Elevated extracellular $[K^+]$ inhibits death-receptor- and chemical-mediated apoptosis prior to caspase activation and cytochrome *c* release

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Efflux of intracellular K^+ and cell shrinkage are features of apoptosis in many experimental systems, and a regulatory role has been proposed for cytoplasmic $[K^+]$ in initiating apoptosis. We have investigated this in both death-receptor-mediated and chemical-induced apoptosis. Using Jurkat T cells pre-loaded with the K^+ ion surrogate $^{86}Rb^+$, we have demonstrated an efflux of intracellular K^+ during apoptosis that was concomitant with, but did not precede, other apoptotic changes, including phosphatidylserine externalization, mitochondrial depolarization and cell shrinkage. To further clarify the role of K^+ ions in apoptosis, cytoprotection by elevated extracellular $[K^+]$ was studied. Induction of apoptosis by diverse death-receptor and chemical stimuli in two cell lines was inhibited prior to phosphatidylserine externalization, mitochondrial depolarization,

cytochrome *c* release and caspase activation. Using a cell-free system, we have demonstrated a novel mechanism by which increasing $[K^+]$ inhibited caspase activation. In control dATP-activated lysates, Apaf-1 oligomerized to a biologically active caspase processing ≈ 700 kDa complex and an inactive ≈ 1.4 MDa complex. Increasing $[K^+]$ inhibited caspase activation by preventing formation of the ≈ 700 kDa complex, but not of the inactive complex. Thus intracellular and extracellular $[K^+]$ markedly affect caspase activation and the initiation of apoptosis induced by both death-receptor ligation and chemical stress.

Key words: Apaf-1, apoptosome, CD95, cytoprotection, Jurkat cells.

INTRODUCTION

Apoptosis is a major form of cell death that can be induced by many factors, including ligation of death receptors, DNA-damaging agents, withdrawal of growth factors or cellular stress. It is characterized by a number of distinctive morphological changes, including cell shrinkage, membrane blebbing and nuclear condensation and fragmentation [1]. Cell shrinkage is almost certainly mediated by an efflux of the principal cytoplasmic ions, K^+ and Cl^- , and the associated osmotic efflux of water molecules. Efflux of cytoplasmic K^+ has been described as an important event in the progression of apoptosis [2–4], and prevention of this efflux has been reported to inhibit death-receptor- and chemical-induced apoptosis [5,6]. Efflux of K^+ may be required during apoptosis, since normal intracellular $[K^+]$ ($[K^+]_i$) inhibits some of the key enzymic events involved in the execution phase of apoptosis [7,8]. These include activation of caspases, a family of cysteine proteases that cleaves many structural and enzymic substrates during apoptosis [9], and endonucleases, which digest chromatin to produce oligonucleosomal DNA fragments [10].

Protection of neuronal cultures [11] and lymphocyte cell lines by elevated extracellular $[K^+]$ ($[K^+]_{ext}$) when exposed to pro-apoptotic stimuli provides further support for a role for K^+ in apoptosis. Elevated $[K^+]_{ext}$ inhibits anti-CD95-antibody-induced apoptosis in Jurkat T cells [2,12], and both cyclohexi-

mid- and anti-CD95-antibody-induced apoptosis in polymorphonuclear leucocytes [13]. Inhibition of apoptosis might be achieved by the prevention of intracellular K^+ efflux from the cell by greatly reducing the electrochemical driving force of K^+ , with the maintained normal cytoplasmic $[K^+]$ preventing the activation of apoptotic effector proteins. However, elevated $[K^+]_{ext}$ will also have profound electrical effects on the plasma membrane and associated proteins.

Members of the tumour-necrosis-factor (TNF) family, which includes CD95 (Fas/Apo-1), are potent inducers of apoptosis in many cell lines. Binding of CD95 ligand or anti-CD95 antibody to the CD95 receptor leads to recruitment of Fas-associated death domain (FADD/MORT-1) to the death domain of the CD95 receptor, which in turn recruits procaspase 8 to its death-effector domain. This protein complex is termed the 'death-inducing signalling complex' (DISC) and mediates auto-activation of procaspase 8 [14,15]. Active caspase 8 either activates effector caspases directly or indirectly [16]. In some cells, such as Jurkat and MCF7 cells, as well as possibly hepatocytes, this indirect activation may be due to caspase 8 cleaving Bid, a pro-apoptotic Bcl-2 family member, resulting in the formation of a truncated Bid, which is a potent inducer of the release of mitochondrial cytochrome *c*, thereby initiating a caspase cascade [17,18].

Chemicals or stress-inducing apoptotic stimuli, such as the DNA topoisomerase II inhibitor etoposide, appear to perturb

Abbreviations used: Apaf-1, apoptotic protease-activating factor 1; DISC, death-inducing signalling complex; $[K^+]_{ext}$, extracellular K^+ concentration; $[K^+]_i$, intracellular K^+ concentration; MG132, carbobenzoxy-Leu-Leu-leucinal; $[Na^+]_{ext}$, extracellular Na^+ concentration; NMDG, *N*-methyl-D-glucamine; Ψ_m , mitochondrial membrane potential; PtdSer, phosphatidylserine; TMRE, tetramethylrhodamine ethyl ester; TNF, tumour-necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DEVDase, Asp-Glu-Val-Asp-ase; Z-DEVD.AFC, benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp *O*-methyl ester fluoromethylketone; FCS, fetal-calf serum.

