second, we show that commitment and postmitochondrial execution of BCR-mediated apoptosis are not dependent on caspase activation by demonstrating that such apoptotic signaling does not induce release of cytochrome c from the mitochondria or activation of effector caspases, as evidenced by poly(ADP-ribose) polymerase or Bcl-x\textsubscript{L} cleavage. Indeed, apoptotic signaling via the BCR in WEHI-231 B cells does not stimulate the activation of caspase-3 and, consistent with this, BCR-mediated disruption of $\Delta\psi_{\text{mt}}$ and commitment to apoptosis take place in the presence of caspase inhibitors. In contrast, BCR signaling induces the postmitochondrial activation of cathepsin B, and resultant apoptosis is blocked by the cathepsin B inhibitor, (23,35\textsuperscript{trans}-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST) suggesting a key role for this executioner protease in Ag receptor-driven apoptosis of WEHI-231 immature B cells. \textit{The Journal of Immunology}, 2001, 166: 137–147.

A

Apoptosis plays a key role in the regulation of the immune system. For example, the immune system has evolved selection processes that result in clonal deletion, by apoptosis, of autoreactive B and T lymphocytes during their development in the bone marrow and thymus, respectively. Moreover, apoptosis provides a major molecular mechanism not only for the induction of peripheral tolerance and cytotoxic T cell killing, but also for the termination of normal immune responses (1–3). Whereas cells that die from damage typically swell and burst (necrosis), apoptosis is a rigorously controlled and highly ordered process that is characterized by dramatic morphological changes in the cell, including shrinkage, chromatin condensation and cleavage, and disassembly into membrane-enclosed vesicles called apoptotic bodies that are rapidly phagocytosed by neighboring cells to prevent induction of inflammation and autoimmune responses (4, 5). This systematic disassembly of the apoptotic cell appears generally to be executed by caspases, cysteinyI aspartate-specific proteases, which have recently been identified as mammalian homologues of the cell death protein, CED-3, from the nematode \textit{Caenorhabditis elegans} (6, 7). Indeed, molecular studies have shown that overexpression of caspases is sufficient to cause apoptosis, and caspase-deficient mice have reduced levels of apoptosis (8, 9). However, examples of caspase-independent commitment to cell death can be found in several of the classic models of apoptosis, including glucocorticoid-induced death of thymocytes and lymphoid cells, death of hemopoietic cell lines induced by growth factor withdrawal, and Bax-mediated cell death (10–12).

It has recently emerged that mitochondrial function plays a pivotal role in determining cellular commitment to survival or apoptosis (12–14). Thus, during apoptotic signaling, mitochondrial changes result in enhanced production of reactive oxygen species (ROS),\textsuperscript{3} calcium cycling, and disruption of the inner mitochondrial potential (15). Collapse of the mitochondrial potential has been thought to represent a point of no return in committing the cell to apoptosis as the resulting increase in the permeability of the outer mitochondrial membrane leads to the release of caspases and factors that promote activation of effector caspases (cytochrome c)

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and/or induce apoptosis (apoptosis-inducing factor and a caspase-independent endonuclease) (16–21). Mitochondrial integrity has been proposed to be regulated by pro- and anti-apoptotic members of the Bcl-2 family (18, 22), as these regulators of cell survival or apoptosis appear to target a number of aspects of mitochondrial function, including mitochondrial permeability and homeostasis of ROS status and calcium cycling.

Cell death receptors such as TNF-R and Fas (also known as CD95 or APO-1) mediate much of the rapid apoptotic cell death required by the immune system (23). They initiate apoptosis by directly recruiting pro-caspases belonging to the IL-1-converting enzyme-like family, such as caspase-1 or -8, to their accessory death domain-transducing molecules, Fas-associated death domain protein, TNF-R-associated death domain protein, receptor-interacting protein, and RIP-associated ICH-1/CED-3-homologous protein with a death domain to induce proteolytic activation of effector caspasess (caspase-3 (CPP32)-like subfamily) that have proved to be important for the execution of the later stages of apoptosis (23). However, repertoire selection during lymphocyte development is mediated via the Ag receptors (1–3, 23). We now show that Ag receptor-driven commitment to apoptosis in the immature B cell lymphoma, WEHI-231, is associated with mitochondrial phospholipase A₂ (PLA₂) activation, disruption of mitochondrial potential, and profound depletion of cellular ATP levels, but does not cause release of cytochrome c from mitochondria and is independent of caspase activation. Although mitochondrial potential disruption is uncoupled from caspase activation, activation of proteases such as cathepsins does appear to play a role in the postmitochondrial execution of apoptosis by WEHI-231 immature B cells. The physiological relevance of this novel mechanism of lymphocyte apoptosis to the negative selection of autoreactive B cells is supported by recent studies on germinal center B cells that require cathepsin activity for apoptosis (24) and by caspase-deficient mice that were shown to have no substantial defects in B cell selection and development (8, 9, 25).

Materials and Methods

Cells, reagents, and Abs

The murine B cell lymphoma, WEHI-231, and the human leukemic T cell line, Jurkat, were cultured in RPMI 1640 medium containing 5% FCS, t-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (RPMI 1640 complete) at 37°C in 5% CO₂. RPMI complete media for WEHI-231 B cells were additionally supplemented with 2-ME (50 µM). Caspase inhibitors N-benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk), acetyl-Asp(OMe)-Glu-Val-Asp(OMe)-fluoromethylketone (z-VEID-fmk) and (25,35)trans-epoxysuccinyl-l-leucylamido-3-methylbutanethylester (VATEF), antymycin A, and oligomycin were obtained from Calbiochem (Cambridge, MA). Purified anti-IgM mAbs (anti-mouse µ-chain), anti-CD40, and anti-human CD3 Abs were produced from the B7.6, EGK45, and OKT3 hybridomas, respectively, as described previously (26, 27). Additional Abs for Western blotting were obtained as follows: anti-cytosolic phospholipase A₂ (PLA₂) (26) and anti-cytochrome c from PharMingen (San Diego, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA), anti-poly(A)-ribosome (PARP) from Santa Cruz Biotechnology, and anti-cleaved PARP from New England Biolabs (Hitchin, Herts, U.K.).

