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Induction of apoptosis by valinomycin: mitochondrial permeability transition causes intracellular acidification

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Abstract

In order to determine whether disruption of mitochondrial function could trigger apoptosis in murine haematopoietic cells, we used the potassium ionophore valinomycin. Valinomycin induces apoptosis in the murine pre-B cell line BAF3, which cannot be inhibited by interleukin-3 addition or Bcl-2 over-expression. Valinomycin triggers rapid loss of mitochondrial membrane potential. This precedes cytoplasmic acidification, which leads to cysteine-active-site protease activation, DNA fragmentation and cell death. Bongkrekic acid, an inhibitor of the mitochondrial permeability transition, prevents acidification and subsequent induction of apoptosis by valinomycin.

Keywords: apoptosis; valinomycin; intracellular pH; mitochondrial permeability transition

Abbreviations: IL-3, interleukin 3; ANT, adenine nucleotide translocator; PARP, poly (ADP-ribose) polymerase; BA, bongkrekic acid; PT, permeability transition; TNF, tumor necrosis factor; NGF, nerve growth factor

Introduction

In many cell types, including those of the haematopoietic system, removal of essential growth factors results in apoptosis (Collins *et al*, 1994). The late, irreversible steps of the death programme in most cells include activation of a family of cysteine-active-site proteases (Martin and Green, 1995), which degrade a number of abundant nuclear proteins (Kaufmann *et al*, 1993; Song *et al*, 1996; Voelkel *et al*, 1995; Tewari *et al*, 1995; Casciola-Rosen *et al*, 1994; Lazebnik *et al*, 1995), and the digestion of chromatin by an endonuclease (Wyllie, 1980). Cell extracts containing activated cysteine-active-site proteases can stimulate apoptotic changes, including chromatin digestion, in isolated nuclei (Lazebnik *et al*, 1993; Solary *et al*, 1993; Martin *et al*, 1995; Enari *et al*, 1995a). Also, inhibition of cysteine-active-site protease activity in intact cells, by cell permeable peptide inhibitors (Fearnhead *et al*, 1995; Zhu *et al*, 1995; Chow *et al*, 1995; Jacobson *et al*, 1996) or expression of viral protease-inhibitory proteins (Gagliardini *et al*, 1994; Tewari and Dixit, 1995; Enari *et al*, 1995b; Los *et al*, 1995; Camita *et al*, 1993), prevents chromatin fragmentation and delays loss of cell viability. Thus, regulation of cysteine-active-site proteases is critical for control of entry into apoptosis. These proteases are synthesised as pro-enzymes and cleaved to form the active enzyme (Nicholson *et al*, 1995; Tewari *et al*, 1995); *in vitro* this cleavage can be auto-catalytic or catalysed by other cysteine-active-site proteases (Xue and Horvitz, 1995; Kumar and Harvey, 1995) and *in vivo* there is evidence for a proteolytic cascade (Enari *et al*, 1996). Fas or TNF receptor-induced apoptosis is triggered by recruitment of an cysteine-active-site protease to the activated receptor (Boldin *et al*, 1996; Muzio *et al*., 1996). However, the mechanism by which the cysteine-active-site protease cascade is activated during apoptosis following signals such as growth factor deprivation is not known.

The bone marrow cell line, BAF3, is dependent on IL-3 for growth in culture and enters apoptosis on IL-3 removal (Rodriguez-Tarduchy *et al*, 1990). DNA fragmentation and loss of cell viability occur when IL-3 is removed, but only after a delay of at least 8 h (Rodriguez-Tarduchy *et al*, 1990). New gene expression is not required for entry into apoptosis and IL-3 can rescue cells when readded close to the time of fragmentation (Rodriguez-Tarduchy *et al*, 1990; Collins *et al*, 1992). Apoptosis following factor removal is inhibited by over-expression of the bcl-2 protein, which allows cell survival, but does not promote cell growth (Marvel *et al*, 1993). Bcl-2 protein is associated with a variety of intracellular membranes and homodimerises and heterodimerises with a family of bcl-2 related proteins to inhibit apoptosis (Korsmeyer, 1995). Apoptosis following IL-3 removal requires cysteine-active-site protease activation, which is prevented in BAF-3 cells over-expressing bcl-2 (Furlong *et al*, 1997).