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mitochondria by release of cytochrome *c* [19,20]. Cytochrome *c*, together with dATP and Apaf-1 (apoptotic protease-activating factor 1), oligomerizes into a caspase-activating apoptosome complex [21]. An ≈ 1.4 MDa apoptosome complex has been reconstituted from purified recombinant proteins [22,23]. Using dATP-activated lysates, we have described a similar apoptosome complex that recruits and activates procaspase 9, which, in turn, activates the effector caspases 3 and 7, so initiating a post-mitochondrial caspase cascade [24]. We have also demonstrated that, following dATP activation, two complexes were formed: a biologically active ≈ 700 kDa complex which could activate effector caspases, and a biologically inactive ≈ 1.4 MDa complex [25].

We demonstrate an efflux of intracellular K^+ concomitant with other markers of apoptosis that was dependent on caspase activation in CD95- but not chemical-mediated apoptosis. Elevated $[K^+]_{ext}$ potentially inhibited apoptosis upstream of caspase activation and mitochondrial perturbation. In addition, we present evidence for K^+ ion regulation of chemically mediated apoptosis by inhibiting formation of the ≈ 700 kDa apoptosome complex.

EXPERIMENTAL

Materials

Anti-CD95 antibody (CH-11 clone) was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Anti-(cytochrome *c*) antibody was from Becton Dickinson (Franklin Lakes, NJ, U.S.A.). Antibodies to caspases 3 and 8 were from sources previously described [26]. Peroxidase-linked goat anti-mouse and FITC-linked goat anti-mouse antibodies were from Sigma (Poole, Dorset, U.K.). Peroxidase-linked goat anti-rabbit antibody was from Dako (Cambridge, U.K.). $^{86}RbCl$ and anti- α -tubulin antibody were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Benzyloxycarbonyl-Val-Ala-Asp *O*-methyl ester fluoromethylketone (Z-VAD.fmk) and benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD.AFC) were from Enzyme System Products (Livermore, CA, U.S.A.). Annexin V/FITC was from Bender Medsystems (Vienna, Austria). Tetramethylrhodamine ethyl ester (TMRE) was from Molecular Probes (Eugene, OR, U.S.A.). Carbobenzoxy-Leu-Leu-leucinal (MG132) was from Calbiochem (Nottingham, U.K.). TNF-related apoptosis-inducing ligand (TRAIL) was prepared as described previously [27].

Methods

Cell culture

Jurkat E6.1 and human monocytic THP.1 tumour cells were cultured at 37 °C/5% CO_2 in RPMI 1640 (Life Technologies, Inc., Paisley, Renfrewshire, Scotland, U.K.) containing 1% (v/v) Glutamax and 10% (v/v) fetal-calf serum (FCS; Life Technologies). Jurkat medium was supplemented with 1% (v/v) non-essential amino acids and 50 μM 2-mercaptoethanol. Cells used in experiments were in the exponential growth phase.

Ionically and osmotically defined media

Experimental media were made up of solution A (500 μM $CaCl_2$ /500 μM $MgSO_4$ /10 mM glucose/10 mM Hepes, pH 7.4), 10% (v/v) dialysed FCS, with NaCl and KCl to a total concentration of 140 mM. The osmolarity was checked in each case and was within the range of 275–300 mOsm. Media containing *N*-methyl-D-glucamine (NMDG) was made up as

described above, but with NMDG substituting for Na^+ . Dialysis of FCS to remove K^+ and Na^+ was performed for two periods of 1 h in solution A containing 20 mM glucose.

Induction of apoptosis

Cells $[(5-8) \times 10^5/ml]$ were incubated in RPMI 1640 medium with vehicle (DMSO), 50 ng/ml anti-CD95 antibody, 1 $\mu g/ml$ TRAIL, 50 μM etoposide or 1 μM MG132. For experiments involving elevated or normal $[K^+]$ media, cells were washed once in experimental medium and then re-suspended to $(5-8) \times 10^5$ cells/ml. Apoptotic stimuli were added 10 min after resuspension. Where appropriate, Z-VAD.fmk (20 μM) was added to cells 30 min prior to the addition of apoptotic stimuli.

Flow cytometry

A Becton Dickinson FACScan apparatus was used in conjunction with CELLQuest software, with 5000 events recorded per assay. For analysis, non-cellular events were gated out by forward and side scatter. Phosphatidylserine (PtdSer) externalization [28] was quantified using FITC-labelled annexin V, in conjunction with propidium iodide to measure membrane integrity. To investigate loss of mitochondrial membrane potential (Ψ_m), cells were incubated with 100 nM TMRE for 10 min at 37 °C before being assayed [29]. The mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone was used to verify TMRE measured changes in Ψ_m and that the signal was not influenced by changes in plasma-membrane potential.

Western-blot analysis

SDS/PAGE immunoblotting for detection of proteins was performed as described previously [30].

