

Calcium induces apoptosis and necrosis in hematopoietic malignant cells: Evidence for caspase-8 dependent and FADD-autonomous pathway

Research Article

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Summary

One of the killing mechanisms employed by Natural Killer (NK) cells and Lymphokine-Activated Killer (LAK) cells is the perforation of the cellular membrane that causes the increase of cytoplasmic calcium concentration and disturbs further the homeostasis of other ions. Cytoplasmic calcium influx, exceeding the tolerated physiologic threshold in cell signaling events, can induce either apoptosis or necrosis depending on its final concentration. Despite several years of intensive research and identification of some molecular targets of action like e.g. calpains, calcineurin or calreticulin, the exact mechanism of calcium-induced cell death is not known in detail. We show here that death pathways triggered by calcium rely on a novel, caspase-8-dependent and Bcl-2-inhibitable pathway that is FADD-adaptor molecule -independent. This is shown in a leukemic cell model. The experimental system employs either cells that lack the expression of caspase-8 or cells genetically modified to overexpress, Bcl-2, or a FADD-dominant negative mutant (FADD-DN).

I. Introduction

Calcium is one of the most versatile and powerful small molecules applied by a cell to regulate its biologic functions. It can either protect from or induce cell death, depending on concentration and cell type (Franklin and Johnson, 1992; Barros et al, 2002). Although the mechanism of calcium triggered death has been investigated for years, the exact mechanism(s) responsible for this process are not known in detail. Dying cells enter either apoptosis, necrosis or an intermediate form of cell death, depending on the death stimulus, its intensity and the level of intracellular ATP (Leist and Jaattela, 2001; Los et al, 2002). In accordance, calcium can induce both forms of cell death as well as an intermediate process, depending on available intracellular concentration and cell type (Gwag et al, 1999; Barros et al, 2002). Calcium-related cell death is best described in neurones (Gwag et al, 1999; Xu et al, 2001), however, detailed studies in lymphatic tissue, from recent date are scarce. Calcium ionophores, such as ionomycin or A-23187 are frequently

applied to manipulate intracellular Ca²⁺ concentration and thus to mimic signaling events or to induce cell death (Errasfa and Stern, 1994; Nakamura, 1996). Several authors provide observations that various tumor cell lines exposed to A-23187 or ionomycin undergo either non-apoptotic degeneration (Duke et al, 1994; Kressel and Groscurth, 1994), or classical apoptosis (Ojcius et al, 1991; Ning and Murphy, 1993).

Caspases (cysteine-dependent aspartases) are crucial apoptotic executioner proteases (Los et al, 1995; Herr and Debatin, 2001). They are members of the C14 protease family according to the Barrett and Rawlings classification (Los et al, 1999; Barrett and Rawlings, 2001). All caspases are characterized by a nearly absolute specificity for substrates containing aspartic acid in the P1 cleavage position and a cysteine in the active center of the enzyme (Stennicke et al, 2002). There are currently 12 known caspases in humans. Caspases-1, -4 and -5 mainly play a role in the regulation of inflammatory response, by proteolytic activation of inflammatory cytokines (Cassens et al, 2003). Caspases-2, -3, -6, -7, -8, -9 and -10 are

considered to be involved predominantly in apoptotic signalling (Sadowski-Debbing et al, 2002). In addition to the role in apoptosis and inflammation, an involvement of caspases in other processes, like cell cycle regulation, hematopoiesis and signal transduction in the immune system have been proposed (Denis et al, 1998; Los et al, 2001). All caspases are synthesized as inactive zymogens that are activated through proteolytic cleavage. Among the caspase activation pathways, the best described ones are the death-receptor dependent signalling cascades, with FADD adaptor molecule and caspase-8 as the key players, and the mitochondria/apoptosome dependent pathway that relies on Apaf-1 and caspase-9 (Krammer, 2000; Walczak and Krammer, 2000; Zheng and Flavell, 2000; Renz et al, 2001). Both pathways are interconnected, thus amplification loops may take place (Sadowski-Debbing et al, 2002). The mitochondrial pathway is largely controlled by Bcl-2 family members. Bcl-2 family proteins exert its pro- and antiapoptotic action partially by influencing calcium homeostasis of mitochondria and endoplasmic reticulum (ER) (reviewed in Hajnóczky et al, 2003).