It has been proposed that mitochondrial disfunction can be a trigger of the late stages of apoptosis, since isolated mitochondria can induce apoptotic events in nuclei (Marchetti *et al*, 1996). Furthermore, bongkrekic acid (BA), which is a ligand for the adenine nucleotide translocator (ANT) and blocks mitochondrial permeability transition (Zoratti and Szabo, 1995), can be used to inhibit apoptosis induced by many agents whose primary site of action is mitochondria (Zamzami *et al*, 1996; Marchetti *et al*, 1996). In order to determine whether mitochodrial disfunction could trigger apoptosis in growth factor dependent cells, BAF3 cells were treated with the potassium ionophore valinomycin. Here we report that valinomycin induces apoptosis by triggering mitochondrial permeability

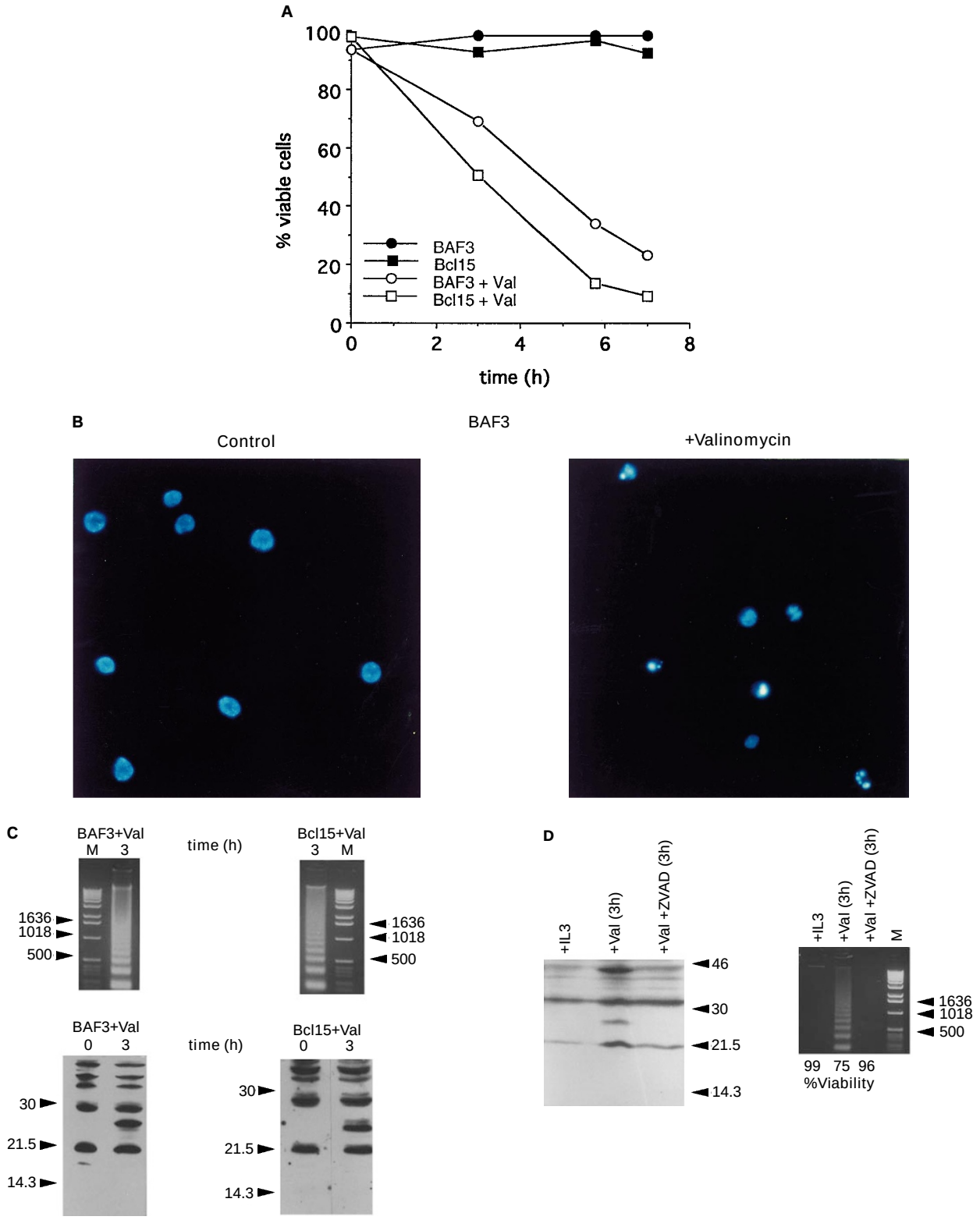


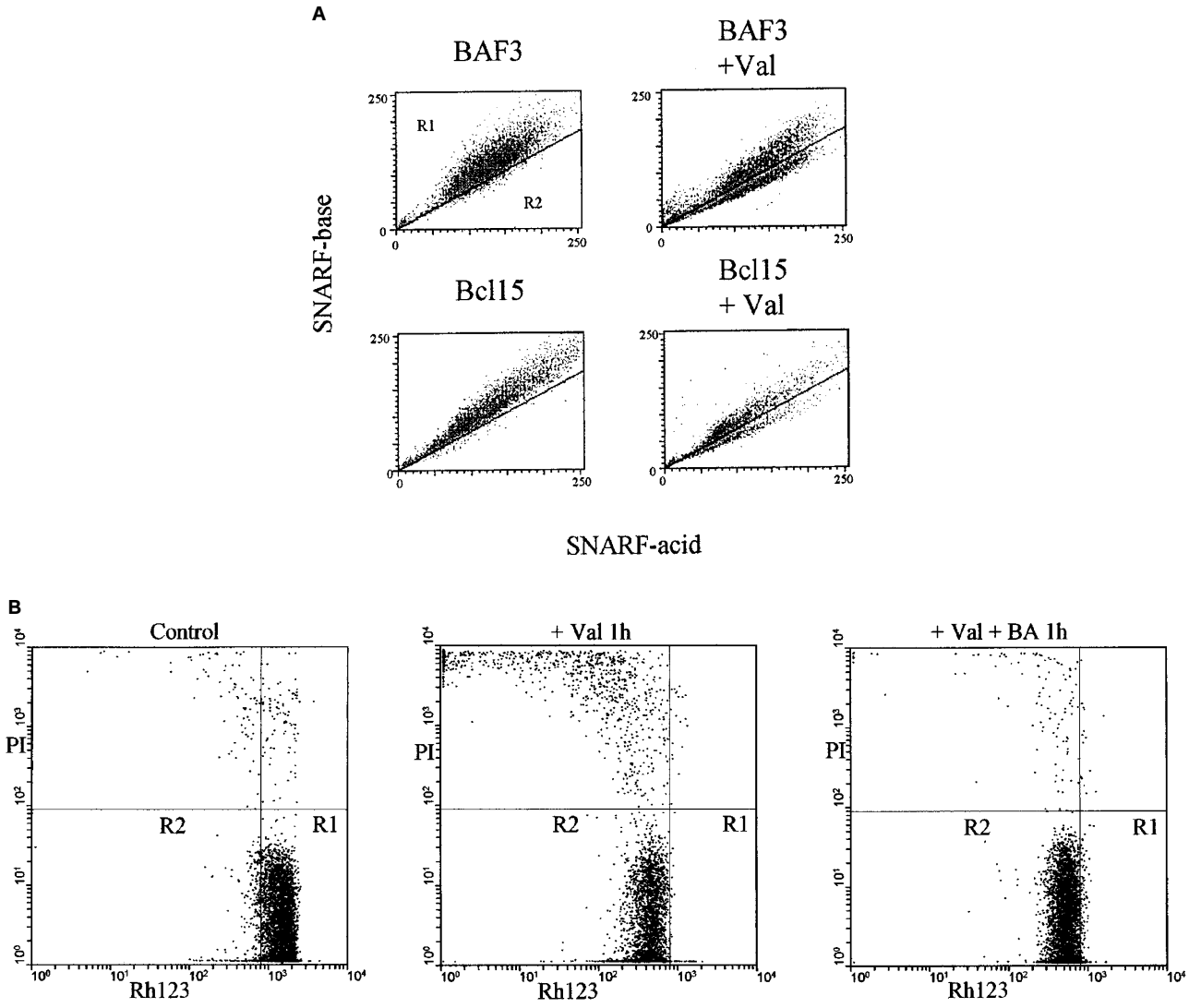
Figure 1 Valinomycin induces apoptosis by activating cysteine-active-site proteases. (**A**) BAF3 and Bcl-15 cells were incubated with 10 μM valinomycin as indicated, for the time shown, in the presence of IL-3. Samples of at least 100 cells were assessed for viability by trypan blue staining. Data are representative of three separate experiments. (**B**) BAF3 cells, untreated or after 3 h treatment with 10 μM valinomycin in the presence of IL-3, were stained with Hoechst 33342 and visualised by fluorescence microscopy using a $\times$63 objective. (**C**) Cell extracts were prepared from BAF3 or Bcl-15 cells, either untreated (0) or incubated with 10 μM valinomycin for 3 h in the presence of IL-3 (3). They were then analyzed for DNA fragmentation (size markers in bp), or PARP cleavage (size markers in kDa), as described in Materials and Methods. (**D**) BAF3 cells were incubated for 1 h with 200 μM ZVAD, prior to treatment with 10 μM valinomycin for 3 h, in the presence of IL-3. Samples were then analyzed for PARP cleavage, DNA fragmentation and cell viability