DNA synthesis (thymidine uptake)

Exponentially growing cells (10⁴ cells/well) in RPMI 1640 complete medium supplemented with 2-ME (50 µM), sodium pyruvate (1 mM), and nonessential amino acids (1%) were stimulated for 44 h at 37°C in RPMI complete media for an additional 4 h. The cells were harvested, and the level of [³H]thymidine incorporated into DNA was measured by liquid scintillation counting (26, 27).

Analysis of apoptosis

Flow cytometry analysis of annexin V binding to phosphatidylserine on the cell surface.

Cells to be examined for annexin V expression were washed in PBS and incubated with annexin V-biotin conjugate, in defined calcium and magnesium concentrations, according to the manufacturer’s instructions (Boehringer Mannheim, Lewes, East Sussex, U.K.). The cells were washed and then incubated with streptavidin-FITC for 15 min and washed by centrifugation. Cells were immediately analyzed using a Becton Dickinson FACScan using Lysis II software (Becton Dickinson, Mountain View, CA) for analysis (27).

Flow cytometry analysis of DNA content and cell cycle analysis.

Cells were analyzed for propidium iodide (PI) incorporation as described previously (27, 28). At least 10⁴ stained cells were analyzed for PI fluorescence at an excitation wavelength of 488 nm on a Coulter Epics XL flow cytometer (Coulter, Luton, U.K.).

Flow cytometry analysis of mitochondrial potential.

Incorporation of the cationic lipophilic dye DiOC₆ into the mitochondria is proportional to the mitochondrial transmembrane potential (ΔΨm) (29). Cells were incubated for 30 min with 50 nM DiOC₆ (3) (Molecular Probes, Eugene, OR) and then washed once in PBS. At least 10⁴ stained cells were analyzed using a Becton Dickinson FACScan using Lysis II software for analysis.

APF determinations

ATP levels were measured using a commercial luciferase kit, ViaLight HS (Lumitech, Nottingham, U.K.), and a TD-20e luminometer (Turner, Mountain View, CA).

Cell stimulation and lyse preparation

WEHI-231 cells (10⁶ cells) were stimulated as indicated, reactions terminated by the addition of 2× ice-cold lysis buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 2% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate, 0.5 mM PMSF, chymostatin (10 µg/ml), leupeptin (10 µg/ml), antipain (10 µg/ml), and pepstatin A (10 µg/ml)), and lysates solubilized for 30 min on ice before centrifugation at 12,000 rpm for 15 min. The resulting supernatants were used for Western blot analysis (26).

Preparation of mitochondria-free extracts

Mitochondria-free extracts were prepared as described previously (30). Briefly, 10⁶ cells were washed in PBS and resuspended in extraction buffer (50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 220 mM mannitol, 68 mM sucrose, 1 mM DTT, 10 µM cytochalasin B, and protease inhibitors). Cells were left on ice for 30 min and then lysed with a glass homogenizer, with 40 strokes of the B pestle. Finally, cells were centrifuged at 14,000 × g for 15 min, and the mitochondria-free supernatant and mitochondrial pellets were taken for Western blot analysis of cytochrome c release. For isolated mitochondria, mitochondrial pellets were resuspended in mitochondria physiological buffer (MPB) comprising 20 mM HEPES containing 250 mM sucrose, 1 mM EGTA, 5 mM succinate, 3 mM K₂HPO₄, 1.5 mM MgCl₂, 10 mM KCl, and 5 µM Rotenone. Western blotting

Protein lysates (100 µg/well; Pierce MicroBCA protein assay (Rockford, IL)) were resolved by SDS-PAGE (12%) before electrotransfer onto polyvinylidene difluoride (Millipore, Watford, U.K.). Nonspecific binding was blocked at room temperature with 10% nonfat dried milk in PBS/Tween 20 (0.1%) under constant agitation. The primary Ab was incubated with the blot for at least 1 h at room temperature before addition of the appropriate alkaline phosphatase-conjugated secondary Ab (Sigma, St. Louis, MO) in 5% nonfat dried milk for 1 h with constant agitation. The blots were developed with enhanced chemiluminescence substrate (Amersham Life Sciences) or ImmunoStar reagent (Bio-Rad, Hercules, CA) and exposed to film. Prestained molecular weight markers were used to elucidate the molecular weight of unknown proteins. Even protein loading/sample recovery of gels was determined by Poncze Red (Sigma) staining (26).
RT-PCR