The family comprises both antiapoptotic and proapoptotic proteins. All antiapoptotic family members (e.g. Bcl-2, Bcl-X_L) share three or four Bcl-2 homology (BH) regions, and they localize to the cytoplasmic side of intracellular membranes (Bouillet and Strasser, 2002). The proapoptotic Bcl-2 family members can be further divided into two subgroups. Members of the first subgroup, best represented by Bax and Bak (reviewed in Bouillet and Strasser, 2002) have two or three BH regions and appear to be structurally similar to their prosurvival relatives (Suzuki et al, 2000). The second subgroup of proapoptotic Bcl-2-related proteins, (e.g. Bad, Bid, Bim) share only the short BH3 region (reviewed in Bouillet and Strasser, 2002). The exact mechanism of apoptosis regulation by Bcl-2 family members is not fully understood (Strasser et al, 2000). It is widely believed that Bcl-2 functions to preserve the mitochondrial membrane integrity, mitochondrial and ER calcium homeostasis and prevent the release of cytochrome c and other proapoptotic molecules from the mitochondria. BH3-only proteins appear to sense stimuli that cause cellular stress and initiate the death cascade. Proapoptotic Bax and Bak are essential for cell killing governed by BH3-only proteins, and this form of cell death is antagonized by overexpression of Bcl-2 (reviewed in Hajnóczky et al, 2003; Marsden and Strasser, 2003).

To gain insight into the mechanisms that govern calcium triggered cell death we have used a T-cell-leukemia based model and calcium ionophores as modulators of intracellular Ca²⁺ level. We show here that the calcium activated apoptotic pathway rely on yet-to-be-defined, caspase-8-dependent and Bcl-2-inhibitable pathway. Interestingly, the pathway does not rely on FADD-adaptor molecule. Thus, we provide further evidences for an intrinsic (death receptor-independent) death pathway that relies on caspase-8.

II. Materials and methods

A. Materials and cell culture

All cell lines were grown in 5% CO₂ at 37°C using a RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (GIBCO, Eggenstein, Germany). A23187 was purchased from Sigma (Deisenhofen, Germany). The caspase inhibitor zVADfmk (benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone) was purchased from Enzyme Systems Products (Dublin, CA), and staurosporine from Roche Biochemicals (Mannheim, Germany). All other chemicals were from Merck KG (Darmstadt, Germany) or Roth (Karlsruhe, Germany). Stable transfectants of Jurkat cells overexpressing Bcl-2 and Jurkat clone that was deficient in caspase-8 were a kind gift of Dr. J. Blenis, (Harvard Medical School, Boston, Massachusetts, USA).

B. Cell extracts and immunoblotting

The proteolytic processing of caspase-3 and caspase-8 was detected by immunoblotting. Briefly, 5 x 10⁵ cells were seeded in 6-well plates and treated with the apoptotic stimuli. After the indicated time, cells were washed in cold PBS and lysed in 1% Triton X-100, 50 mM Tris-HCl, pH 7.6 and 150 mM NaCl containing 3 µg/ml aprotinin, 3 µg/ml leupeptin, 3 µg/ml pepstatin A and 2 mM phenylmethylsulfonyl fluoride (PMSF). Subsequently, the proteins were separated under reducing conditions by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to a polyvinylidene difluoride membrane (Amersham, Braunschweig, Germany). The equal loading of protein was controlled by measuring the protein concentration using the Bradford assay (BioRad, Munich, Germany). Membranes were blocked for 1 h with 5% non-fat dry milk powder in TBS and then incubated for 1 h with murine monoclonal antibodies directed against caspase-3 (Transduction Laboratory, Heidelberg, Germany). Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with the respective peroxidase-conjugated affinity-purified secondary antibody for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham).

C. Fluorimetric assay of caspase activity - DEVD-ase assay

Cytosolic cell extracts were prepared by lysing cells in a buffer containing 0.5% NP-40, 20 mM HEPES pH 7.4, 84 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM PMSF. Caspase activity was determined by the incubation of cell lysates with 50 µM of the fluorogenic substrate DEVD-AMC (N-acetyl-Asp-Glu-Val-Aspaminomethylcoumarin, Bachem, Heidelberg, Germany) in 200 µl buffer containing 50 mM HEPES pH 7.3, 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT. The release of aminomethylcoumarin was measured by fluorometry using an excitation wavelength of 360 nm and an emission wavelength of 475 nm.