transition, leading to cytoplasmic acidification, cysteine-active-site protease activation and DNA fragmentation.

Results

Viability of BAF3 cells and in their derivative Bcl-15, which overexpress Bcl-2, after treatment with valinomycin was determined by trypan blue exclusion. Figure 1A shows that in the presence of IL-3, valinomycin induced a decrease in viability of both cell lines. Loss of viability was rapid, with most cells being permeable to trypan blue after 7 h. This time course was not changed by treatment with valinomycin in the absence of IL-3 (data not shown). In order to determine whether this loss of viability was due to apoptosis, the nuclei of valinomycin-treated cells were stained with Hoescht 33342. Figure 1B shows that pycnotic nuclei, with the condensed chromatin characteristic of apoptotic nuclei, were observed 3 h after valinomycin addition. Valinomycin also triggered biochemical changes characteristic of apoptosis. Figure 1C shows that PARP cleavage as a result of cysteine-active-site protease activation was observed in both BAF3 and Bcl-15 cells after valinomycin treatment. Valinomycin treated BAF3 and Bcl-15 cells also showed DNA fragmentation to internucleosome fragments (Figure 1C). Valinomycin has also been shown to induce apoptosis in T-cell lines CTLL-2 and EL-4 (Duke *et al*, 1994). Here we demonstrate that induction of apoptosis by valinomycin could not be inhibited by the presence of IL-3 or by Bcl-2 overexpression.