Cells (5–10 × 10⁶/sample) were treated as required, then RNA was extracted using RNazol B (Biogenesis, Bournemouth, U.K.), and reverse transcription of total RNA (5 μg) was performed using SuperScript II RT and priming with random hexamers (Life Technologies, Paisley, U.K.) for 50 min at 42°C. Reactions were terminated by heating for 15 min at 70°C. PCR was performed in a total volume of 50 μl containing 1 μl of the RT reaction mixture, 0.2 mM dNTPs, 0.35 μM of each primer, and 1 U of Taq polymerase (Sigma) in a Technne Cyclogene thermal cycler using the following primers for cPLA₂: left primer, 5'-AAATGTCAGCCACACACCTC-3' and right primer, 5'-GGGAGACAGTGAAAGAGGCGG-3' (PCR product of 227 bp) for a total of 32 cycles using the following protocol: (94°C, 5 min) × 1, (94°C, 30 s/56°C, 1 min) × 2, (94°C, 30 s/60°C, 1 min/72°C, 1 min) × 2, (94°C, 30 s/60°C, 1 min/72°C, 1 min) × 30, (72°C, 5 min) × 1. Primers specific for murine β-actin were used as a control: left primer, 5'-GGGCTATGCTCTTCCCTACCCGCTTCCTGCG-3' and right primer, 5'-TTTGCGATAGAGGTCTTACCGGAATGCAGG-3' (PCR product of 389 bp). The PCR products were then resolved by DNA-agarose (1.5%) gel electrophoresis.

cPLA₂ assay

cPLA₂ activity in whole cell lysates or mitochondrial fractions was determined using a commercial cPLA₂ assay kit (Cayman Chemical, Ann Arbor, MI) based on spectrophotometric detection of free thiol by Ellman’s reagent (5,5'-dithio-bis(2-nitrobenzoic acid) following hydrolysis of the arachidonyl thioester bond at the sn-2 position of the cPLA₂ substrate, arachidonyl thio-phosphatidylcholine. A role for calcium-independent phospholipase A₂ (iPLA₂) activity was excluded by the use of the selective inhibitor bromoenol lactone and the requirement for calcium for PLA₂ activity.

In addition, in some experiments, cPLA₂ activity was assessed by measurement of [³H]arachidonic acid release, as described previously (26). This activity was blocked by the inhibitor arachidonitrilfluoromethyl ketone (selective for iPLA₂) and cPLA₂, excluding a role for secretory phospholipase A₂. Briefly, before each experiment, cells were washed, resuspended in fresh isotope-free medium, and cultured for an additional hour at 37°C. The cells were then washed three times in HBSS (pH 7.4) containing 2% (w/v) BSA and 10 mM glucose, resuspended in this buffer at 10⁶ cells/ml, and equilibrated for 30 min at 37°C. Cells (10⁶/sample) were stimulated with anti-Ig (10 μg/ml) or ceramide (25 μM) for 3 or 24 h. WEHI-231 B cells were washed and fixed (5 min) in PBS (pH 7.4) containing 4% (w/v) paraformaldehyde. The cells were then washed in PBS and permeabilized (5 min) in PBS containing 2% FCS, 2 mM EDTA, and 0.1% (w/v) saponin before incubation with the appropriate primary Abs (1 μg/ml) for 30 min at 4°C. The cells were washed again before staining with the relevant FITC-conjugated or biotinylated secondary Ab. Following further washing, the cells were stained simultaneously with streptavidin-Texas Red (Vector Laboratories, Burlingame, CA), 4',6'-diamidino-2-phenylindole (Vector Laboratories), and, in some experiments, the mitochondrial-selective dye rhodamine 123 (500 nM; Molecular Probes, Eugene, OR). In some experiments, the mitochondria were identified by staining with an anti-adenine dinucleotide transporter Ab (a kind gift from P. Schmid, Hornel Institute, University of Minnesota, Austin, MN). In situ immunofluorescence microscopy was performed using an Axiomicroscope microscope (Zeiss, Oberkochen, Germany), charge-coupled device camera, and digital capture program (Signal/Analytics, IP Lab, Vienna, Austria).

Protease activity assays

WEHI-231 cells (5 × 10⁶ cells/sample) were stimulated with anti-Ig (10 μg/ml) or ceramide (25 μM) for 2, 5, 8, or 24 h at 37°C before cell lysates were prepared in 50 mM Tris buffer (pH 7.4) containing 150 mM sodium chloride, 2% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate, and 0.5 mM PMSF. Samples were then incubated for 30 min at room temperature with 100 μM of either cathepsin B substrate, z-Arg-Arg-pNA (zRR-pNA; Calbiochem), or caspase-3 substrate, N-Acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA; Calbiochem), and the resultant generation of cleaved substrate was measured by reading absorbance at 405 nm (31, 32).

Results

Ligation of the Ag receptors on the immature B cell line, WEHI-231, induces growth arrest and apoptosis

Cross-linking of the Ag receptors with anti-Ig leads to a concentration-dependent induction of growth arrest and apoptosis in the B cell lymphoma, WEHI-231 (Fig. 1), which is a widely used model system for investigating the signaling mechanisms underlying growth arrest and apoptosis.
clonal selection of normal IgM^+ IgD^- immature B cells. Growth arrest was assessed by the anti-Ig-mediated suppression of DNA synthesis in WEHI-231 B cells (Fig. 1A), which showed that maximal growth inhibition was essentially achieved at concentrations of anti-Ig between 0.1 and 1 μg/ml. This growth arrest was confirmed by cell cycle analysis, which showed the anti-Ig-driven accumulation of WEHI-231 cells in the G_0-G_1 phase of the cell cycle (70 ± 7% of stimulated live cells vs 47 ± 10% for control live WEHI-231 cells 24 h poststimulation with anti-Ig (1 μg/ml), n = 4 independent experiments). Commitment to apoptosis was assessed by examining the anti-Ig-mediated disruption of mitochondrial membrane potential (as indicated by a decrease in the fluorescence of the mitochondrial dye, DiOC_6, Fig. 1B), expression of phosphatidylserine (annexin V binding, Fig. 1C) at the cell surface, DNA laddering (Fig. 1D), and PI staining of hypodiploid DNA content during cell cycle analysis (Fig. 1C). These studies showed that commitment to apoptosis required higher concentrations of anti-Ig (1–10 μg/ml; Fig. 1D and results not shown) than those needed for induction of growth arrest following cross-linking of the Ag receptors.