D. Measurement of cell death and apoptosis

Cell death was measured either by the detection of hypodiploid nuclei (Nicoletti method) (Renz et al, 2001) or by the uptake of propidium iodide (PI) (Stroh et al, 2002). Briefly, for the measurement of hypodiploid DNA, nuclei were prepared by lysing 10⁴ cells in 100 µl of hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, and 50 µg/ml PI). The nuclei

were subsequently analyzed by flow cytometry, using a FACScalibur (Becton Dickinson, Heidelberg, Germany) and CellQuest analysis software. To assess PI uptake, cells were harvested after the indicated times and incubated with PI (2 $\mu\text{g/ml}$). The uptake of PI into nonfixed cells was measured by flow cytometry, using the FSC/FL2 profile.

III. Results

A. Calcium influx induces apoptotic and necrotic cell death in a dose dependent manner

In order to get insight into the mechanism(s) of calcium induced cell death we have performed time-, and concentration- kinetic studies. Jurkat human T-leukemia cells were treated with increasing concentrations of the A23187 calcium ionophore. A23187 induces cell death in a dose dependent manner (**Figure 1**). Higher concentrations of intracellular calcium induce cell death with faster kinetics. At the concentration of 800 ng/ml A23187 induces a maximum cell death at 18 h, whereas lower concentrations of the ionophore show slower kinetics. The assessment of data obtained by the measurement of PI uptake and apoptosis-specific measurement by the detection of hypodiploid nuclei ("Nicoletti" method) indicates that higher concentrations induce not only apoptotic, but also necrosis in the experimental system (**Figure 1C**). Since contrary to necrosis the apoptotic cell death relies on caspases, we repeated the series of experiments employing the broad-spectrum caspase inhibitor zVADfmk (**Figure 2**). Thus zVAD-fmk inhibitable cell death represents the apoptotic fraction. The zVADfmk based approach largely confirms the data obtained by the combination of the PI-uptake based- and the "Nicoletti" method (**Figure 1C**). Unlike the Nicoletti method that detects (lack of) the intactness of nuclear DNA (hypodiploidy), PI-uptake stains cells with permeable cell membranes (necrotic and late apoptotic cells). zVADfmk inhibits the proteolytic caspase activity and, therefore, it blocks the apoptotic fraction of cell death. The experiments involving the caspase inhibitor indicate the highest zVADfmk-independent (presumably necrotic) fraction of cell death upon the treatment with intermediate (200 ng/ml) concentrations of A23187 calcium ionophore (**Figure 2C**). These method-related differential results are explained in detail in the discussion-part of the paper.

B. Caspase-8 deficiency impairs calcium induced cell death

The broad-spectrum caspase inhibitor zVADfmk was largely protective against calcium induced cell death. To examine further the role of caspases in the death mechanism triggered by calcium we have employed a Jurkat cell clone that lacks caspase-8 activity. Calcium induced cell death measured by PI uptake was significantly impaired in cells lacking caspase-8 activity (**Figure 3A**). The observed effect could be detected at several time points and it was most pronounced after 18 h.