The cell permeable inhibitor of cysteine-active-site proteases, ZVAD, was used to determine the effect of protease inhibition on events induced by valinomycin. Prior to treatment with valinomycin, cells were incubated for 2 h in the presence of 200 μM ZVAD. Figure 1D shows that, in the presence 200 μM ZVAD, the PARP cleavage product was not detected and thus cysteine-active-site protease activity was inhibited. Figure 1D also shows that, under these conditions of inhibited protease activity, chromatin fragmentation and loss of viability induced by valinomycin were also inhibited. In the absence of ZVAD, chromatin laddering was induced after 3 h of valinomycin treatment. In the presence of ZVAD chromatin laddering was blocked and only 75% of valinomycin-treated cells



remained viable compared to almost 100% viability in ZVAD-inhibited cells. Cells remained over 90% viable for up to 10 h in the presence of ZVAD and valinomycin (data not shown). Therefore, cysteine-active-site protease activation was necessary for induction of apoptosis by valinomycin.

We have previously reported that a decrease in intracellular pH precedes cysteine-active-site protease activation in BAF3 cells upon IL-3 removal and that decreasing intracellular pH with the ionophore nigericin triggers cysteine-active-site protease activation and apoptosis (Furlong *et al*, 1997). In order to examine whether valinomycin treatment also caused intracellular acidification, cells treated with valinomycin were labelled with SNARF-1-AM and displayed on a two-dimensional scatter plot. Figure 2A shows that after a 30 min incubation with valinomycin in the presence of IL-3, 50.4% of BAF3 cells appeared in a low pH population R2. Cells in R2 represented those whose intracellular pH had dropped to 0.4 units below that in control cells (pH 7.3). This rapid intracellular acidification was also observed in the Bcl-2 overexpressors Bcl-15. After 30 min, 42.2% of Bcl-15 cells, treated identically, had decreased intracellular pH (Figure 2A).

One site of action of valinomycin is mitochondria, where K^+ transport through the inner mitochondrial membrane by valinomycin has the effect of decreasing $\Delta\Psi$ (Sharpe *et al*,