**Disruption of the inner mitochondrial potential (ΔΨ_m) plays a key role in Ag receptor-driven apoptosis of WEHI-231 cells:** signals that rescue WEHI-231 B cells from apoptosis stabilize ΔΨ_m

Disruption of mitochondrial function and integrity has been shown to play a central role not only in the commitment to apoptosis, but also in the initiation of the execution of the later stages of apoptosis in many cell systems (12–14). We therefore investigated the role of mitochondrial disruption in Ag receptor-driven apoptosis by characterizing the effects of anti-Ig on the ΔΨ_m. To do this quantitatively, we analyzed the incorporation of the cationic lipophilic dye DiOC_6 staining to demonstrate the extent of depolarization, whereas in B, these data are presented as percentage of cells of low mitochondrial potential as indicated in Fig. 1. Both parameters are presented in D, with the open bars representing the mean fluorescence intensity (y-axis) and the filled bars representing the percentage of cells of low mitochondrial potential (y-axis). The concentration of stimuli and mitochondrial inhibitors used was: anti-IgM, for WEHI-231 cells 10 μg/ml, for splenic B cells 50 μg/ml; anti-CD40, 10 μg/ml; antimycin, 50 ng/ml; and oligomycin, 8 ng/ml. All data are from single experiments representative of at least two other independent experiments.

**Anti-Ig induces a decrease in the ΔΨ_m of WEHI-231 immature B cells, but not mature splenic B cells.** WEHI-231 immature B cells (A, B, and D) or mature splenic B cells (C) were treated with the indicated stimuli, and mitochondrial potential was assessed using the dye DiOC_6 staining. In A, the data are represented as the mean fluorescence intensity of DiOC_6 staining to demonstrate the extent of depolarization, whereas in B, these data are presented as percentage of cells of low mitochondrial potential as indicated in Fig. 1. Both parameters are presented in D, with the open bars representing the mean fluorescence intensity (y-axis) and the filled bars representing the percentage of cells of low mitochondrial potential (y-axis). The concentration of stimuli and mitochondrial inhibitors used was: anti-IgM, for WEHI-231 cells 10 μg/ml, for splenic B cells 50 μg/ml; anti-CD40, 10 μg/ml; antimycin, 50 ng/ml; and oligomycin, 8 ng/ml. All data are from single experiments representative of at least two other independent experiments.

**Anti-Ig-driven apoptosis of WEHI-231 immature B cells is further supported by the finding that co-stimulation via CD40, which has been widely shown to rescue Ag receptor-driven apoptosis (nuclear DNA loss; Fig. 3A) and growth arrest in G_0-G_1 (Fig. 3B), acts to stabilize ΔΨ_m (albeit at an intermediate ΔΨ_m) and prevent the profound dissipation of ΔΨ_m observed in anti-Ig-treated cells (Fig. 2, A and B).** Furthermore, the mitochondrial inhibitors, antimycin (an inhibitor of respiratory chain complex III that blocks the transfer of electrons to cytochrome c, and hence suppresses respiration by blocking proton pumping, ATP synthesis, and oxygen uptake) and oligomycin (an inhibitor of respiratory chain complex V, the F_0F_1-ATPase: oligomycin inhibits proton transport back into the mitochondria by blocking the proton channel, F_0 protein), which can be used to stabilize the mitochondrial potential (33), maintain ATP levels (34), and protect against apoptosis (35), are not only capable of maintaining almost normal ΔΨ_m levels even in the presence of anti-Ig (Fig. 2D), but are also able to protect against the surface Ig(sIg)-driven commitment of WEHI-231 immature B cells to apoptosis (nuclear DNA loss; Fig. 3A). Like CD40 signaling, the mitochondrial inhibitor antimycin, and oligomycin to a very much lesser extent, is also able to prevent anti-Ig-mediated arrest in G_0-G_1, as indicated by its ability to protect entry into S phase (Fig. 3B). However, unlike the CD40 rescue signals, they are unable to allow cell cycle progression through G_2-M (Fig. 3B). Together, these data suggest that mitochondrial function not only plays a central role in the commitment to death signaling in B cells, but also in the reversible induction of growth arrest by anti-IgM (36).

**Ag receptor-mediated disruption of the mitochondrial potential results in cellular ATP depletion and is associated with induction of mitochondrial PLA2 activity**

Loss of mitochondrial function and integrity has been shown to contribute to the effector stages of apoptosis via production of ROS, ATP depletion, calcium cycling, and release of cytochrome...
caspases, and apoptosis-inducing factors. Since our data clearly showed disruption of mitochondrial function, we investigated the potential downstream effector mechanisms involved in the execution of Ag receptor-driven apoptosis of WEHI-231 immature B cells. First, we investigated the role of ATP depletion in Ag-driven apoptosis of WEHI-231 cells: cross-linking of the Ag receptors induces a profound depletion of cellular ATP (Fig. 4A). The kinetics of ATP depletion, which showed a lag before onset (5–10 h), are consistent with ATP depletion resulting from mitochondrial disruption. Moreover, as ATP depletion was apparent before the appearance of DNA ladders (≥16 h), this depletion did not simply reflect cell necrosis resulting from apoptosis. Importantly, such
ATP depletion and apoptosis could be blocked not only by the CD40 rescue signal, but also by the mitochondrial inhibitors, antimycin and oligomycin (Fig. 4, A and B). Taken together, these results suggest that ATP depletion resulting from mitochondrial disruption plays a key role in Ag receptor-driven apoptosis of WEHI-231 immature B cells.