Analysis of cytochrome *c* release

Cells were harvested after 5 h incubation with vehicle, anti-CD95 antibody or etoposide and washed twice in ice-cold PBS. Cells were resuspended in cytosol extraction buffer and cytosolic lysates were prepared as described previously [31]. Protein content of lysates was determined by the Bradford method. Cytochrome *c* release was assessed by Western-blot analysis of lysates.

$^{86}Rb^+$ efflux assay

Jurkat T cells were loaded for 6 h at 2.5×10^6 cells/ml in complete RPMI medium containing 2 $\mu Ci/ml$ $^{86}RbCl$. Cells were washed twice in PBS, resuspended at 8×10^5 cells/ml, plated and treated appropriately. At the indicated times, cells were pelleted and the media transferred to a liquid-scintillation tube. Cells were washed in ice-cold PBS and then transferred to a liquid-scintillation tube. Counting was performed in a Wallac CompuGamma counter for 50 min per sample. The relative $^{86}Rb^+$ activity in the cell pellet was calculated as a percentage of total assay activity and compared with the control value at each time point. Results are represented as a ratio of experimental against control-cell-pellet $[^{86}Rb^+]$, a parameter termed K_R . The background count was negligible and was discounted.

Preparation of cell lysates for caspase activation and assay of caspase activity

Jurkat lysates (100 000 *g* supernatants) were prepared as described previously [25]. The lysates (10 mg/ml) were activated by

the addition of 2 mM dATP/MgCl₂ together with cytochrome *c* (0.5 mg/ml) and incubated at 37 °C for 1 h. The (DEVDase) activity (primarily a measure of caspases 3 and 7) of lysates or column fractions was measured using the fluorogenic substrate Z-DEVD.AFC, as described previously [25]. The cleavage rate of the fluorogenic substrate was determined by linear regression and activities were expressed as either pmol/min per mg of protein or pmol/min per fraction.

Analysis of apoptosome formation

Cytosolic lysates were separated by size-exclusion chromatography using a FPLC protein-purification system on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) as described previously [25]. Samples ($\approx$ 2.5 mg of protein) were applied to the column, eluted at 0.4 ml/min, and 0.5 ml fractions were collected. Caspase assays and Western blotting were performed on the collected fractions. The ability of the $\approx$ 700 kDa and $\approx$ 1.4 MDa apoptosome complexes to process and activate effector procaspases was assessed using the assay described previously [25]. Briefly, apoptosome complexes were isolated from dATP-treated lysates by Superose 6 filtration as described above. The appropriate column fractions were then incubated for 30 min with procaspases (25 μ g of protein) obtained following fractionation of control lysates, before being assayed for DEVDase activity. Thus this assay measures the ability of an assembled Apaf-1 complex to both process and activate effector caspases 3 and 7.

RESULTS

K^+ efflux accompanies the increase in PtdSer externalization, cell shrinkage and loss of Ψ_m during the induction of apoptosis

Induction of apoptosis in Jurkat T cells was analysed by three measures of apoptosis, which assess different features of the apoptotic phenotype, namely annexin V/FITC fluorescence to measure PtdSer externalization, decrease in TMRE fluorescence as an indicator of loss of Ψ_m and decrease in forward light scatter as a measure of cell shrinkage. In control cells, spontaneous apoptosis was $\approx$ 5%, as assessed by these three parameters (Figure 1A). Both anti-CD95 antibody and etoposide induced apoptosis in a time-dependent manner as assessed by these three parameters, with the induction of apoptosis being more rapid with anti-CD95 antibody (Figures 1B and 1C).

We wished to monitor $[K^+]_i$ and the efflux of intracellular K^+ in relation to induction of apoptosis over time and expression of the apoptotic parameters measured. To assess this, cells were preloaded with $^{86}Rb^+$ and, at the indicated times following treatment with the different apoptotic stimuli, $[K^+]_i$ was determined indirectly by measuring the retention of $^{86}Rb^+$ in the cell pellet. The change in $[K^+]_i$ was represented by the ratio of experimental against control cell-pellet $[^{86}Rb^+]$, a parameter termed K_R (see the Experimental section). Induction of apoptosis led to a decrease in K_R (Figures 1B and 1C), which indicated a decrease in $[K^+]_i$. This K^+ efflux accompanied PtdSer externalization, cell shrinkage and loss of Ψ_m (Figures 1B and 1C), which suggested that loss of $[K^+]_i$ might also be utilized as an indicator of apoptosis in some cellular systems. To our knowledge this is the first demonstration of a time-dependent efflux of K^+ during apoptosis, although other studies have reported its efflux. The decrease in K_R was not due to cell lysis, as at all times $< 3\%$ of cells stained positive for propidium iodide, a marker that indicates loss of plasma-membrane integrity.