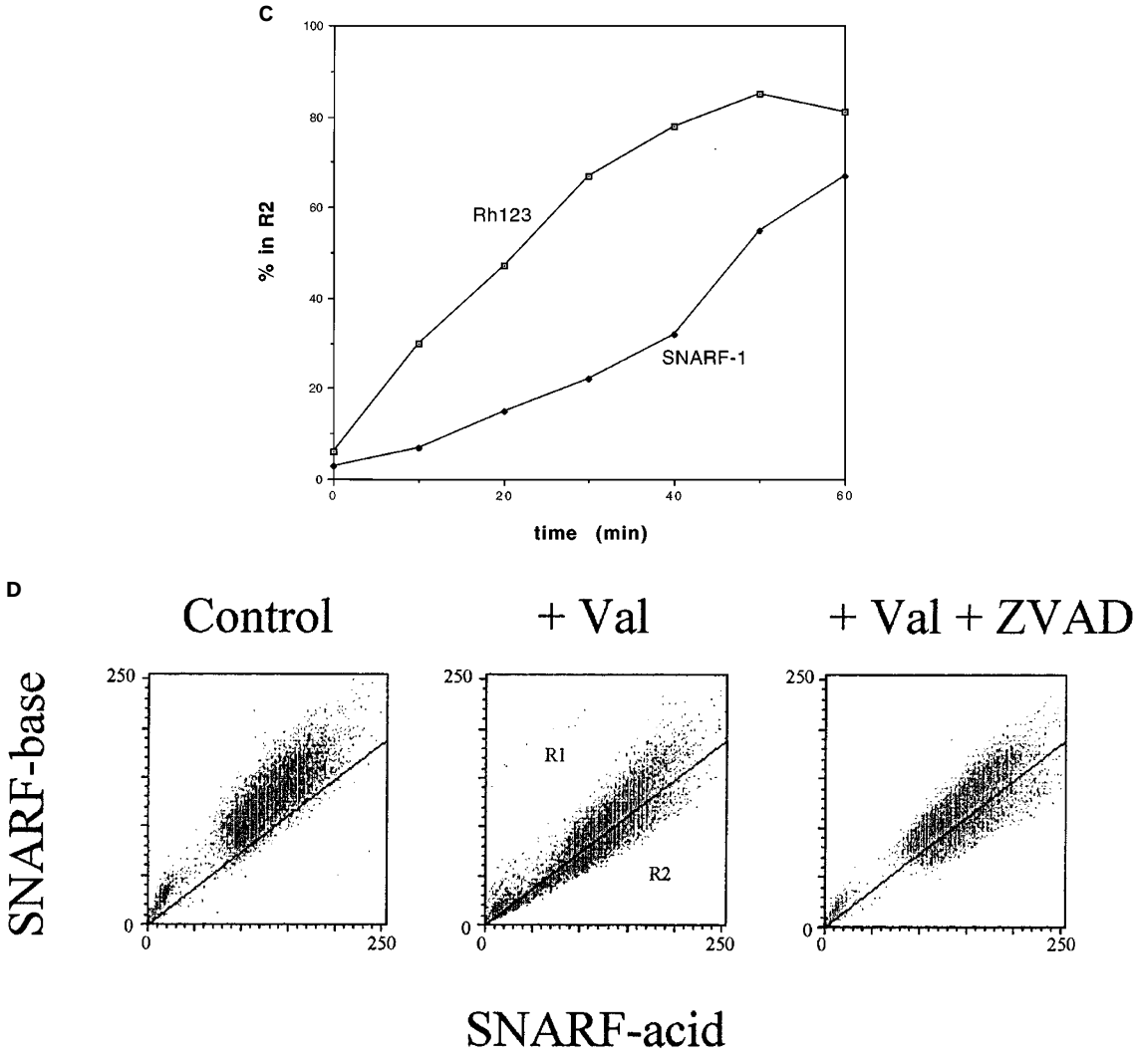


Figure 2 Valinomycin induces rapid cytoplasmic acidification. (**A**) BAF3 or Bcl-15 cells were incubated for 30 min with 25 μM valinomycin in the presence of IL-3, labelled with SNARF-1-AM as described in Materials and Methods, then analyzed by FACScan and displayed on a two dimensional scatter plot. Representative plots from one of three separate experiments are shown. Comparison with a callibration curve, described in Materials and Methods, demonstrated that cells in R1, defined as that which contains 90% of untreated cells, had an average intracellular pH of 7.30 $\pm$ 0.06 (mean $\pm$ S.E., three experiments) and cells in R2 had an average pH of 6.90 $\pm$ 0.03. In the presence of valinomycin 50.4 $\pm$ 5.0% of BAF3 cells and 42.2 $\pm$ 21.3% of Bcl-15 cells were in R2. (**B**) BAF3 cells were treated for 1 h with 25 μM valinomycin and 100 μM bongkrekic acid (BA) and in the presence of IL-3, then stained with Rh123 and PI, analyzed by FACScan and displayed on a two dimensional scatter plot, as described in Materials and Methods. R1 is defined to contain 90% of untreated cells. (**C**) Change in SNARF and Rh123 fluorescence after BAF3 cells were treated for the time shown with 25 μM valinomycin in the presence of IL-3. (**D**) BAF3 cells were incubated for 1 h with 200 μM ZVAD, prior to treatment with 25 μM valinomycin for 30 min, in the presence of IL-3. In the experiment shown, 52% of cells were in R2 after treatment with valinomycin and 22% were in R2 after treatment with valinomycin in the presence of ZVAD

1995). $\Delta\Psi$ in whole cells can be measured by staining with rhodamine 123, which diffuses into mitochondria where its retention depends on $\Delta\Psi$ (Kinally *et al*, 1978). Figure 2B shows that a population of BAF3 cells with decreased rhodamine 123 fluorescence could be detected after valinomycin treatment. Bongkrekic acid (BA), which is a ligand for the adenine nucleotide translocator (ANT) and blocks mitochondrial permeability transition (PT) (Zoratti and Szabo, 1995), did not inhibit accumulation of cells with decreased rhodamine 123 fluorescence (Figure 2B). This suggested that the primary action of valinomycin was to decrease $\Delta\Psi$. Figure 2C further supports this idea by showing that the population of cells with decreased pH appeared after those with decreased $\Delta\Psi$. It has previously been reported, in apoptosis induced by anti-Fas antibody, that cysteine-active-site protease activation precedes intracellular acidification (Meisenholder *et al*, 1996). However, we have demonstrated that intracellular acidification precedes cysteine-active-site protease activation in BAF3 cells deprived of IL-3 (Furlong *et al*, 1997). Likewise, when apoptosis was induced by valinomycin, inhibition of cysteine-active-site protease activation by ZVAD did not prevent acidification (Figure 2D). These data support the idea that a rapid decrease in $\Delta\Psi$ followed by a pH decrease was stimulated by valinomycin, which preceded the irreversible onset of apoptosis.