Generation of unsaturated fatty acids, such as arachidonic acid, by a mitochondrial PLA2 activity has been reported to alter the permeability of the mitochondrial inner membrane resulting in the collapse of Δψm (15, 37–39). Interestingly, we have recently shown (26) that cPLA2 is only expressed and coupled to the Ag receptors on B cells under conditions of apoptotic signaling. Indeed, whereas apoptotic signaling via sIg strongly stimulates cPLA2 activity, and growth-promoting signals via IL-4R or CD40 are not coupled to this activity (26), rescue from sIg-mediated apoptosis by costimulation via CD40 uncouples sIg from cPLA2 signaling in WEHI-231 immature B cells (Fig. 5A). Moreover, consistent with a role for cPLA2 in sIg-mediated apoptosis, addition of exogenous arachidonic acid induces a dose-dependent state of profound growth arrest (26) and apoptosis, as indicated by DNA laddering (Fig. 5B) and induction of loss of nuclear DNA content (Fig. 5C) in WEHI-231 immature B cells. We therefore investigated whether cPLA2 played a role in the sIg-mediated collapse of Δψm and ATP depletion by determining whether signaling via sIg induced mitochondria-associated cPLA2 activity under conditions that correlated with the collapse of Δψm, ATP depletion, and commitment to apoptosis: anti-Ig not only stimulated total cellular PLA2 activity under apoptotic conditions, but some 20–25% of this activity was found in purified isolated mitochondrial preparations (Fig. 5D).

The sIg-coupled PLA2 activity is 1) calcium-dependent, 2) blocked by the cPLA2/iPLA2-selective inhibitor, arachidonyl triluciferol-ethyl ketone, and 3) not inhibited by the iPLA2-selective inhibitor, bromoelanol lactone, ruling out a role for either iPLA2 or secretory phospholipase A2 in such arachidonic acid generation (results not shown).

In addition, in situ immunofluorescence analysis of permeabilized cells indicates that cPLA2 translocates to the mitochondria and the nucleus within 3 h of stimulation of WEHI-231 B cells via the B cell receptor (BCR), and this is at least partially reversed/prevented by costimulation via CD40 (results not shown). Moreover, addition of exogenous arachidonic acid elicited Δψm collapse in a manner analogous to that observed with anti-Ig (Fig. 5C). Furthermore, although anti-Ig treatment was found to up-regulate PLA2 mRNA (Fig. 6A; 24 h) and protein (Fig. 6B; 2.25-fold basal levels at 48 h) levels, costimulation with anti-CD40 was found to prevent/reverse this up-regulation (Fig. 6, A and B; 0.65-fold basal levels of cPLA2 protein at 48 h). Taken together, these results suggest that sIg-mediated induction of mitochondrial cPLA2 activation may play a key role in the collapse of Δψm and commitment to apoptosis in WEHI-231 immature B cells.

Ag receptor-mediated disruption of the mitochondrial membrane potential is not associated with cytochrome c release from the mitochondria or activation of caspase-3

To determine whether the observed Ag receptor-driven mitochondrial disruption also results in caspase-dependent execution of apoptosis of WEHI-231 immature B cells, we investigated whether the loss of mitochondrial potential correlated with activation of effector caspases as evidenced by release of cytochrome c from the mitochondria and cleavage of the caspase-3 substrates, PARP and Bcl-xL (40, 41). Examination of mitochondria-free extracts showed that Ag receptor-mediated stimulation of WEHI-231 immature B cells failed to induce any release of cytochrome c into the cytosol over the 48-h time course of apoptosis measurements (Fig. 6C), results consistent with a recent report that BCR-mediated apoptosis in WEHI-231 cells is independent of cytochrome c translocation from the mitochondria (42). In contrast, cytochrome c release to such cytosolic fractions was easily detectable within 4 h following treatment of Jurkat cells with anti-Fas Abs. Moreover, in situ immunofluorescence analysis of intact cells showed that while cytochrome c remained localized to the mitochondria following stimulation of WEHI-231 cells with anti-Ig, cytochrome c release to the cytosol could be strongly detected (data not shown) following stimulation with the cell-permeant sphingolipid, C2-ceramide (25 µM; lane 5), and arachidonic acid (25 µM; lane 6). All experiments are single experiments representative of at least two other independent experiments.

FIGURE 6. Anti-Ig modulates PLA2 expression, but does not induce mitochondrial release of cytochrome c nor PARP cleavage in WEHI-231 B cells. Expression of cPLA2 on the mRNA (RT-PCR; A) and protein (Western blot; B) levels is modulated during apoptotic signaling in WEHI-231 immature B cells: WEHI-231 cells were incubated with media (lanes 1 and 5), 10 µg/ml anti-IgM (lanes 2 and 6), 10 µg/ml anti-CD40 (lanes 3 and 7), or a combination of these stimuli (lanes 4 and 8) for 1 h (lanes 1–4) or 24 h (A, lanes 5–8) or 48 h (B, lanes 5–8). Lane 9 in A represents the positive control RT-PCR product of cPLA2 obtained from the J774 monocyte cell line. Equivalent loading of PCR products was determined by simultaneous analysis of β-actin expression (results not shown). Mitochondrial release of cytochrome c was assessed by Western blot analysis of whole cell lysate and mitochondria-free fractions of WEHI-231 B cells (C). WEHI-231 cells were incubated with media (lanes 5 and 9), 10 µg/ml anti-IgM (lanes 2, 6, and 10), 10 µg/ml anti-CD40 (lanes 3, 7, and 11), or a combination of these stimuli (lanes 4, 8, and 12) for 8 h (lanes 2–4), 24 h (lanes 5–8) or 48 h (lanes 9–12). Lanes 1 and 13 are whole cell lysates of untreated WEHI-231 cells at 0 and 48 h, respectively. Lanes 2–12 are mitochondria-free extracts prepared as described in Materials and Methods. Lane +, Purified cytochrome c (from chicken; Sigma). Effector caspase activation, as assessed by PARP cleavage, was determined in WEHI-231 cells (D and E). In D, WEHI-231 B cells were incubated with 10 µg/ml anti-IgM (lanes 2 and 5), 10 µg/ml anti-CD40 (lanes 3 and 6), or a combination of these stimuli (lanes 4 and 7) for 24 h (lanes 2–4) or 48 h (lanes 5–7). Lanes 1 and 8 are untreated WEHI-231 cells at 0 and 48 h, respectively. In E, WEHI-231 cells were treated for 48 h with media (lane 1), anti-Ig (10 µg/ml; lane 2), anti-CD40 (10 µg/ml; lane 3), anti-Ig plus anti-CD40 (lane 4), C2-ceramide (25 µM; lane 5), and arachidonic acid (25 µM; lane 6). All experiments are single experiments representative of at least two other independent experiments.
Caspase inhibitors do not relieve Ag receptor-mediated growth arrest or apoptosis in WEHI-231 immature B cells