K^+ efflux is not dependent upon effector caspase activity in etoposide-induced apoptosis

In order to determine the relationship between these measures of apoptosis and caspase activity, the broad-spectrum caspase inhibitor Z-VAD.fmk (20 μ M) was used [32]. In control cells, Z-VAD.fmk did not affect any of the four measures of apoptosis (Figure 1D), whereas it completely inhibited the changes in the apoptotic measures induced by anti-CD95 antibody (compare Figures 1B and 1E). By contrast, in cells treated with etoposide, Z-VAD.fmk completely blocked externalization of PtdSer, whereas loss of Ψ_m and efflux of intracellular K^+ were only partially inhibited and cell shrinkage was unaffected (Figure 1F). Taken together, these data are compatible with the hypothesis that Z-VAD.fmk inhibits an initiator caspase in anti-CD95-antibody-induced apoptosis, as expression of apoptotic markers was fully blocked. In etoposide-induced apoptosis, some of the markers of apoptosis, i.e. shrinkage, loss of Ψ_m and K^+ efflux, were either upstream or independent of postmitochondrial caspase activation. This is in agreement with, and further extends, our previous studies [26], and accentuates differences in execution of the apoptotic program induced by death receptors or chemical agents.

Elevated $[K^+]_{ext}$ inhibits apoptosis upstream of caspase activation

Several groups have reported that elevated $[K^+]_{ext}$ inhibits the induction of apoptosis [2,12,13]. In order to investigate whether elevated $[K^+]_{ext}$ would affect apoptosis in our system, Jurkat T cells were exposed to anti-CD95 antibody or etoposide for 5 h in either normal (5 mM K^+ /135 mM Na^+) or elevated (135 mM K^+ /5 mM Na^+) $[K^+]_{ext}$. Both stimuli induced apoptosis in normal $[K^+]_{ext}$, which was almost completely inhibited in elevated $[K^+]_{ext}$ (Figure 2C). Control cells showed a low level of spontaneous apoptosis in both normal and elevated $[K^+]_{ext}$ (Figure 2C), though at later times (10–12 h) cells in elevated $[K^+]_{ext}$ exhibited an increase in apoptosis, most probably as a result of continuous depolarization of the cells (results not shown).

Activated caspases play a major role in the execution phase of apoptosis induced by many stimuli [9]. To investigate whether elevated $[K^+]_{ext}$ inhibited apoptosis prior to caspase activation, cell pellets were analysed by Western blot for processing of caspases 8 and 3. In control cells, both caspases 3 and 8 were present entirely in their proforms (Figures 2A and 2B, lanes 1 and 4). Induction of apoptosis by both stimuli in normal $[K^+]_{ext}$ was accompanied by processing of both these caspases, with more extensive processing observed following anti-CD95 antibody treatment, commensurate with its greater induction of apoptosis (Figures 2A and 2B, lanes 2 and 3). Caspase 8 was processed to its p43 and p41 fragments, which arise following initial cleavage of both caspases 8a and 8b between the large and small subunits [33]. Caspase 3 was processed to its p20, p19 and p17 fragments [34]. The inhibition by elevated $[K^+]_{ext}$ of apoptosis induced by anti-CD95 antibody and etoposide resulted in total inhibition of processing of both caspases 3 and 8, which remained entirely as their intact zymogens (Figures 2A and 2B, lanes 5 and 6), except for a small amount of an uncharacterized caspase-3-immunoreactive product (Figure 2B, lane 6). This indicated for the first time that inhibition of apoptosis by elevated $[K^+]_{ext}$ in intact cells, rather than cell lysates, was upstream of caspase activation.

It was not possible to examine the interaction between cell shrinkage and inhibition of apoptosis by elevated $[K^+]_{ext}$, as both control and treated cells in this medium were shrunken compared with cells in normal $[K^+]_{ext}$, possibly due to interference with regulatory volume mechanisms. However, no further change in

Figure 1 Efflux of intracellular K^+ from apoptotic cells is concomitant with PtdSer externalization, loss of Ψ_m and cell shrinkage

Jurkat T cells, either loaded with $^{86}\text{Rb}^+$ or sham-loaded, were incubated with vehicle (**A** and **D**), anti-CD95 antibody (50 ng/ml; **B** and **E**) or etoposide (50 μM ; **C** and **F**) for 5 h in standard RPMI medium. At the times shown, assays were performed for $^{86}\text{Rb}^+$ efflux ($\bullet$), PtdSer externalization ($\blacksquare$), cell shrinkage ($\square$) and loss of Ψ_m ($\circ$). Experiments were also performed in the presence of Z-VAD.fmk (20 μM ; **D–F**). The right-hand y-axis displays cell-pellet [$^{86}\text{Rb}^+$] as a ratio of control (K_R). Data are representative of three independent experiments.

Figure 2 Elevated [K^+]_{ext} inhibits apoptosis induced by anti-CD95 antibody and etoposide upstream of caspase activation

Jurkat T cells were incubated for 5 h with anti-CD95 antibody (50 ng/ml) or etoposide (50 μM) in either normal or elevated [K^+]_{ext}. Processing of caspase 8 (**A**) and caspase 3 (**B**) was analysed by Western blot. Apoptosis was assessed by PtdSer externalization (**C**). Data are representative of three independent experiments.

volume was observed in cells in elevated [K^+]_{ext} upon treatment with apoptotic stimuli.

Elevated [K^+]_{ext} inhibits apoptosis upstream of cytochrome *c* release and loss of Ψ_m

Release of cytochrome *c* from the mitochondria is considered a critical step in chemical-induced apoptosis [19,20]. Correspondingly, death-receptor-induced apoptosis in some, but not all, cell types involves a signal amplification step involving release of cytochrome *c* mediated by cleaved Bid, thus activating a postmitochondrial caspase cascade. Presently the release of cytochrome *c* represents the most upstream event identified that is common to apoptosis induced by both death receptors and stress.