Disruption of $\Delta\Psi$ can be responsible for mitochondrial PT, which is the opening of protaceous pores in the inner mitochondrial membrane allowing free distributions of solutes between matrix and cytosol (Bernardi *et al*, 1993). It has been proposed that PT is a critical event in activating late stages of apoptosis since isolated mitochondria undergoing PT can induce apoptotic events in nuclei in a cell free system (Marchetti *et al*, 1996). PT can only be directly measured *in vitro*, by swelling of permeable mitochondria in hypotonic buffer. However, in whole cells, bongkrekic acid (BA), which is a ligand for the mitochondrial adenine nucleotide translocator (ANT) responsible for the PT (Zoratti and Szabo, 1995), can be used to prevent the PT and inhibit apoptosis induced by many agents whose primary site of action is mitochondria (Zamzami *et al*, 1996; Marchetti *et al*, 1996). Cells were therefore treated with valinomcyin in the absence or presence of BA then incubated with SNARF-1-AM to determine the effect of BA inhibition on intracellular pH changes. Figure 3A shows that intracellular pH was not affected by incubation of cells in 100 μM BA. However, after valinomycin treatment, the appearance of cells in the low pH population R2 is effectively blocked by the presence of BA. In the presence of valinomycin alone, 53.6% of cells were in R2 whereas only 5.6% of BA treated cells had an intracellular pH below control cells. Figure 3B and C shows that in the presence of 100 μM BA, the DNA fragmentation and loss of cell viability induced by valinomycin were also substantially inhibited. BA alone had no effect on cell viability (data not shown), however after 6 h treatment with valinomycin, viability was reduced to 20% but maintained at 70% by the addition of BA. This suggests that the effect of valinomycin following its decrease of $\Delta\Psi$ is to trigger the mitochondrial PT, which then leads to acidification and the subsequent stages of apoptosis.

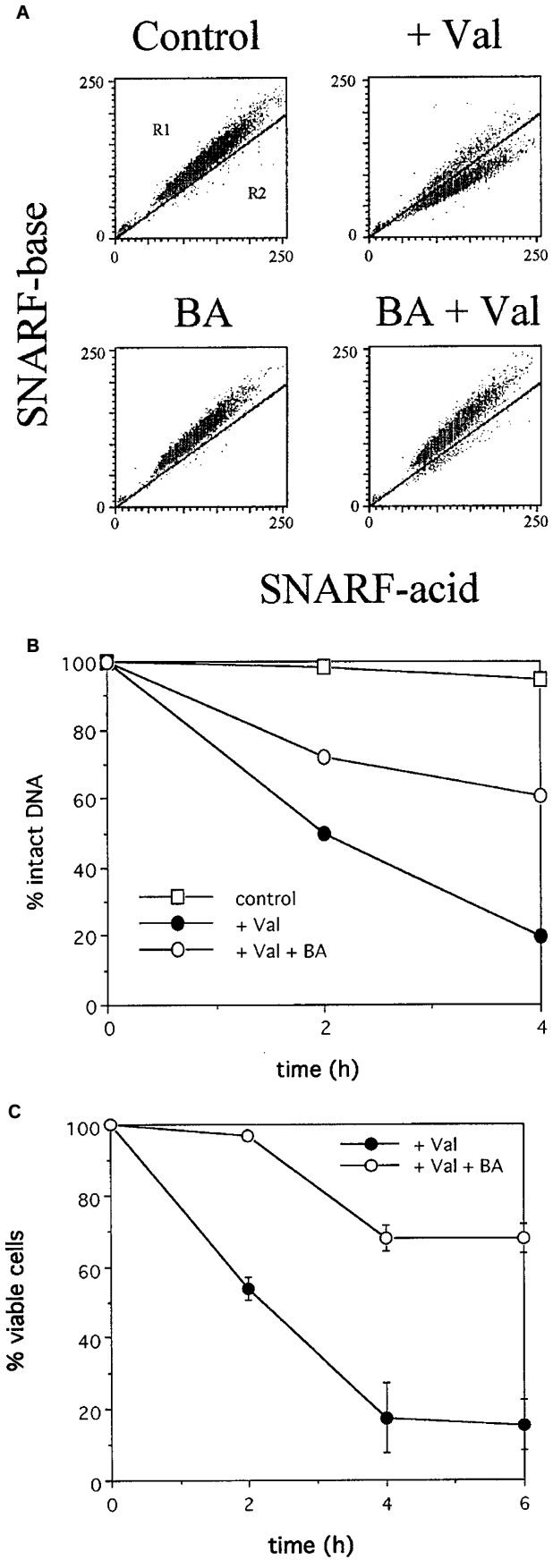
Discussion

A drop in intracellular pH can be induced in BAF3 cells by treatment with the potassium ionophore valinomycin. Following this acidification, cells die by apoptosis displaying pycnotic nuclei, cysteine-active-site protease activation and chromatin fragmentation. These effects are rapid and occur in the presence of IL-3 or Bcl-2 overexpression. Acidification and apoptosis induced by valinomycin are blocked by bongkrekic acid (BA), an inhibitor of the mitochondrial permeability transition (PT).