The failure of anti-Ig to induce a PARP-cleaving caspase activity or the release of cytochrome c from the mitochondria of WEHI-231 immature B cells suggested that Ag receptor-driven apoptosis of these cells may occur in a caspase-independent manner. To investigate this possibility further, we examined the effect of caspase inhibitors on the Ag receptor-driven growth arrest and apoptosis of WEHI-231 immature B cells. To control for the efficacy of these reagents, we conducted parallel experiments demonstrating their ability to protect against the caspase-dependent growth arrest and programmed cell death of Jurkat T cells resulting from stimulation via the Ag receptors (anti-CD3 or PHA), TNF-α, or Fas death receptors to determine whether the Ag receptors on WEHI-231 immature B cells (BCR) and Jurkat T cells (TCR-CD3 complex) utilized different apoptosis pathways.

The role of individual caspase subtypes in these models of lymphocyte apoptosis was assessed by the use of selective caspase inhibitors: while z-VAD-fmk is considered to be a pan-caspase inhibitor (44) with a high affinity for the caspase-1-like subfamily and a lower affinity for the caspase-3-like subfamily (45), Ac-DEVD-CHO shows greater affinity for caspase-3 than for the caspases-1, -4, and -7, and z-VEID-fmk is a potent caspase-6 inhibitor that has little effect on caspase-3, -4, -7, and -8 (46). We found that z-VAD-fmk was able to completely reverse Fas-mediated growth arrest and partially overcome anti-CD3 and, to a lesser extent, TNF-α-induced growth arrest (Fig. 7A, and results not shown), findings consistent with previously published studies (40). However, Ac-DEVD-CHO and z-VEID-fmk were unable to rescue either Fas- or TNF-mediated growth arrest (Fig. 7A, and results not shown). Nevertheless, and in agreement with previous studies (47, 48), all three inhibitors, albeit to a lesser extent, z-VEID-fmk, were able to prevent Fas-mediated apoptosis of Jurkat T cells (Fig. 7A). In contrast, none of these inhibitors had any effect on anti-Ig-mediated growth arrest, annexin V binding, cell cycle progression, or nuclear DNA loss (subdiploid cells) of WEHI-231 immature B cells (Figs. 7, B–D, and 8, A–C, and results not shown). However, z-VAD-fmk (1 nM to 10 μM, maximal 1 nM), Ac-DEVD-CHO (maximal >100 pM), and z-VEID-fmk (≥10 μM) were able to partially block C2-ceramide induction of nuclear DNA loss but not growth arrest, Fig. 7, B–D) in WEHI-231 B cells (Fig. 8, A and B, and results not shown), and both z-VAD-fmk and Ac-DEVD-CHO were able to inhibit apoptosis of germinal center (peanut agglutinin (PNA)−) B cells (Fig. 8D), results demonstrating the efficacy of these inhibitors in our B cell systems. In addition, while treatment of WEHI-231 cells with C2-ceramide can induce activation of a caspase-3-like activity, which can be blocked by the caspase-3 inhibitor, Ac-DEVD-CHO, cross-linking of the BCR for up to 24 h (2, 5, 8, and 24 h) does not induce activation of this caspase (Fig. 8E, and results not shown).

In direct contrast, the cathepsin B inhibitor, EST (24), strongly inhibits sIg-mediated apoptosis of WEHI-231 B cells (as indicated by loss of nuclear DNA content, Fig. 9A). In addition, although cross-linking of the BCR does not induce activation of caspase-3 activity, it does stimulate the cleavage of the cathepsin B substrate zRR-pNA (Fig. 9B). Moreover, although inhibition of sIg-mediated apoptosis by EST was maximal by 1 nM, the percentage of subdiploid cells resulting from stimulation with C2-ceramide was not reduced by this reagent at concentrations of EST ≤ 10 μM (Fig. 9A), providing further evidence that anti-Ig and C2-ceramide stimulate apoptosis of WEHI-231 cells by distinct mechanisms.

Despite preventing BCR-driven apoptosis, EST did not block anti-
Commitment to Ag receptor-driven apoptosis of the B cell lymphoma, WEHI-231, correlates with mitochondrial PLA$_2$ activation, leading to disruption of mitochondrial function and ATP depletion. Rather surprisingly, however, such apoptosis is executed independently of caspase activation and instead, the execution phase appears to involve the BCR-driven, postmitochondrial activation of the protease, cathepsin B. This novel executioner phase appears to involve the BCR-driven, postmitochondrial activation of the protease, cathepsin B (or similar proteases) rather than by effector caspases.