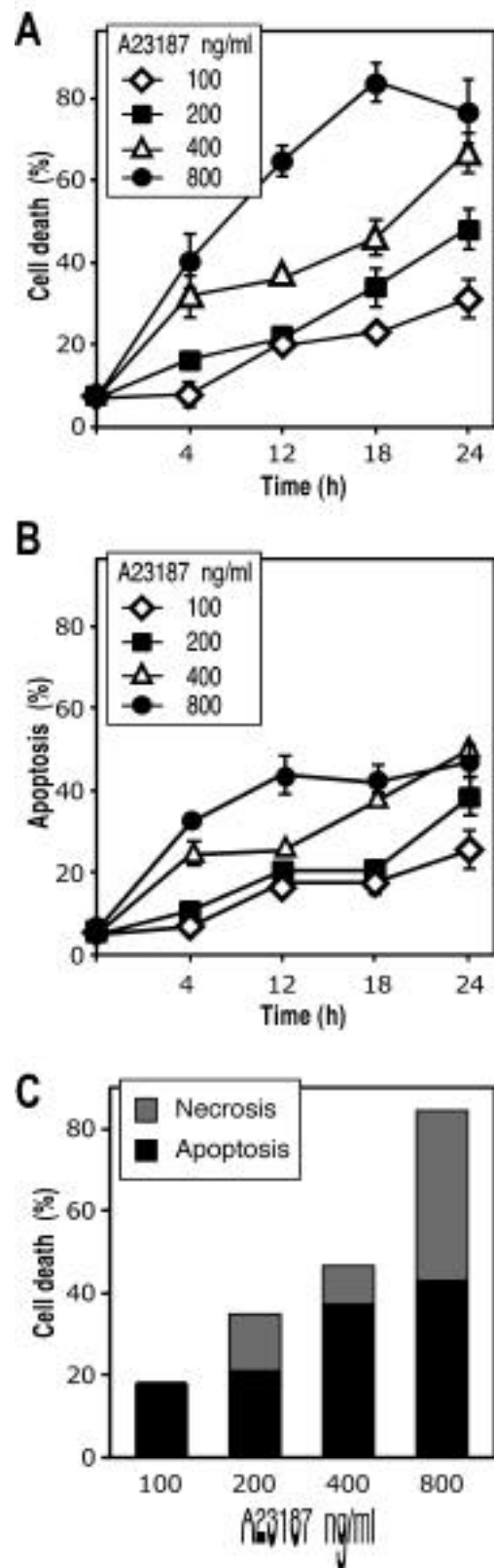


Figure 1. Induction of cell death by calcium ionophore in Jurkat cells. (A and B) show parallel-, time-kinetic experiments evaluated either by PI-uptake, a method that unspecifically detects cell death (A), or by apoptosis-specific "Nicoletti" method that measures hypodiploid, apoptotic nuclei (B). The standard deviation of four independent experiments shown here did not exceeded 11 %. The percentage representation of both death modes, that occurred after 18 h are visualized in the panel (C).