Several studies have shown that loss of Ψ_m is a key event in apoptosis induced by diverse stimuli such as UV and γ -irradiation, growth-factor withdrawal, DNA damage, chemical stress, Bax overexpression and ligation of death receptors [35]. Putative interactions between loss of Ψ_m and cytochrome *c* release are nonetheless controversial and are currently under intense investigation. In order to investigate the effect of alterations in [K^+]_{ext} on cytochrome *c* release, cytosolic lysates were prepared from cells treated with anti-CD95 antibody and

Figure 3 Elevated $[K^+]_{ext}$ inhibits apoptosis induced by anti-CD95 antibody and etoposide prior to mitochondrial perturbation

Jurkat T cells were incubated for 5 h with anti-CD95 antibody (50 ng/ml) or etoposide (50 μ M) in either normal or elevated $[K^+]_{ext}$. Western blots were performed to investigate cytochrome *c* release (A), and also for α -tubulin, to demonstrate equal protein loading (B). Cells were assessed by flow cytometry for PtdSer (PS) externalization (C) and loss of Ψ_m (D).

etoposide and incubated in normal and elevated $[K^+]_{ext}$ for 5 h. Control cells in both media showed no release of cytochrome *c* from the mitochondria (Figure 3A, lanes 1 and 4). Both apoptotic stimuli induced release of cytochrome *c* in normal $[K^+]_{ext}$, with a greater release induced by anti-CD95 antibody, consistent with its greater induction of apoptosis as assessed by PtdSer externalization (Figures 3A and 3C, lanes 2 and 3). This increase in cytosolic cytochrome *c* was almost completely inhibited in elevated $[K^+]_{ext}$ (Figure 3A, lanes 5 and 6). The small quantity of cytochrome *c* released by the anti-CD95 antibody correlated with the incomplete inhibition of apoptosis by elevated $[K^+]_{ext}$ (Figures 3A and 3C, lane 5). Equal protein loading was demonstrated by Western blotting for α -tubulin (Figure 3B).

Treatment of cells in normal $[K^+]_{ext}$ with anti-CD95 antibody or etoposide also induced loss of Ψ_m (Figure 3D, lanes 2 and 3), though with etoposide treatment in normal $[K^+]_{ext}$, fewer cells exhibited loss of Ψ_m than PtdSer externalization. In elevated $[K^+]_{ext}$, loss of Ψ_m was almost totally inhibited (Figure 3D, lanes 5 and 6), indicating that apoptosis induced by both stimuli was inhibited prior to loss of Ψ_m .

Elevated $[K^+]_{ext}$ does not inhibit binding of anti-CD95 antibody

The possibility that elevated $[K^+]_{ext}$ might block CD95-mediated apoptosis in Jurkat T cells by preventing binding of anti-CD95 antibody was investigated by utilizing a FITC-conjugated goat anti-mouse secondary antibody immunoreactive to the mouse anti-CD95 antibody used. Flow-cytometric analysis of binding of FITC-conjugated antibody to cells demonstrated that there was little difference in anti-CD95 antibody binding between normal and elevated $[K^+]_{ext}$ (Figure 4). Therefore the point of inhibition of apoptosis by elevated $[K^+]_{ext}$ was subsequent to binding of anti-CD95 antibody to the receptor. This result was confirmed by treating cells in elevated $[K^+]_{ext}$ with anti-CD95 antibody, then washing and resuspending the cells in normal $[K^+]_{ext}$. The induction of apoptosis in these cells was unaffected, confirming that the elevated $[K^+]_{ext}$ did not inhibit the binding of the anti-CD95 antibody, and demonstrating that the ligated

Figure 4 Elevated $[K^+]_{ext}$ does not prevent the binding of anti-CD95 antibody to Jurkat T cells

Cells in experimental media were treated with anti-CD95 antibody (50 ng/ml), then incubated for 15 min at 37 °C, pelleted and washed in PBS. After re-suspension in PBS/10% goat serum, cells were placed on ice for 1 h with FITC-conjugated goat anti-mouse antibody. Cells were washed once in PBS/10% goat serum and then analysed by flow cytometry. Histograms indicate FITC fluorescence of cells treated with anti-CD95 antibody in normal and elevated $[K^+]_{ext}$.

Figure 5 Treatment of cells with anti-CD95 antibody and etoposide in $[K^+]_{ext}$ at various values

Jurkat T cells were incubated with vehicle (○), 50 ng/ml anti-CD95 antibody (■) or 50 μ M etoposide (▲) for 5 h in $[K^+]_{ext}$ at various values, with $[Na^+]_{ext}$ correspondingly decreased to maintain a univalent-cation concentration of 140 mM. Apoptosis was assessed by PtdSer externalization. Results are means $\pm$ S.E.M. for four independent experiments.

receptor could still activate the caspase cascade when the $[K^+]_{ext}$ was returned to normal (results not shown).