Discussion
Commitment to Ag receptor-driven apoptosis of the B cell lymphoma, WEHI-231, correlates with mitochondrial PLA$_2$ activation, leading to disruption of mitochondrial function and ATP depletion. Rather surprisingly, however, such apoptosis is executed independently of caspase activation and instead, the execution phase appears to involve the BCR-driven, postmitochondrial activation of the protease, cathepsin B. This novel mechanism of B cell apoptosis is supported by several lines of evidence: first, we demonstrate a pivotal role for mitochondrial function in determining commitment to B cell survival or apoptosis by showing cPLA$_2$ expression and mitochondrial activity are up-regulated under conditions of sIg-driven apoptosis and down-regulated during CD40-mediated rescue (Figs. 5 and 6). Second, the $\Delta \psi_{mi}$ is disrupted, resulting in depletion of cellular ATP under conditions of apoptotic, but not proliferative, signaling via the BCR (Figs. 1–4). Importantly, such collapse of $\Delta \psi_{mi}$ can be mimicked by addition of exogenous arachidonic acid, while disruption of $\Delta \psi_{mi}$, ATP depletion, and apoptosis can be blocked by rescue signals via CD40 or by $\Delta \psi_{mi}$ stabilizers such as antimycin or oligomycin (Figs. 2–4). Third, we show that activation of caspases is not essential for commitment and postmitochondrial execution of BCR-mediated apoptosis by demonstrating that apoptotic signaling via the BCR or following addition of exogenous arachidonic acid does not induce either the release of cytochrome c from mitochondria, which is required for the activation of the caspase-3 cascade (49, 50), nor does it cause cleavage of PARP (Fig. 6) or Bcl-x$_L$ (results not shown), which have been reported to be suitable markers of caspase-3-like effector activity in various cell types (40, 41). Indeed, apoptotic signaling via the BCR in WEHI-231 B cells does not appear to stimulate the activation of caspase-3 (Fig. 8). Consistent with this, BCR-mediated disruption of $\Delta \psi_{mi}$ and commitment to apoptosis take place in the presence of caspase inhibitors (Figs. 7–9), indicating that none of the known initiators or effectors caspases are essential for the commitment or execution of BCR-induced apoptosis of WEHI-231 immature B cells. Rather, apoptotic signaling via the BCR stimulates the postmitochondrial activation of the protease, cathepsin B (Fig. 9), which has previously been shown to be a key executioner protease in alternative mechanisms of apoptosis that have recently been described (51). That this PLA$_2$-dependent mechanism of mitochondrial function is likely to be a key checkpoint of Ag-driven cell fate is evidenced by the downregulation of PLA$_2$ activity and expression, and protection of mitochondrial function, resulting from signaling via CD40, which effects rescue from BCR-mediated apoptosis in these WEHI-231 immature B cells.

ATP depletion resulting from oxidant-induced calcium release from mitochondria followed by excessive calcium cycling and collapse of $\Delta \psi_{mi}$ has been proposed to be a hallmark of apoptosis (15). Interestingly, production of ROS has previously been shown to

FIGURE 8. Caspase inhibitors do not block anti-Ig-mediated apoptosis in WEHI-231 cells, but do relieve BCR-mediated apoptosis of germinal center B cells and ceramide-induced apoptosis in WEHI-231 B cells. In A and B, WEHI-231 B cells were cultured with media or anti-Ig (10 $\mu$g/ml) or ceramide (25 $\mu$M) for 48 h in the presence and absence of the indicated concentration of z-VAD-fmk (A) or 100 $\mu$M Ac-DEVD-CHO or 10 $\mu$M z-VEID-fmk (B) before assessing DNA content (PI staining, A and B). In C, WEHI-231 B cells were cultured with media or anti-Ig (10 $\mu$g/ml) for 48 h in the presence and absence of of 10 $\mu$M z-VAD-fmk or 2 nM Ac-DEVDis CHO or 100 $\mu$M z-VEID-fmk (B) before staining with annexin V. In D, spleen cells were cultured with anti-Ig (50 $\mu$g/ml for 48 h) and apoptosis (subdiploid DNA content) of germinal center B cells (PNA+/B220-gated population) assessed by PI staining. Concentrations used for caspase inhibitors in D were: Ac-DEVDis CHO, 2 nM, and z-VAD-fmk, 10 $\mu$M. In E, WEHI-231 B cells were incubated with media, anti-Ig (10 $\mu$g/ml), or ceramide (25 $\mu$M) in the presence or absence of the caspase-3 inhibitor Ac-DEVDis CHO (1 nM or 10 $\mu$M) for 24 h, and cell lysates were prepared. Caspase-3 activity, as evidenced by the cleavage of the caspase-3 substrate, Ac-DEVD-pNA (Calbiochem), was then assayed as described in Materials and Methods. All data are from single experiments representative of at least two other independent experiments.