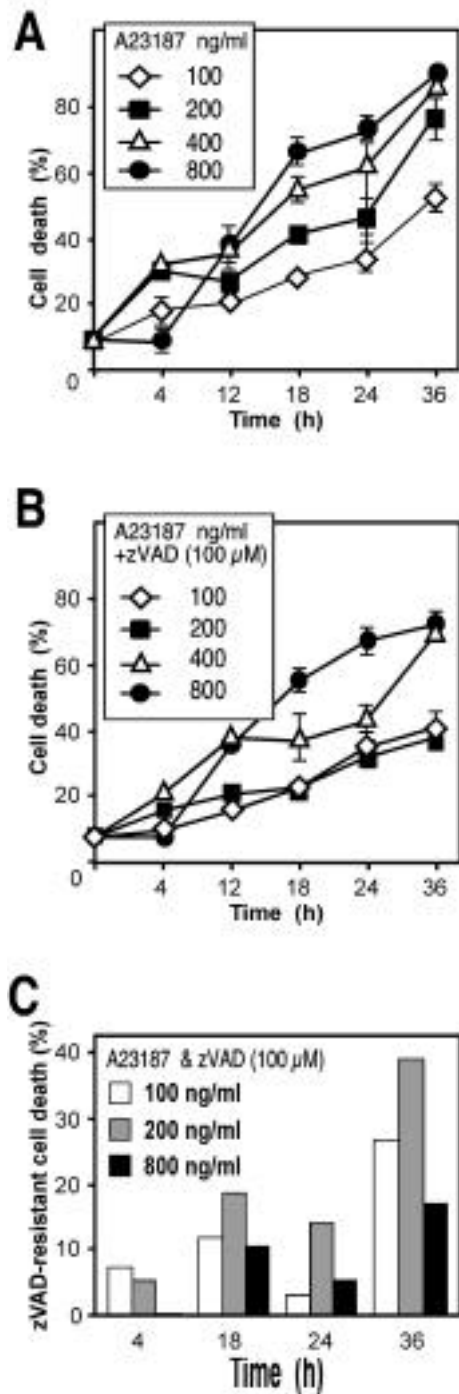


Figure 2. Delineation of caspase-dependent (zVADfmk-inhibitable) and caspase-independent components of calcium-induced cell death. Jurkat cells were treated with different concentrations of A23187 as indicated in **A** and **B**. The application of zVADfmk, the broad-spectrum caspase inhibitor has significantly, but only partially blocked cell death events (**B**). The panel (**B**) shows data from four independent experiments. The standard deviation did not exceeded 9 %. The “zVADfmk” resistant cell death component is depicted in the panel (**C**). Cell death was measured by PI-uptake.

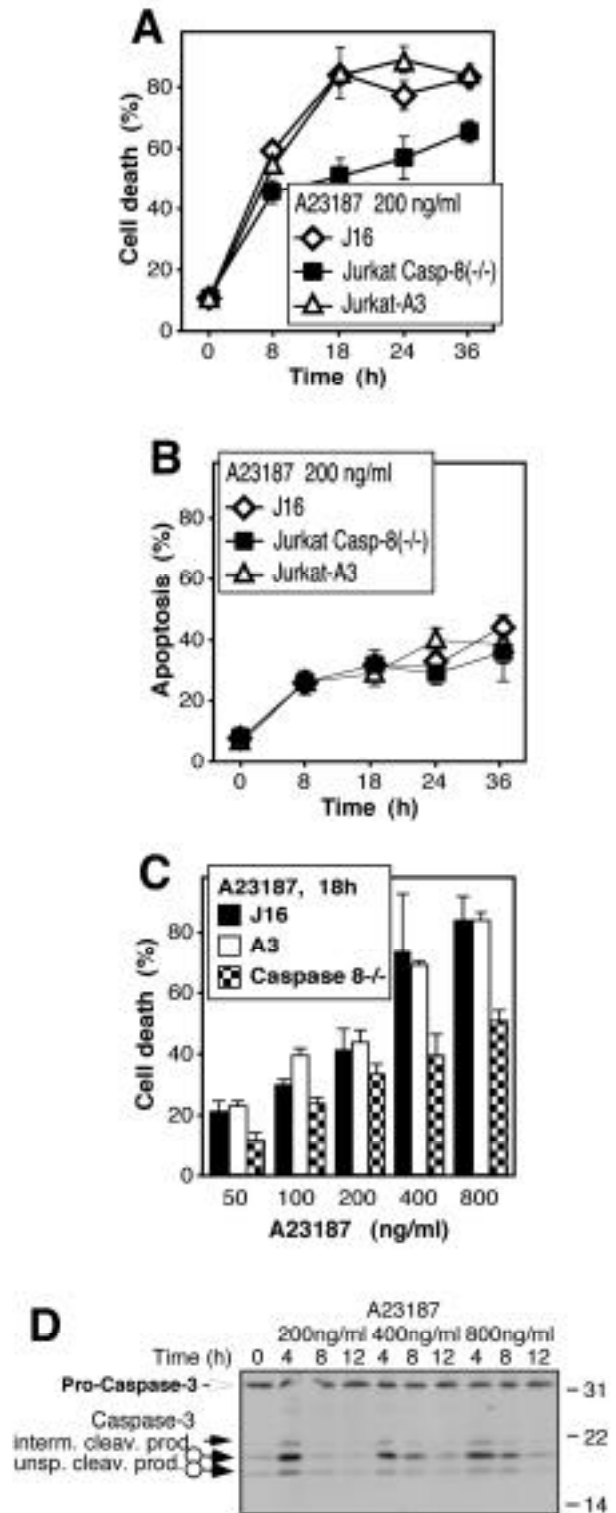


Figure 3. Caspase-8 activity deficiency protects from necrotic component, but not from the apoptotic constituent of calcium-induced cell death. Jurkat cells were induced to die by the addition of 200 ng/ml of A23187. Cell death was measured in parallel by PI-uptake (**A**), and by the assessment of nuclear hypodiploidy that corresponds to apoptotic cell death (**B**). To get the confirmation of the data, we conducted a kinetic study using increasing concentrations of the calcium ionophore A23187 (**C**). The cell death was measured by PI-uptake. The activation of caspase cascade was assessed by Western blot detection of caspase-3 cleavage (**D**).