$[K^+]_{ext}$ below 95 mM is ineffective in inhibiting CD95-mediated apoptosis

As 135 mM $[K^+]_{ext}$ almost completely inhibited apoptosis, we wished to investigate the concentration-dependence of this inhibition. Jurkat T cells were incubated for 5 h in media of differing $[K^+]_{ext}$, where $[Na^+]_{ext}$ was varied reciprocally in order to maintain osmolarity and a univalent-cation concentration of 140 mM. Cells treated with vehicle alone exhibited a low level of background apoptosis that did not vary greatly with changes in $[Na^+]_{ext}$ and $[K^+]_{ext}$. In anti-CD95-antibody-treated cells, increasing $[K^+]_{ext}$ up to 95 mM had little effect on the induction of

Figure 6 Cytoprotection by elevated $[K^+]_{ext}$ is independent of $[Na^+]_{ext}$ for anti-CD95-antibody- but not etoposide-induced apoptosis

Jurkat T cells in media of the indicated K^+ , Na^+ and NMDG concentrations were incubated with vehicle, anti-CD95 antibody (50 ng/ml) or etoposide (50 μ M) for 5 h. Apoptosis was assessed by PtdSer externalization. Results are means $\pm$ S.E.M. for three independent experiments.

apoptosis, whereas $[K^+]_{ext}$ above this inhibited apoptosis in a concentration-dependent fashion (Figure 5). Interestingly, apoptosis induced by etoposide was significantly inhibited by lower $[K^+]_{ext}$ than anti-CD95-antibody-induced apoptosis, and was almost completely inhibited at 120 mM $[K^+]$. These results imply that elevated $[K^+]_{ext}$ may act at different targets to inhibit the two apoptotic stimuli.

Cytoprotection by elevated $[K^+]_{ext}$ is not due to decreased extracellular $[Na^+]$

In experiments investigating the effect of increasing $[K^+]_{ext}$ on apoptosis, extracellular $[Na^+]$ ($[Na^+]_{ext}$) had been simultaneously decreased in order to maintain osmolarity and univalent-cation concentration. Although we had assumed that the inhibition of apoptosis was due to an increase in $[K^+]_{ext}$, it was possible that a decrease in $[Na^+]_{ext}$ was responsible. In order to ascertain the basis of the effects observed, the consequences of decreasing $[Na^+]_{ext}$ while keeping $[K^+]_{ext}$ constant were investigated. Experimental media were made up with the cation substitute NMDG replacing most or all of the Na^+ . Induction of apoptosis by anti-CD95 antibody was not affected when $[K^+]_{ext}$ was maintained at 5 mM and $[Na^+]_{ext}$ was decreased to 5 mM by replacement with NMDG (Figure 6). This demonstrated that protection against induction of apoptosis by elevated $[K^+]_{ext}$ was due to the increase in $[K^+]_{ext}$ and not decreased $[Na^+]_{ext}$. Induction of apoptosis by anti-CD95 antibody was also unaffected when Na^+ was completely substituted for by NMDG (5 mM K^+ /135 mM NMDG). Interestingly, etoposide-induced apoptosis was inhibited by the Na^+ -depleted media, but not to the same extent as elevated $[K^+]_{ext}$. This result implied that induction of apoptosis by etoposide, but not anti-CD95 antibody, was partially dependent upon the presence of Na^+ in the medium.

Elevated $[K^+]_{ext}$ inhibits apoptosis induced by a range of stimuli

We wished to investigate whether elevated $[K^+]_{ext}$ protected against other apoptotic stimuli. Both TRAIL, another member of the TNF family, and the proteasomal inhibitor lactacystin induce apoptosis in Jurkat T cells [36]. Treatment of Jurkat T cells in normal $[K^+]_{ext}$ with TRAIL or MG132, another proteasomal inhibitor, induced significant levels of apoptosis as assessed

Table 1 Inhibition of TRAIL- and MG132-induced apoptosis by elevated $[K^+]_{ext}$

Jurkat T cells in normal or elevated $[K^+]_{ext}$ were treated with vehicle, TRAIL (1 μ g/ml) or MG132 (1 μ M), incubated for 5 h, and then apoptosis was assessed by PtdSer exposure. Results are means $\pm$ S.E.M. for three independent experiments.

$[K^+]_{ext}$	Treatment ...	Apoptosis (%)		
		Control	TRAIL	MG132
Normal		3.1 $\pm$ 0.3	24.9 $\pm$ 3.0	15.4 $\pm$ 1.0
Elevated		2.5 $\pm$ 0.5	7.8 $\pm$ 0.3	2.7 $\pm$ 0.5

Table 2 Elevated $[K^+]_{ext}$ inhibits induction of apoptosis in THP.1 cells

THP.1 cells in normal or elevated $[K^+]_{ext}$ were treated with vehicle, etoposide (50 μ M) or MG132 (1 μ M), incubated for 8 h, and apoptosis was then assessed by PS exposure. Results are means $\pm$ S.E.M. for three independent experiments.

$[K^+]_{ext}$	Treatment ...	Apoptosis (%)		
		Control	Etoposide	MG132
Normal		2.8 $\pm$ 0.2	47.6 $\pm$ 2.6	31.0 $\pm$ 1.2
Elevated		4.3 $\pm$ 0.5	7.8 $\pm$ 1.0	7.1 $\pm$ 1.4

by PtdSer externalization (Table 1). Induction of apoptosis by both these stimuli was markedly inhibited by elevated $[K^+]_{ext}$.