Ig-stimulated growth arrest (Fig. 9C), nor did it prevent anti-Ig-driven collapse of $\Delta \psi_{mi}$ (Fig. 9D). In contrast, z-VAD (1 nM-100 $\mu$M) was able to block C$_2$-ceramide, but not anti-Ig-mediated disruption of $\Delta \psi_{mi}$ in WEHI-231 cells. Taken together, these results suggest that the execution phase of BCR-driven apoptosis is mediated by cathepsin B (or similar proteases) rather than by effector caspases.
increase following apoptotic signaling via slg on WEHI-231 immature B cells (52). Moreover, CD40 signaling, which rescues these cells from apoptosis, leads to an increase in the expression of Bcl-xL (53, and our unpublished observations), which prevents accumulation of intracellular oxidants (52, 53), blocks thapsigargin-induced intracellular mobilization of calcium from the endoplasmic reticulum (54), and stabilizes Δψm and mitochondrial homeostasis (22). However, our findings that the mitochondrial inhibitors, antimycin and oligomycin, which will induce the production of ROS (55), protect against slg-mediated ATP depletion and apoptosis (Fig. 3), together with our observed lack of effect of ROS inhibitors on BCR-mediated growth arrest and apoptosis of WEHI-231 cells (results not shown), argue against a role for ROS in BCR-driven collapse of Δψm in WEHI-231 B cells. Consistent with this, it has recently been shown that rather than induce apoptosis, low levels of ROS appear to exert mitogenic or antiapoptotic effects (56, 57).

An alternative candidate apoptotic pathway for the collapse of Δψm and ATP depletion involves the stimulation of mitochondrial PLA2 activity, resulting in the accumulation of unsaturated fatty acids (arachidonic acid) that have been reported to alter the permeability of the mitochondrial inner membrane, resulting in the collapse of Δψm (15, 37–39). Indeed, we have found that whereas apoptotic signaling via slg up-regulates cPLA2 expression, induces its translocation to the mitochondria, and strongly stimulates cPLA2 activity (Figs. 5 and 6, and results not shown), rescue from BCR-mediated apoptosis by costimulation via CD40 down-regulates cPLA2 expression and uncouples slg from cPLA2 translocation and signaling in WEHI-231 immature B cells (Figs. 5 and 6, and results not shown). In addition, and consistent with a role for cPLA2 in this slg-mediated, mitochondrial-dependent mechanism of apoptosis, addition of exogeneous arachidonic acid induces a profound collapse of Δψm and resultant induction of growth arrest (26) and apoptosis (Fig. 5) in WEHI-231 immature B cells. That arachidonic acid is the active lipid moiety is supported by our preliminary findings that while cyclooxygenase inhibitors promote BCR-mediated apoptosis, signaling via CD40 acts to promote intracellular PGE2 production (our unpublished results). Taken together, these results suggest that slg-mediated induction of mitochondrial PLA2 and generation of arachidonic acid may play a key role in the collapse of Δψm and commitment to apoptosis in WEHI-231 immature B cells.

The mechanisms downstream of mitochondrial membrane depolarization and ATP depletion have not, as yet, been delineated, but the failure of anti-Ig to induce a PARP-cleaving caspase activity or the release of cytochrome c from the mitochondria of WEHI-231 immature B cells (Fig. 6) suggested that BCR-driven apoptosis of these cells is not dependent on caspase activation, a proposal supported by our findings that signaling via slg does not induce caspase-3 activation and that caspase inhibitors did not prevent BCR-mediated growth arrest and apoptosis in such cells (Fig. 8). At first sight, therefore, these results appear to conflict with previously published reports that showed that slg was coupled to the activation of caspases and that such caspase activation played a role in slg-mediated apoptosis of WEHI-231 (43, 58) and primary immature B cells (59). However, by using z-VAD-fmk at high concentrations (≥100 μM), we have been able to reproduce some of the effects of caspase inhibitors on slg-mediated apoptosis.
presented in the earlier papers: these apparent differences may therefore simply reflect the use of high concentrations (≥12.5 μM) of caspase inhibitors in the earlier studies, as it is now widely recognized that at concentrations >10 μM, z-VAD-fmk will inhibit other cysteine proteases such as calpain and cathepsin B (51). Consistent with this, we find that apoptotic signaling via the BCR stimulates cathepsin B activity (Fig. 9) and that the postmitochondrial stages of Ag receptor-driven apoptosis in WEHI-231 immature B cells are blocked by the cathepsin B inhibitor EST (Fig. 9). Similarly, BCR-mediated apoptosis can also be prevented by the serine and cysteine protease-selective, caspase-independent inhibitor, Nec-(p-tosyl)lysylchloromethyl ketone (46, 60), and partially by the calpain and/or cathepsin-selective inhibitors, leupeptin, antipain, and pepstatin (results not shown) (60–62).

Interestingly, recent evidence now suggests that ATP depletion can lead to the induction of DNA fragmentation and consequent apoptosis in a caspase-independent, but Nec-(p-tosyl)lysylchloromethyl ketone- or 1–I-tosylamido-2-phenylchloromethyl ketone-sensitive, protease-dependent manner (63). Taken together, these findings suggest that, in addition to an inability to drive the energetically unfavorable reactions involved in the metabolic, biosynthetic, and signal transduction processes required for cell survival and cell cycle progression (12, 14, 56, 64, 65), ATP depletion can trigger the postmitochondrial activation of a cathepsin-B-like protease-dependent mechanism of DNA fragmentation and apoptosis in WEHI-231 B cells stimulated via the BCR.

Finally, our results show that caspase inhibitors can block signal-mediated apoptosis of germinal center (PNA+1) B cells, suggesting that B cells may employ distinct maturation stage-specific mechanisms of apoptosis. However, this rescue by caspase inhibitors is only partial and may reflect the results of a recent study that show that apoptosis of human germinal center B cells requires the activation of both caspase and cathepsin activities, the cathepsin activity being downstream of caspase-3 and responsible for exonuclease activity and execution of apoptosis (24). Taken together with caspase-dependent processes of apoptosis observed in Jurkat T cells, therefore, our results may suggest that there is a fundamental difference in the way that the Ag receptors on B and T cells signal commitment to growth arrest and apoptosis, and this proposal could reflect recent reports that while caspase-deficient mice exhibit aberrant T cell development, they did not appear to have significant defects in B cell selection and development (8, 9, 25).

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References
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