Interestingly, despite having a strong effect on cell death (Figure 3A), caspase-8 deficient cells were equally sensitive towards the apoptotic form of cell death (Figure 3B), measured by the “Nicoletti” method. To further confirm the observation, the A23187 concentration kinetics at 18 h was performed (Figure 3C). Similarly as in Figure 3A, here the cell death was measured by PI-uptake that cannot discriminate well between apoptosis and necrosis. Also this data fully confirmed the observations that caspase-8 deficiency significantly protects from the ionophore-triggered death. To get further insight into the death mechanisms induced by calcium influx we have examined caspase-3 cleavage (activation) by Western blot (Figure 3D). To our surprise a significant portion of caspase-3 was cleaved unspecifically, yielding non-active proteolytic fragments. The subsequent enzymatic measurement of caspase-3 (DEVDase) activity fully confirmed the Western blot data, showing only a very moderate increase in activity (data not shown).

C. Calcium induced cell death is FADD-independent, and it is inhibitable by Bcl-2

Since caspase-8 deficiency was largely protective against calcium-induced cell death in our experimental system, we next tested the effect of FADD, the adaptor molecule that is necessary for caspase-8 recruitment to death receptors. In addition, we examined the possible involvement of apoptosome/mitochondrial death pathway employing Jurkat cells overexpressing Bcl-2 proteins. Cells overexpressing a mutated form of the FADD molecule, that lack the death effector domain required for the interaction with caspase-8, were as equally sensitive as the control Jurkat cell line (Figure 4A). Thus, although caspase-8 deficiency significantly impairs death triggered by calcium, the adaptor molecule FADD plays no role in the system. Whereas, Bcl-2 overexpression was fully protective against low concentrations (200 ng/ml) of the calcium ionophore A23187 (Figure 4B). Higher concentrations of A23187 (e.g. 400 ng/ml) partially overcame the Bcl-2 protective effect, but still about 50 % more of the Jurkat-Bcl-2 cells survived the forced calcium influx as compared to the control Jurkat clone.

IV. Discussion

The presented study identifies a novel, caspase-8 dependent, calcium-triggered pathway involved in the propagation of cell death. The pathway differs significantly from the classical, death receptor-triggered apoptotic signaling cascades since it is FADD-independent. Caspase-8 requires adaptor molecules for its activation. This requirement can be fulfilled by the ER-localized protein Bap31 that binds caspase-8 (Breckenridge et al, 2002; Ducret et al, 2003). The observed sensitivity towards overexpression of Bcl-2 may be indicative for the involvement of mitochondrial/apoptosome-dependent signaling events. The Bcl-2 sensitivity of the pathway can also be explained alternatively. It has been described previously (Foyouzi-Youssefi et al, 2000; Vanden Abeele et al, 2002) that some