Valinomycin, an antibiotic ionophore produced by Streptomycetes, is a cyclic poly-peptide-like molecule whose folded conformation forms an inner cavity that can accommodate K^+ but not other ions. Since the outer surface is hydrophobic, the molecule is soluble in lipid bilayers and can diffuse across membranes. When added to intact cells, it can transport K^+ ions both outwards across the plasma membrane and inwards across the mitochondrial inner membrane, driven by K^+ gradients. By collapsing the K^+ gradient across the mitochondrial inner membrane, valinomycin decreases $\Delta\Psi$ (the mitochondrial membrane potential). A decrease in $\Delta\Psi$ occurs during apoptosis in dexamethasone and etoposide-treated T and B lymphocytes (Zamzami *et al*, 1996), during positive and negative selection (Zamzami *et al*, 1995), in rat neurones deprived of NGF (Deckwerth and Johnson, 1993) and TNF-stimulated U937 cells (Cossarizza *et al*, 1995). A decrease in rhodamine 123 fluorescence, a measurement of $\Delta\Psi$, was also detected after 10 min in 30% of BAF3 cells treated with valinomycin.

The next effect of valinomycin was intracellular acidification, detectable in 30% of cells after 40 min, which we have previously demonstrated induces cysteine-active-site protease activation and apoptosis in BAF3 cells (Furlong *et al*, 1997). BA inhibited this decrease in intracellular pH induced by valinomycin but not the decrease in rhodamine 123 fluorescence. This suggests that the effect of valinomycin on $\Delta\Psi$ can trigger PT (Bernardi *et al*, 1993), which then leads to acidification. Isolated mitochondria over-expressing Bcl-2 have been shown to be inhibited in the PT induced by atractyloside which binds the mitochondrial adenine nucleotide translocator (ANT). However, Bcl-2 could not inhibit the effect of some other PT inducers including calcium and diamide (Marchetti *et al*, 1996). This paralleled the ability of Bcl-2 to inhibit apoptosis induced by atractyloside, but not calcium or diamide, in T cell hybridomas (Zamzami *et al*, 1996). Likewise, Bcl-2 overexpression could not inhibit acidification induced by valinomycin.

How PT induced by valinomycin causes a drop in intracellular pH remains to be determined. We have previously described acidification induced by IL-3 removal, etoposide treatment, or the ionophore nigericin (Furlong *et al*, 1997). However, BA had no effect on acidification or apoptosis following these treatments ((Furlong *et al*, 1997) and data not shown). This suggests that acidification can



be induced by a number of pathways and that it provides a central stimulus for cysteine-active-site protease activation which in turn stimulates DNA fragmentation.

Materials and Methods

Cell culture

BAF3 cells (obtained from Dr Ronald Palacios, Basel Institute for Immunology) (Palacios, 1985) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Europe) containing 10% foetal calf serum (FCS) (Gibco Europe) and 5% cell-conditioned medium from the IL-3-producing cell line Wehi-3B. This was used as a source of IL-3 throughout. To remove IL-3, cells were washed twice in DMEM. BAF3 cells expressing human Bcl-2 (clone Bcl-15) were previously described (Marvel *et al*, 1993). BAF3 and Bcl-15 cells were grown at a density of 5×10^4 to 5×10^5 cells/ml at 37°C in a humidified atmosphere with 10% CO_2. Valinomycin was obtained from Sigma and ZVAD from Enzyme Systems Products, Dublin, USA. Bongkrekic acid was the gift of Professor J.A. Duine (Department of Microbiology/ Enzymology, Delft University of Technology, Netherlands).

Cell counting and morphology

Cell numbers were determined by counting on a haemocytometer. Cell viability was determined by staining cells with trypan blue and counting the percentage of cells excluding stain. To examine nuclear morphology, cells were pelleted and resuspended at 5×10^6 cells/ml in phosphate buffered saline (PBS)+2.5 μg/ml Hoechst 33342 (Sigma) before examination by fluorescence microscopy (UV-light source plus filter) using a Zeiss Axioskop microscope.