Elevated $[K^+]_{ext}$ inhibits apoptosis in THP.1 cells

To investigate whether inhibition by elevated $[K^+]_{ext}$ extended to other cell lines, selected experiments were repeated using THP.1 cells, a human monocytic tumour-cell line. Induction of apoptosis in THP.1 cells by etoposide or MG132 over 8 h was greatly inhibited by elevated $[K^+]_{ext}$ (Table 2). Thus elevated $[K^+]_{ext}$ inhibited a range of stimuli in two cell lines, which suggested that either it inhibited a fundamental process common to all stimuli or that it inhibited apoptosis at multiple loci.

Addition of K^+ to cytosolic lysates inhibits caspase activation and formation of the $\approx$ 700 kDa apoptosome complex

We have shown that elevated $[K^+]_{ext}$ inhibits both anti-CD95-antibody- and etoposide-induced apoptosis in cultured Jurkat T cells upstream of mitochondrial cytochrome *c* release. However, we also wished to examine the action of raised ionic concentration on aspects of the apoptotic process downstream of the release of cytochrome *c*, namely activation of caspases and formation and function of the apoptosome, a process that is critical in chemically induced apoptosis. This seemed important, as previous studies have highlighted the ability of K^+ to inhibit dATP activation of caspases in cell lysates [2,7].

The formation of the apoptosome can be modelled *in vitro* by adding dATP and cytochrome *c* to cell lysates [24,25]. Since caspase activity was almost completely inhibited by 50 mM KCl (results not shown), we investigated the effect of this ionic concentration on apoptosome formation. Cytosolic lysates from Jurkat cells were separated on gel-filtration columns and the individual fractions immunoblotted for Apaf-1. In unactivated

Figure 7 KCl inhibits formation of the ≈ 700 kDa apoptosome in Jurkat T cell lysates

Lysates (10 mg/ml) from Jurkat T cells were incubated for 1 h at 37° without dATP ($\circ$), with dATP ($\bullet$) and with dATP, having been pretreated with 50 mM KCl at 4° C for 30 min ($\blacktriangle$). Lysates (≈ 2.5 mg of protein) were separated by Superose 6 gel filtration and the fractions immunoblotted for Apaf-1 (A) and assayed for their caspase processing activity by measuring DEVDase (B). Fraction numbers and the position of the high-molecular-mass standards are shown in the centre of the Figure.

lysates, Apaf-1 was eluted in its monomeric form, with an estimated M_r of ≤ 158000 (Figure 7A). After dATP activation, three different peaks of Apaf-1 were seen at ≈ 1.4 MDa, ≈ 700 kDa and 130 kDa (Figure 7A), corresponding to the previously described apoptosome complexes, and the monomeric form of Apaf-1 [25]. Addition of 50 mM KCl prior to dATP activation of the lysates completely suppressed caspase activation, and Apaf-1 was only eluted in the ≈ 1.4 MDa and 130 kDa fractions (Figure 7A). This strongly suggested that the oligomerization of Apaf-1 to form the biologically active ≈ 700 kDa apoptosome complex was inhibited by 50 mM KCl.

The caspase processing activity of these two apoptosome complexes was assessed using Z-DEVD.AFC. Fractions corresponding to the ≈ 700 kDa complex (Figure 7B, fractions 9–16) exhibited increased caspase-processing activity in lysates treated with dATP alone. The ≈ 1.4 MDa apoptosome complex (fractions 5–7) was much less active, despite containing more Apaf-1 (Figure 7A). In the presence of 50 mM KCl, little or no caspase-processing activity was seen in the ≈ 700 kDa fractions [9–16], consistent with formation of the caspase-activating ≈ 700 kDa apoptosome complex being suppressed under these conditions.

The inhibition of apoptosome formation and function by relatively low concentrations of K^+ suggests that normal $[K^+]_i$ may prevent inappropriate formation of the apoptosome complex and activation of the death pathway. This may, as suggested

previously [7], act as a safety measure for preventing inadvertent activation of caspases and it implies that other factors in the local cytoplasmic environment need to change before apoptosis is able to proceed.

DISCUSSION

In the present study we have demonstrated a time-dependent efflux of intracellular K^+ during apoptosis that was concomitant with other markers of apoptosis, including PtdSer externalization, cell shrinkage and loss of Ψ_m (Figure 1). Our data did not support the recent suggestions that efflux of K^+ is a very early event [6,7,37,38], nor a late-stage secondary event, but did indicate that K^+ efflux contributes to the apoptotic phenotype. Efflux of intracellular K^+ closely reflected cell shrinkage, suggesting they might be intimately related, with water leaving the cell as an osmotic consequence of an efflux of K^+ ions. Inhibition of caspase activity by Z-VAD.fmk showed that CD95- but not chemical-mediated efflux of intracellular K^+ was dependent upon caspase activity, further emphasizing the different mechanisms for induction of apoptosis by these different stimuli (Figure 1).