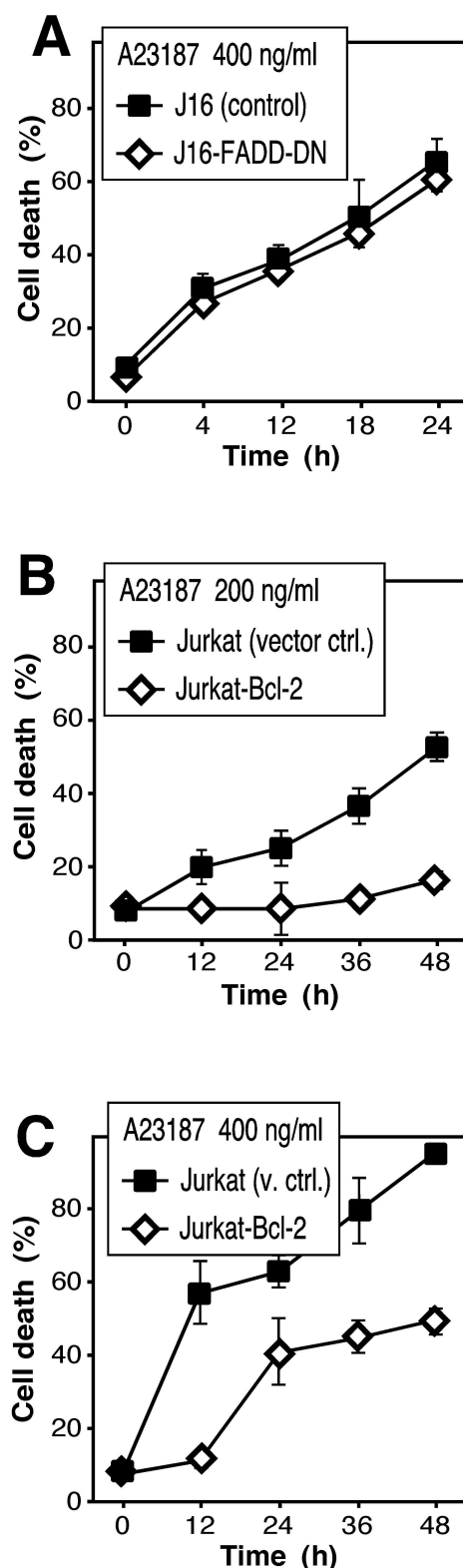


Figure 4. The effect of FADD death receptor adaptor molecule and Bcl-2 on calcium triggered apoptosis. FADD-negative- and control (J16) cells were treated with A23187 (400 ng/ml) over different time points and cell death was measured by PI-uptake (A). To examine the effect of Bcl-2 on calcium induced death we have used a Jurkat cell clone that overexpress the protein. Time kinetics were done with two different concentrations of A23187. Bcl-2 almost completely inhibited cell death induced by 200 ng/ml of A23187 (B), and it was about 40-50 % protective upon treatment with 400 ng/ml of the ionophore (C). Cell death was measured by PI-uptake.

antiapoptotic Bcl-2 family members including Bcl-2 itself and Bcl-X_L, protect cells from calcium by lowering the Ca²⁺-storage capacity of ER. Thus, the death stimuli that cause the release of calcium from ER will be less efficient in elevating the cytoplasmic calcium concentration and therefore, will less effectively activate the calcium-dependent signaling pathways.

The death induced by the calcium ionophore A23187 was a mixture of necrosis and apoptosis. A critical factor that influences the form of cell death (apoptotic or necrotic) is the cellular ATP content. Stimuli that under normal condition induce apoptosis will cause classical necrotic cell death if the cellular concentration of ATP drops below 10-15 % of the normal level (Nieminen et al, 1994; Los et al, 2002). One of the mechanisms that cause severe ATP depletion is the uncoupling of phosphorylative oxidation and ATP production caused by mitochondrial permeability transition (MPT). MPT may be triggered by a rising Ca²⁺ level and the subsequent activation of the hypothetical permeability transition pore component cyclophilin D. Once the pH and electrical gradient across the inner mitochondrial membrane collapses the final enzyme of the mitochondrial respiratory chain, the F₁F₀-ATPase, that normally converts ADP to ATP, reverses and consumes ATP while trying to restore the gradient. This mechanism is among the strongest depletors of cellular ATP, since it also consumes ATP produced by the compensatory, glycolytic pathway (reviewed in Lemasters et al, 2002; Hajnoczky et al, 2003). The above mechanism permits both necrotic- and apoptotic death. A strong increase of Ca²⁺ concentration would cause a significant portion of mitochondria to collapse, massive ATP depletion would follow, thus, cells would die by necrosis. A less pronounced rise of calcium concentration would result in a slow and asynchronous MPT occurrence. Affected mitochondria would release proapoptotic molecules like cytochrome c, AIF and endonuclease G. While the depletion of ATP would not be significant, the cell would have enough energy to die in an orderly, apoptotic fashion. This is exactly what we observed in our experimental system. While low concentrations of the calcium ionophore A23187 induce apoptosis, intermediate and higher concentrations of it cause substantial necrosis.

In summary, we are presenting here evidence for a new caspase-8-dependent calcium-induced death pathway. Since it is FADD-independent, we hypothesize that the Bap31 ER-localized adaptor molecule is involved in the pathway. In addition to the ER-compartment, the mitochondrial death pathways are important mediators of death induced by an elevated cellular calcium level.

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