Intracellular pH and $\Delta\Psi$ measurements by FACScan

Intracellular pH measurements were performed according to the method detailed in (Rabinovitch, 1990). Briefly, for the final 15 min of each experiment, 1×10^6 cells were incubated with 20 μM Carboxy-SNARF-1-AM Acetate (Calbiochem) in 200 μl complete medium at 37°C in a humidified 10% CO_2 atmosphere. Loaded cells were then washed in Electrolyte Solution (5 mM Pipes/Tris pH 7.2, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-Glucose), resuspended in fresh electrolyte solution and incubated at 37°C for 5 min before analysis on FACScan. Intracellular carboxy-SNARF-1 was excited at 488 nM and emission measured at 575 and 670 nM using linear amplifiers. Intracellular pH values were determined by

Figure 3 Bongkrekic acid inhibits apoptosis induced by valinomycin. (**A**) BAF3 cells were pre-incubated for 1 h with 100 μM bongkrekic acid (BA), before treatment for 30 min with 25 μM valinomycin in the presence of BA and IL-3, as indicated. SNARF-1 labelled cells were then analyzed by FACScan and displayed on a two dimensional scatter plot. In the presence of valinomycin 53.6±5.5% (mean±S.E., three experiments) of cells were in R2, with BA alone 4.5±3.1% were in R2 and with valinomycin+BA 5.6±3.1% were in R2. (**B**) ^{3}H-thymidine labelled BAF3 cells were pre-incubated for 1 h with 100 μM bongkrekic acid (BA), before treatment with 10 μM valinomycin in the presence of BA and IL-3, as indicated, for the time shown. Release of DNA was measured as described in Materials and Methods. (**C**) BAF3 cells were pre-incubated for 1 h with 100 μM bongkrekic acid (BA), before treatment with 10 μM valinomycin in the presence of BA and IL-3, as indicated, for the time shown. Viability was assessed by trypan blue staining and counting at least 100 cells, each point represents the mean±S.E. of two experiments

comparing mean 575/670 nM fluorescence ratio values to a calibration curve constructed by incubating carboxy-SNARF-1-loaded BAF3 cells in a range of pH buffers in the presence of the ionophore nigericin (Sigma) at 2 μg/ml. pH calibration buffers were made by mixing 20 mM PIPES or 20 mM Tris in 130 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-Glucose to give a range of pH 5–8 at 37°C. To represent the fluorescence of each SNARF-1 labelled cell, a 2-dimensional dot plot with 575 nM fluorescence on the x-axis (SNARF-acid) and 670 nM fluorescence on the y-axis (SNARF-base) was displayed. On this plot, the distance of each cell from the origin is directly proportional to the amount of SNARF-1 loaded in the cell and the ratio of 575/670 nM fluorescence is a measure of intracellular pH.

$\Delta\Psi$ was measured by incubating 4×10^5 cells with 100 nM rhodamine 123 (Sigma) in 2 ml complete medium at 37°C in a humidified 10% CO_2 atmosphere, for the final 10 min of each experiment. Cells were pelleted at 4°C then resuspended in 1 ml ice cold PBS containing 1 μg/ml propidium iodide, before analysis on FACScan. A 2-dimensional dot plot with rhodamine 123 fluorescence on the x-axis and propidium iodide fluorescence on the y-axis was displayed.

Chromatin fragmentation

In order to isolate apoptotic DNA fragments from treated cells, the method of Herrmann *et al* was followed (Herrmann *et al*, 1994). 10^6 cells were washed in PBS and pelleted by centrifugation. Cell pellets were then lysed for 10 s with 50 μl DNA lysis buffer (1% NP40 in 20 mM EDTA, 50 mM Tris-HCl pH 7.5). After centrifugation at 13 000 g for 20 s, the supernatant was removed and extraction from the pellet repeated. Apoptotic DNA fragments were recovered from the combined supernatants by adding SDS to 1% and treating with 5 mg/ml RNaseA for 2 h at 56°C followed by digestion with proteinase K (2.5 mg/ml) for 2 h at 37°C. DNA was precipitated with 0.5 vol 10 M ammonium acetate and 2.5 vol ethanol, dissolved in gel loading buffer and separated by electrophoresis in 1.2% agarose gels. 1 μg of commercial λ DNA ladder (Gibco BRL) was run in parallel.

For measurement of chromatin fragmentation in intact cells by [^{3}H]thymidine release, cells (2×10^5 cells/ml) were incubated overnight with 1 μCi/ml of [^{3}H]thymidine (20 Ci/mmol Amersham International) then washed twice before incubation for the time shown. Cells were harvested using a Dynatech cell harvester (Dynatech Labs Ltd. Sussex, UK). ^{3}H counts were measured by scintillation counting. Chromatin fragmentation was calculated as retained ^{3}H-labelled DNA as a percentage of total (t=0 counts).

Analysis of PARP cleavage

Cells (1×10^6 per track) were lysed in gel sample buffer, boiled and sonicated before size fractionation of proteins on 12.5% SDS–PAGE. Proteins were transferred to nitro-cellulose membranes by electroblotting, blocked and incubated for 2 h at room temperature with the anti-PARP antibody anti-FII (gift of Dr. G. de Murcia (CNRS Illkirch, Strasbourg) diluted 1 : 1000 in blocking buffer. Anti-FII is a polyclonal rabbit serum raised against a synthetic polypeptide corresponding to the second zinc finger of the DNA binding domain of human PARP that detects a 25 kDa fragment after PARP cleavage. HRP-conjugated goat anti-rabbit and ECL were used for detection.

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