Elevated $[K^+]_{ext}$ inhibited both death-receptor- and chemical-mediated apoptosis at a very early stage, as caspase activation, cytochrome *c* release and loss of Ψ_m were almost completely inhibited (Figures 2 and 3). However, observation of the direct interaction between elevated $[K^+]_{ext}$ and apoptotic cell shrinkage was not possible, as elevated $[K^+]_{ext}$ induced shrinkage in all cells, possibly due to sustained depolarization of the plasma membrane disrupting regulatory-volume-decrease mechanisms. Since activation of procaspase 8 was inhibited by elevated $[K^+]_{ext}$, we deduce that CD95-mediated apoptosis was inhibited at the level of the plasma membrane, either by prevention of the formation of the DISC or the activity of the DISC.

By contrast, the plasma membrane is not known to be involved in initiating etoposide-induced apoptosis, which strongly suggests that elevated $[K^+]_{ext}$ had different loci of action for the two apoptotic stimuli. Release of cytochrome *c* in etoposide-mediated apoptosis can occur independently of cleaved Bid, whereas such cleavage is required in CD95-mediated apoptosis [26]. Etoposide-induced cytochrome *c* release may be mediated via modulation of the stress-activated protein kinase cascade [39], release of nuclear factors or direct action on the mitochondria [40]. Our data suggest that elevated $[K^+]_{ext}$ inhibited etoposide-induced apoptosis by interfering with the intracellular signaling that would normally trigger cytochrome *c* release in chemical-mediated apoptosis. Interestingly, inhibition of etoposide-induced apoptosis prior to mitochondrial perturbation has also been observed using the K^+ -channel blocker tetrapentylammonium [5].

Previous studies have proposed that elevated $[K^+]_{ext}$ inhibits apoptosis by preventing efflux of intracellular K^+ , thereby preventing activation of caspases [2,12,41]. While this mechanism may have contributed to the observed inhibition, it was not the predominant mechanism by which apoptosis was inhibited. In etoposide-induced apoptosis, efflux of intracellular K^+ was upstream of caspase activation in normal $[K^+]_{ext}$ (Figure 1), consistent with activation of caspases in low $[K^+]_i$. However, elevated $[K^+]_{ext}$ inhibited cytochrome *c* release (Figure 3), thus preventing oligomerization of Apaf-1 and formation of the caspase-activating apoptosome complex, and we therefore conclude that inhibition was prior to any involvement of caspases.

Although we have demonstrated inhibition of apoptosis prior to apoptosome complex formation, we also wished to further investigate previous findings showing inhibition by increased

[K⁺] of caspase 3-like activation but not caspase activity in cell extracts [2,7]. In addition, the cell-free system allowed us to examine actions of K⁺ on cytoplasmic proteins without changes in membrane properties, since elevated [K⁺]_{ext} would depolarize the plasma membrane of cultured cells and also dissipate the electrochemical gradient for the flow of K⁺ out of the cell. Our data showed for the first time that inhibition of caspase activation in cell lysates was mediated by preventing the formation of the biologically active ≈ 700 kDa apoptosome complex (Figure 7). This explains why activation of caspases, but not their activity *per se*, is inhibited by increased [K⁺]. This demonstrates that, independently of the effects of elevated [K⁺]_{ext} on the plasma-membrane potential and electrochemical gradients, maintained cytoplasmic [K⁺] will inhibit the initiation of the postmitochondrial caspase cascade. As caspases are activated during the execution phase of apoptosis, other cellular factors, possibly not present in our cell-free system, must be present to alleviate the inhibition of caspase activation by cytoplasmic [K⁺].

We have established the point at which elevated [K⁺]_{ext} blocked the apoptotic program, but the mechanisms by which apoptosis was inhibited are more difficult to determine. Varying medium [Na⁺] against [K⁺] revealed a sharp increase in inhibition of CD95-mediated apoptosis at approx. 95 mM K⁺ (Figure 5). This may have been mediated by a reversal in ionic driving force preventing K⁺ efflux or even increasing [K⁺]_i. Alternatively, depolarization of the plasma membrane by elevated [K⁺]_{ext} might have disrupted apoptotic signalling events by changing the state of membrane ion channels or by affecting the movement of voltage-sensitive proteins embedded within the membrane. There is some evidence for an early depolarization of the plasma membrane prior to loss of Ψ_m [42], and therefore, as suggested previously [13], elevated [K⁺]_{ext} might inhibit CD95-mediated apoptosis by depolarizing the plasma membrane rather than buffering movement of K⁺ ions. Inhibition of etoposide-induced apoptosis was seen at slightly lower [K⁺]_{ext}. As previously stated, initiation of etoposide-mediated apoptosis has no known involvement at the plasma membrane, leading us to suggest that [K⁺]_{ext} affected [K⁺]_i by altering driving forces of K⁺ across the plasma membrane.

In conclusion, we have demonstrated that manipulation of [K⁺]_{ext} profoundly affects the execution of apoptosis by acting at several loci and by several mechanisms to inhibit induction of apoptosis by different stimuli. Elevated [K⁺]_{ext} acts at the plasma membrane, inhibiting DISC formation or activity or altering the electrochemical properties of the plasma membrane itself. Elevated [K⁺]_{ext} also acts intracellularly to block signals upstream of mitochondrial perturbation, and inhibit caspase activation by preventing formation of the biologically active ≈ 700 kDa apoptosome complex.

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